Roderich Walter
Johannes Meienhofer

PEPTIDES

CHEMISTRY • STRUCTURE • BIOLOGY

Proceedings of the
Fourth American
Peptide Symposium

PEPTIDES: Chemistry, Structure and Biology

Proceedings of the
Fourth American Peptide Symposium

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OPENING REMARKS

by

Roderich Walter

The Fourth American Peptide Symposium is sponsored by The Rockefeller University and the University of Illinois Medical Center. This meeting is a "first" in more ways than one:

1. From now on, the American Peptide Symposium and the European counterpart will be alternating. Perhaps the time will come soon when our Japanese colleagues will consider extending their national peptide meeting to international participation.

2. As another first, we have received the stamp of approval from the National Institutes of Health, reflected in their financial support of this program. Of course, industry has once again come through with significant contributions, all the more appreciated during these difficult economic times.

3. We also have the pleasure to have with us several ambassadors to the U.N., of those countries whose scientists present papers.

Lyman C. Craig is honored in a special program. Many of you know that a month ago we suffered another loss in the untimely death of Josef Rudinger. Dr. Fruton, his friend of many years, will say a few words on Wednesday morning opening the session on neurohypophyseal hormones, which will appropriately be dedicated to his memory.

A word of explanation about the formulation of the program is in order. The initial announcement of the meeting produced an overwhelming response of about 2000 positive answers. Clearly, some parameter had to be set if the meeting was going to be not only manageable but, more importantly, of high scientific quality. The members of the Program Committee deserve my warm thanks and your praise for their successful efforts in hammering out the program as it stands before you. Let me assure you that scientific quality, above all, guided us, overriding such factors as nationality quotas, possible need for financial assistance and number of contributions from any one given laboratory. Nevertheless, to some degree each symposium will reflect the predilections of its organizers, and I am certain we have not escaped unscathed.

The decision to adopt a broader view was a conscious one, designed to present the field against a backdrop of the broad role of peptides in biology.

FOURTH AMERICAN PEPTIDE SYMPOSIUM

New York, New York

June 1-6, 1975

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TABLE OF CONTENTS

SECTION I
SCIENTIFIC PROGRAM IN TRIBUTE TO LYMAN C. CRAIG

SECTION II
CONFORMATIONAL STUDIES

SECTION III
SYNTHETIC STUDIES

SECTION IV
BIOLOGICALLY ACTIVE PEPTIDES

SECTION V
BRAIN SPECIFIC PEPTIDES

SECTION VII
HYPOTHALAMIC PEPTIDES

SECTION VIII
ANTIBIOTICS, ENZYME INHIBITORS, AND TOXINS

SECTION IX
ANALYTICAL AND ISOLATION PROCEDURES

SECTION I

SCIENTIFIC PROGRAM IN TRIBUTE TO LYMAN C. CRAIG

LYMAN C. CRAIG, 1906-1974

LYMAN C. CRAIG, IN MEMORIAM

Stanford Moore. The Rockefeller University, New York, New York 10021

ONE OF THE MOST TANGIBLE evidences for the quality of a scientist's career is the record of those whom he has helped to train in their profession. The post-doctoral fellows and graduate students who worked with Lyman Craig give eloquent testimony to the productive atmosphere which he fostered over the years, and a number of the alumni of the department are in the audience this afternoon. The organizers of this symposium have appropriately arranged for five contributions from this group to this special program dedicated to the recognition of Lyman Craig's fundamental contributions to peptide chemistry.

My aim, in this introductory tribute to Lyman Craig's broad scholarship, is to try to present a brief summary which will portray a gifted experimentalist who gave to all who worked with him an opportunity to gain a firsthand appreciation of the organic chemist's approach to the structural study of compounds of biological origin.

An individual's contributions to the world of science are of course closely interwoven with his role as an individual in the communities of his youth and his adult years. Lyman Craig was born on a farm in Iowa. He once wrote that his early years were those of a typical farm boy; farm work was a normal part of each of the school years. But he also found time and energy to be active in school athletics. (Any of you who saw him play tennis, during his long reign as the tennis champion of the Rockefeller Institute, will remember his skill in that sport.) His father was a farmer who was active in community affairs and a member of the Iowa State Legislature. He encouraged his son to enter graduate school and Lyman's selection of chemistry as a major subject and entomology as his minor

at Iowa State College grew in part from his practical appreciation of the role of insecticides in agriculture. (You will notice, as we move along, that each major step in Lyman's career was characterized by a logical progression from one fund of knowledge to the next; he always built upon a solid intellectual foundation.)

After earning his Ph.D. in 1931 he won a National Research Council Fellowship to extend his studies on insecticides at the department of chemistry at Johns Hopkins. His postdoctoral advisor at Hopkins was E. Emmet Reid. During Lyman's doctoral studies, in collaboration with teachers at Iowa State, and his two years at Hopkins he published twelve papers on the chemistry of nicotine alkaloids and their insecticidal action, particularly toward bean aphids.

This record of accomplishment won him an appointment in 1933 as a research assistant in chemical pharmacology at the Rockefeller Institute for Medical Research with Walter A. Jacobs, who was looking for a young chemist to work on the alkaloids of ergot. Walter Jacobs was a native of New York who earned his Ph.D. in Berlin in 1907 under Emil Fischer. The first paper by Jacobs and Craig[1] was on the characterization of a product obtained upon alkaline hydrolysis of ergotinine; they named the compound lysergic acid, on the basis that it was obtained by the lysis of ergot. They also isolated proline and phenylalanine as hydrolysis products, and this was the beginning of Lyman's experimental concern with amino acids.

In 1936 Lyman published a paper[2] which described what was to be the first in his series of ingenious contributions to chemical instrumentation. (Figure 1) In the alkaloid work he encountered a need to distill very small amounts of liquid in the days when microchemistry was in its infancy. He designed and built this microdistillation apparatus in which bulb A had a capacity of 250 microliters and the distillate was collected from a little inverted cup which would hold about 200 microliters by capillarity. In the next year[3] he extended the idea (Figure 2) to fractional distillation by inserting a fractionating column between the bulb and a cup.

My first consultation with Lyman, after my arrival at the Rockefeller Institute in 1939, was in reference to the use of this particular apparatus. One of my earliest impressions of him came from watching him at the glass-blowing bench. You could observe that as he shaped that glass, the elusive molten medium flowed according to his precise wishes. You would see a new piece of intricate glass equipment, well-

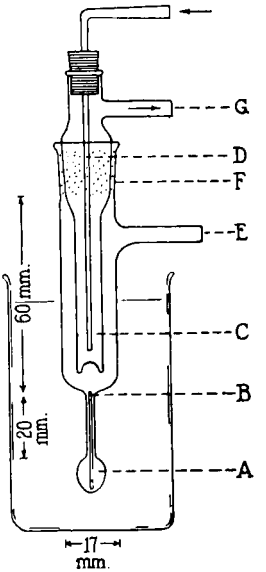

Figure 1: A microdistillation apparatus (1936).[2]

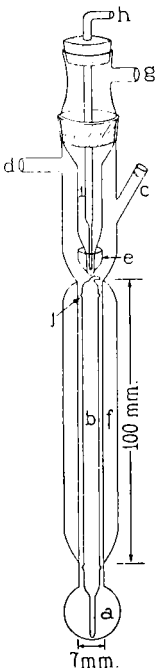

Figure 2: Microapparatus for fractional distillation (1937).[3]

conceived intellectually, grow into functional form much as a sculpture might grow in the hands of a realistic artist.

Over a period of about ten years, steady productivity by Jacobs and Craig yielded sixty fundamental papers on the chemistry of alkaloids.

Then came the war years, when most of the laboratories at the Rockefeller Institute were shifted to work on problems of immediate practical concern. Lyman, as a chemical pharmacologist, was asked to examine the question of whether the antimalarial drug atabrine, or a metabolic product thereof, is the active parasiticide in man. To examine the purity of the isolated metabolic products of atabrine, Lyman decided to explore a suggestion which, he pointed out, was made to him in a conversation with Milton T. Bush of the Vanderbilt University School of Medicine. The thought was that the distribution coefficient of an organic compound between two immiscible liquids might be a useful additional physical constant (along with melting point or boiling point) for the identification of the compound if the measurement could be made with precision on micrograms of material. Lyman[4] enlarged upon this idea by visualizing (Figure 3) a plot of the change in distribution coefficient as the compositions of two liquid phases were varied. He obtained characteristic curves with atabrine and several related compounds. The method was very sensitive since atabrine could be determined fluorometrically.

An unusual feature of this paper in the Journal of Biological Chemistry in 1943 is that it has no bibliography. (It is not often that an author can write an experimental paper that the editors of a journal will agree requires no references to the literature.)

These results on distribution coefficients grew in Lyman's inventive mind into a plan for equipment to use liquid-liquid extraction in a manner different from that of earlier approaches. Countercurrent extraction, with streams of immiscible liquids flowing continuously past one another, was a commonly used method in industry; Martin and Synge had recently introduced partition chromatography in which one of the liquid phases was immobilized in a gel, and their technique was beginning to open new worlds to the biochemist. Lyman had a third idea -- he decided to build a machine which was in effect a series of 20 small separatory funnels with the capability (after each shaking and settling) of sliding the top phase from one funnel onto the lower phase of the next[5] (Figure 4). The first apparatus was built from stainless steel. This step-wise procedure had the advantage

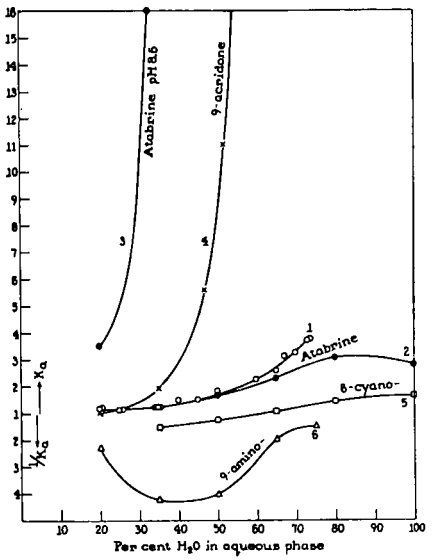

Figure 3: Curves for the apparent distribution coefficients of atabrine and related compounds (1943).[4] Solvent system: ethylene dichloride-water-methanol.

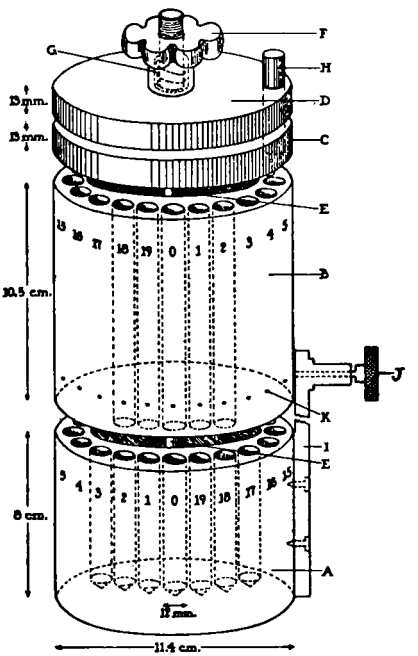

Figure 4: Countercurrent distribution apparatus (1944).[5]

over earlier techniques that the distribution pattern[5] (Figure 5) is theoretically a Gaussian curve which can be calculated precisely by the binomial expansion. His first test with β-naphthoic acid showed that very close to theoretical performance can be attained in practice.

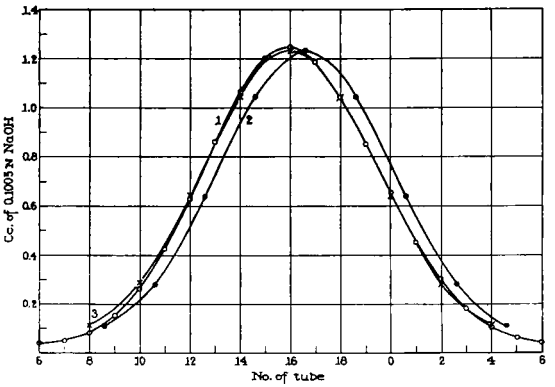

Figure 5: Curve 1 is the experimentally determined distribution of pure β-naphthoic acid in the tubes of the apparatus illustrated in Figure 4 after 59 transfers. Curve 2 is the calculated curve. Curve 3 is the calculated curve with the maximum superimposed on the experimentally determined maximum (1944).[5]

The first major application of this technique, in cooperation with Vincent du Vigneaud, was to the characterization of penicillins[6] (Figure 6) using an ether-aqueous buffer solvent system. From this distribution pattern it was possible, by comparing the theoretical and the found curves, to estimate that this preparation of the antibiotic contained approximately 90% benzyl penicillin. The amount of solute in each tube was estimated by weight (after evaporation of an ether extract); Lyman emphasized that weight was the most fundamental measurement when homogeneity was under test.

The next step in the instrumentation was the invention of an extraction train made from glass rather than metal and an increase in the number of tubes from 20 to 220 or 1000[7] (Figure 7). This type of automated apparatus, built in cooperation with Otto Post and Josef Blum, was based upon cleverly designed glass units[8] which permitted transfer of the upper phase to the next tube by decantation. Such

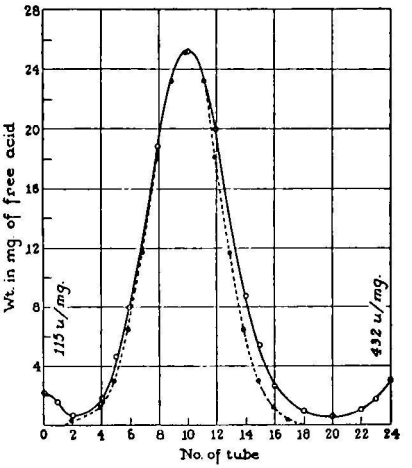

Figure 6: Distribution pattern of a commercial penicillin: O = weight curve, ● = calculated curve (1947).[6]

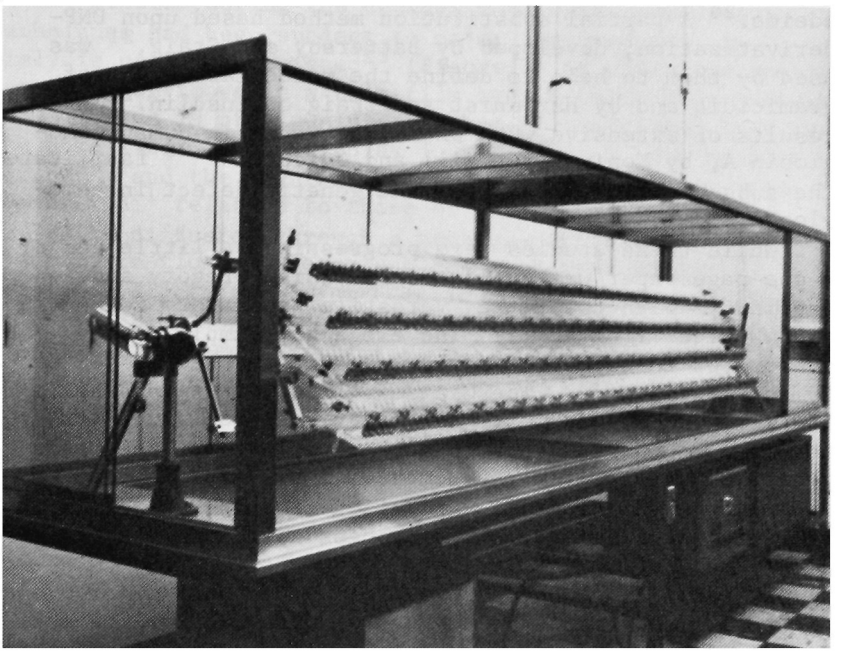

Figure 7: 1000-Tube automatic countercurrent distribution train (1958).[7]

equipment made possible multi-plate separations of high resolving power.

With the aid of such equipment Lyman, his postdoctoral associates, and his students, with great productivity, turned their attention to purification problems of increasing complexity. The substances studied included bile acids,[9] bacitracin,[10] tyrocidine,[11] polymyxin,[12] edeine,[13] insulin,[14] parathyroid hormone,[15] ribonuclease,[16] Bence-Jones proteins,[17] α- and β-chains of hemoglobin,[18] and serum albumin.[19] (It is of course beyond the scope of this review to indicate the myriad applications that countercurrent distribution has had in academic and industrial laboratories around the world, frequently with generous counsel from Lyman in the course of his international travels.)

The availability of pure antibiotic peptides prepared by countercurrent distribution led to major programs in Lyman's laboratory on the determination of the sequences of amino acid residues in a number of them. Countercurrent distribution was employed to advantage in the isolation of amino acids of unusual structure from peptides such as edeine.[20] A partial substitution method based upon DNP-derivatization, developed by Battersby and Craig,[21] was used by them to help to define the molecular weight of gramicidin and by Harfenist and Craig on insulin.[22] And the results of extensive work on the sequence in human hemoglobin A, by Konigsberg, Hill and Guidotti[23-25] facilitated the subsequent definition of the genetic defect in hemoglobin S.

While these studies were progressing, a little gem of a one-page paper was published by Craig, Gregory and Hausmann[26] with the title "Versatile Laboratory Concentration Device" (Figure 8). The elegantly simple rotary evaporator is undoubtedly the most widely used of all of Lyman's inventions. The Claisen flask, with its fragile capillary, a standard item of laboratory equipment for nearly one hundred years, has been replaced by the Craig rotary evaporator for the removal of solvents in most laboratory operations. Evaporation from the thin film in the rotating flask gives the process speed and also eliminates the problem of bumping.

Lyman then decided to explore, as his next major project, possible improvements in the use of dialysis for the separation of compounds on the basis of size. The experiments, begun with T. P. King,[27] have resulted in a new chapter in the science of the use of semipermeable membranes.[28] A fundamental investigation was undertaken of the parameters that

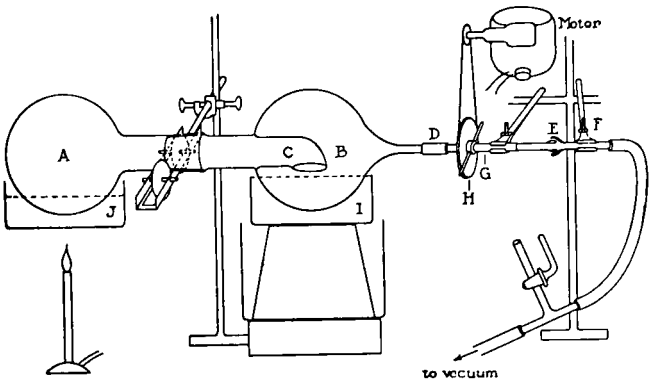

Figure 8: Rotary evaporator (1950).[26]

govern the process of dialysis, which had not been extensively studied during the decades when other separation techniques had been subject to major improvement. A dialysis cell was designed[29] (Figure 9) in which the entering solution flows in a thin film over a cellophane membrane; the membrane is previously stretched or acetylated to vary the pore size. The variables in the process have been defined[30] and the countercurrent possibilities have been considered[31] relative to those of gel filtration.

Since diffusion through a membrane is dependent upon the conformation of the molecule, Lyman also turned to tritium-hydrogen exchange,[32] optical rotatory dispersion,[33] circular dichroism,[34] nuclear magnetic resonance,[35] and fluorescent probes[36] as sources of additional information on the shape of molecules in solution.[37] This broad approach to the subject of the conformation of polypeptides was conducted in cooperation with associates at the Rockefeller University versed in these special techniques and was the principal theme of Lyman's researches in the last decade of his very full life.

In selecting the illustrations for this brief summary I have focused on Lyman's contributions to the experimental techniques of biochemistry, but I trust that I have also indicated that he was a firm believer in the principle that methods are a means to an end and not an end in themselves. For every methodological paper in his bibliography there are twenty papers on applications.

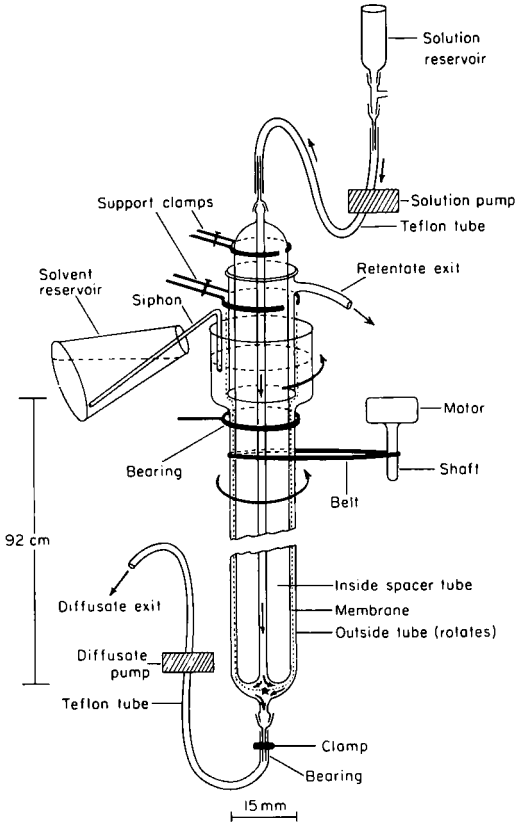

Figure 9: Thin film dialyzer (1965).[29]

Lyman was a highly productive scientist who always retained a modesty and a generosity that endeared him to all who knew him. The full life of a scientist is also intimately interwoven with that of his family. Rachel Parker, when Lyman met her, was an artist-in-residence at the Cold Spring Harbor laboratory, where she drew the scientific illustrations for contributions to the literature by members of the staff. She was an artist who married a scientist, and the success of the Craig family is a tribute to the mutual interests they shared. Her role in making it possible for Lyman to devote such a major part of his life to research in behalf of long-range goals speaks for her deep understanding of the importance of his contributions to human welfare.

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STRUCTURE AND FUNCTION OF SNAKE VENOM TOXINS

David Eaker. Institute of Biochemistry, Uppsala, Sweden

THE MOST LETHAL OF THE TOXIC proteins present in the venoms of elapid (cobras, draits, mambas, tiger snake, and taipan) and hydrophid (sea snakes) snakes are the paralytic neurotoxins which block transmission across the peripheral cholinergic synapse of the neuromuscular junction, thereby arresting respiratory movements and leading to death by asphyxia. The block of transmission can be either presynaptic, post-synaptic, or both, depending on the species of snake.

Post-Synaptic Neurotoxins

All elapid and hydrophid venoms examined so far contain at least one and usually several of the post-synaptic or "curarimimetic" neurotoxins which block the nicotinic acetylcholine receptor of the muscle motor end plate. The major post-synaptic toxins of most venoms can be isolated in pure form in one or two simple chromatographic operations, and with certain easily available venoms (*e.g., Naja naja siamensis*) the yield of an individual toxin can be a very impressive 250-300 mg per gram of dried crude venom.[1] Their ready availability, together with very high specific affinities for the peripheral nicotinic receptor has led to the universal use of such toxins, particularly β-bungarotoxin, *nigricollis* α, and *siamensis* 3, in the form of matrix-bound or radioactive derivatives for the isolation and assay, respectively, of the receptor from electroplax and other tissues. A dissociation constant of 2×10^{-11} has been reported for the complex between neurotoxin *nigricollis* α and the membrane-bound receptor,[2] while values 2-4 orders of magnitude higher are obtained with the solubilized receptor in the presence of detergents.

In the absence of crystallographic data, efforts to relate this high and specific affinity to particular structural features of the neurotoxin molecule have been based on specific chemical modification studies and on structural comparisons among naturally-occurring homologs. At present, the covalent structures of at least 40 curarimimetic neurotoxins are known, all of which fall into one or the other of two structurally and immunologically distinct groups: namely, the "long" neurotoxins containing usually 71-74 amino acids and five disulfide bridges, and the "short" neurotoxins containing 60, 61, or 62 amino acids and four disulfides. In addition to the neurotoxins, cobra and mamba venoms contain non-neurotoxic homologs of the cytotoxin/cardiotoxin/lytic factor group which exhibit to varying degrees cytolytic, cardiotoxic, transport-inhibiting, depolarizing, and hemolytic activities.[3] At least 19 sequences have been reported. A complete compilation of the known neurotoxin and non-neurotoxin sequences is precluded here by limitations of space, but 33 sequences have been compiled in a recent review.[4]

The salient similarities and differences in covalent structure among the neurotoxins and the non-neurotoxic homologs are adequately illustrated by the six sequences listed in Figure 1. All of these proteins are clearly homologous and might have evolved from an ancestral ribonuclease containing some multiple of 30 amino acids.[11] The pairing of the four invariant disulfide bridges is the same in all cases, the fifth or "extra" disulfide in the long neurotoxins serving to pinch off a stretch of sequence that is partly deleted in the short toxins, effectively reducing the second disulfide loop to about the same length as in the short toxins, as illustrated in Figure 2. The common disulfide pairing would seem to require that the three-dimensional structures of all of these toxins should be grossly similar, but immunological evidence indicates that the long neurotoxins, short neurotoxins, and non-neurotoxins must be quite different with regard to surface detail. There is good immunological cross-reactivity among different representatives of the same group in all cases tested, but there is no indication of any cross-reactivity whatever among any representatives of the three different groups.

However, the obvious "two-dimensional" structural similarity among the three groups of toxins determined by the sequence homology and the common disulfide pairing prompted an attempt to find out whether any reasonable hypothesis regarding structure-function relationships could

Homology Alignment
of Neurotoxins, a Cardiotoxin, and a Direct Lytic Factor (DLF)

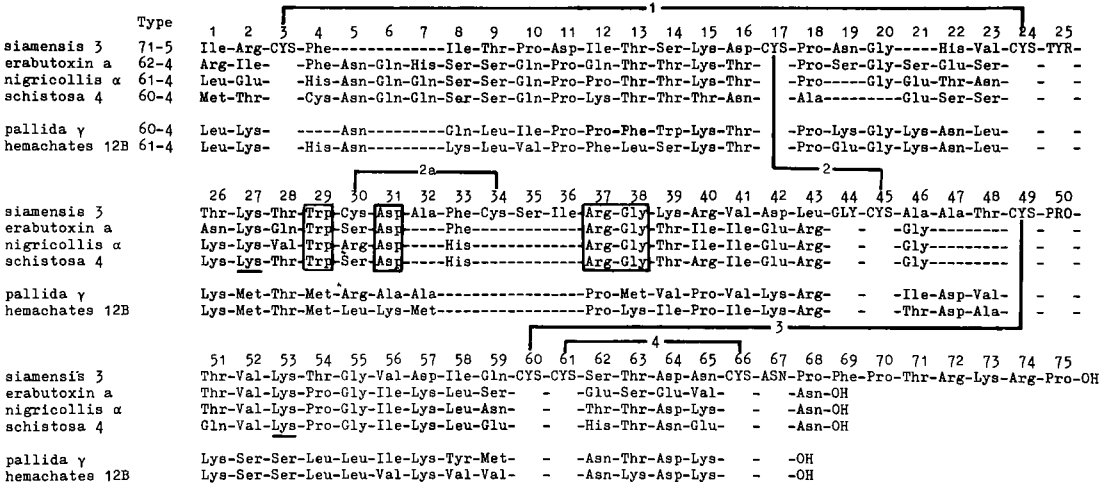

Figure 1: Toxins *siamensis* 3 from the venom of the Thailand cobra, *Naja naja siamensis*,[1] erabutoxin a from the sea snake *Laticauda semifasciata*[5,6], *nigricollis* α from the spitting cobra *Naja nigricollis*,[7] and *schistosa* 4 from the sea snake *Enhydrina schistosa*[8] are curarimimetic neurotoxins. *pallida* γ from the cobra *Naja mossambica pallida* is a cardiotoxin/cytotoxin,[9] and *hemachates* 12B is a direct hemolytic factor (DLF)[10]. The twelve residues written in capital letters are invariant in all known homologous neurotoxins and non-neurotoxins. The residues enclosed in boxes are invariant in the neurotoxins.

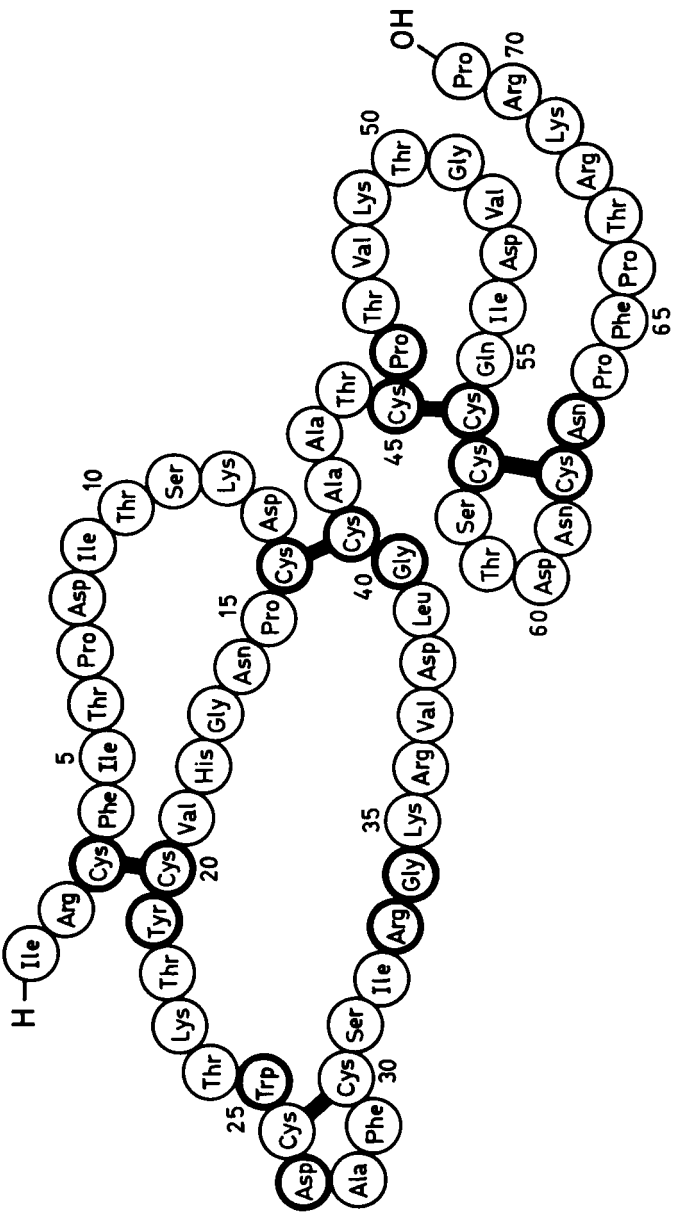

Figure 2: Covalent structure of long neurotoxin *siamensis* 3. The structurally and functionally invariant residues are encircled with heavy lines.

be derived from an examination of the sequences themselves, and the preliminary result was summarized in the form of a three-dimensional model.[12] In connection with the construction of the model, amino acids that are invariant or conservatively substituted (*e.g.*, Ile/Val) in the entire set (which now represents more than 60 sequences) were classified as structurally invariant or structurally conserved, respectively, with the assumption that these residues are part of a common structural core which is necessary for the formation of the folded form with the proper disulfide pairing. The half-cystine residues 3, 17, 24, 45, 49, 60, 61 and 66 (homology numbering, Figure 1) are thus structurally invariant, as are Tyr-25, Gly-44, Pro-50, and Asn-67, each of which is immediately adjacent to a half-cystine residue. Residue 41 is structurally conserved, being Val, Ile or Thr (β-methyl group) in most sequences, as is residue 56 (Val, Ile or Leu in 53 of 60 sequences).

Trp-29 and Arg-Gly 37-38 (homology numbering, Figure 1) are invariant in all known neurotoxins and residue 31 is Asp in all but one sequence, where it is Asn.[13] Residue 9 is Ser in all short neurotoxins and is Thr or Ser in all but two long neurotoxins. Residues 27 and 53 are invariably Lys in the short neurotoxins, but in two long neurotoxins Lys-27 is replaced by Glu and in two other long neurotoxins residue 53 is Glu or Asn instead of the usual Lys or Arg. Residue 52 is Val in all neurotoxins except α-bungarotoxin, where it is Lys, and residue 58 in all neurotoxins is Ile, Leu or Val. Finally, residue 33 is always Phe, Trp or His, and since His is probably not protonated at physiological pH these substitutions might reflect conservation of aromatic character.

In the non-neurotoxic homologs the pattern at the above nine positions is quite different. The conserved Thr/Ser 9 is usually replaced by Leu. The conserved Lys-27 and the invariant Trp-29 are usually replaced by Met. Positions 31, 33, 37-38, and 53 are radically variable, and residue 52 is usually Ser instead of Lys or Arg. The nine positions 9, 27, 29, 31, 33, 37-38, 52 and 53 in the neurotoxins are therefore designated *functionally* invariant or *functionally* conserved because they are invariant or highly conserved in the toxins having the receptor-blocking function contra the non-neurotoxic homologs. This apparent correlation cannot be regarded as evidence for the direct participation of any one of these nine amino acids in the toxin-receptor interaction, and conversely, certain of the residues classified as structurally invariant or structurally conserved might well be directly involved. The latter applies

especially for the conserved hydrophobic amino acids at positions 41 and 56 in the carboxyl-terminal half of the molecules.

Group-specific chemical modification studies with various of the neurotoxins have so far failed to prove the participation of any particular amino acid side chain in the toxin-receptor interaction. Botes[14] showed that the short "extra" disulfide bridge in the long neurotoxin *nivea* α could be selectively reduced and the two resulting cysteine residues alkylated with iodoacetate without abolishing the neurotoxic activity. The purified derivative was about 9 percent as potent as the native toxin. The reduction of the remaining four disulfides was completely cooperative, as is the case with the four invariant disulfides of the short toxins. The completely reduced molecules are non-toxic.

The importance of the modifiable functionally invariant and functionally conserved amino acids has been investigated in our laboratory using mainly the long neurotoxin *siamensis* 3 (Figure 2). Although the fifth disulfide is not essential it does make the molecule less susceptible than the short toxins to structural disturbances that attend certain of the chemical modifications. The invariant tryptophan is exposed and can be modified readily in non-denaturing media. Ozonolysis of the invariant Trp-25 (*siamensis* 3 numbering, Figure 2) reduces the toxicity by 50 percent, as does the introduction of one 2-hydroxy-5-nitrobenzyl group. Derivatives with *two* such groups on the invariant tryptophan were also isolated and showed about 20 percent activity. The sheer bulk of the modifying groups would seem to rule out any essential function of Trp-25 in the toxin-receptor interaction.

Cationic and Anionic Groups

Since the small agonists and antagonists of the nicotinic acetylcholine receptor contain at least one cationic group it was initially taken for granted that one or more of the amino- or guanidino groups in the neurotoxins should play a direct essential role in the toxin-receptor interaction. However, we found that each of the six monoacetylamino (α-amino and 5 lysine residues) derivatives of the *siamensis* 3 toxin is at least 70 percent as toxic as the native protein and binds strongly to the isolated receptor.[16] Enzymatic removal of the C-terminal Arg-Lys-Arg-Pro reduces the toxicity by about 50 percent.[15] Three (Arg-2, His-18, and Arg-36, consecutive numbering, Figure 2) of the remain-

ing four cationic residues in *siamensis* 3 are not conserved in other long neurotoxins and can be excluded from consideration on that basis, leaving the invariant Arg-33 as the only possible candidate for an *essential* cationic group. We have not succeeded to isolate a monomeric derivative modified only at this arginine, but Yang *et al.*[17] have reported that a derivative of cobrotoxin in which the invariant Arg had been modified with phenylglyoxal was 25 percent as potent as the native toxin. This result must be interpreted with some caution, since the phenylglyoxal modification is slowly reversible under physiological conditions. At the moment, however, it seems likely that no individual cationic group in the toxin is *essential* for the toxin-receptor interaction.

In confirmation of the above results suggesting the absence of any single functionally essential amino acid apart from perhaps one or more of the "inaccessible" (to chemical modification) hydrophobic residues, Maeda and Tamiya[13] have recently isolated from *Laticauda semifasciata* venom a "defective" long neurotoxin, Ls III, in which the conserved Thr/Ser-9 (homology numbering, Figure 1) is replaced by Leu, the only conserved and otherwise invariant carboxylic amino acid Asp-31 is replaced by Asn, the conserved Arg/Lys 53 is replaced by Asn, and the basic C-terminal tetrapeptide is absent. The molecule is nevertheless 10-12 percent as potent as erabutoxin a.

Although at least six (9, 27, 29, 31, 37, and 53, homology numbering) functionally invariant or highly conserved residues mentioned above appear not to be individually essential, this does not mean that none of them participates in the toxin-receptor interaction. Unsuspected residues might also be involved. Owing to conformational differences among the various neurotoxins a particular function might be served by amino acids which occur at slightly different positions in the different sequences and which are therefore excluded from consideration by the "homology sieve." Certainly a crystallographic comparison of the long and short neurotoxins would reveal what surface features they have in common, which should permit some useful hypotheses regarding the nature of the surface involved in the toxin-receptor interaction. Several short neurotoxins containing 62 amino acids have been crystallized and crystallographic studies are in progress. Short neurotoxins containing 60 amino acids have also been crystallized, but despite several years of effort in many laboratories no one, to my knowledge, has yet succeeded to crystallize any long neurotoxin or any short neurotoxin containing 61

amino acids. The ideal solution would be to crystallize the toxin-receptor complex, which will be a formidable task if even possible. It is certainly worth trying, since one might then obtain the three-dimensional structure of the receptor as well, which has been all along the major motivation for the neurotoxin work.

Pre-Synaptic Neurotoxins

Neurotoxins which act pre-synaptically, suppressing both spontaneous and nerve-impulse-invoked release of acetylcholine from motor nerve terminals have been isolated from the venoms of the many-banded krait *Bungarus multicinctus* (β-bungarotoxin),[18,19] the Australian Tiger Snake *Notechis scutatus scutatus* (notexin),[20,21] and the Australian taipan *Oxyuranus scutellatus scutellatus* (taipoxin).[22,23] These toxins are, on a weight basis, 4-25 fold more lethal than the most potent post-synaptic neurotoxins. Although the pre-synaptic effects are similar to those produced by the still much more potent toxins of *Clostridium botulinum*,[24] competition experiments indicate that the target sites might overlap somewhat but are not identical.[25]

The most thoroughly characterized of the pre-synaptic toxins from snake venoms is the notexin isolated in Uppsala from the venom of the Tiger Snake.[20] Notexin exhibits three interesting biological activities: 1) it is a phospholipase A, almost certainly A_2; 2) it blocks the release[21] of acetylcholine from motor nerve terminals *without* causing any initial "bursts"; and 3) it has a remarkable dystrophic action on striated muscle cells.[26] Upon exposure to notexin *in vivo* muscle fibers of types I and III degenerate completely within three days. Since the blockage of the nerve terminal recedes *in vivo* within a few days and the microcirculation is apparently unaffected, regeneration and differentiation from myoblasts begins within a week and is essentially complete by 21 days. Notexin shall certainly be a valuable tool for the study of muscle growth and development.

The amino acid sequence of notexin determined recently in the author's laboratory[22,27] is shown in Figure 3, along with the sequences of four other snake venom phospholipases and the phospholipase A_2 from porcine pancreas. The pig enzyme[30] is slightly acidic and has no neurotoxic activity *in vivo*. The three phospholipases from the venom of the cobra *Naja melanoleuca*[28,29] are also moderately acidic and might be weakly neurotoxic. The *Naja nigricollis* enzyme sequenced very recently in the author's laboratory[31] is very basic, has lower phospholipase activity than the four

Homology Alignment of Pancreatic and Snake Venom Phospholipases A

Figure 3: Homology alignment of porcine pancreatic phospholipase A_2 and five phospholipase A enzymes from elapid venoms. The porcine enzyme has six dilulfide bridges, while the venom enzymes have seven. The 43 residues that are invariant among the six sequences are capitalized. Blanks (- -) signify identity with the melanoleuca enzyme DE-III. Deletions --- have been inserted to maximize homology.

enzymes above, but shows both the pre-synaptic and dystrophic effects observed with notexin, albeit about 30-fold less potent. Notexin is slightly less basic than the *nigricollis* phospholipase and is about 10 percent as active in the phospholipase assay, but has a mouse lethal dose of 0.5 μg.

The sequences are clearly homologous, showing identity at 43 of 129 positions (33 percent) in the homology alignment and considerable conservation elsewhere. The homology is especially good in the amino-terminal stretch from position 1-53, wherein 45 percent of the positions are identical. The trend toward decreasing phospholipase A activity with increasing toxicity among these six proteins suggests that the phospholipase activity might have little or no role in the neurotoxic action, and this seems to be the case. We have recently found that p-bromophenacyl bromide reacts specifically with His-48 in notexin, completely abolishing the phospholipase A activity,[32] as was shown earlier by Volwerk *et al.*[33] for the corresponding histidine in the pig enzyme. The modification reaction is strongly inhibited by Ca^{++}, and the latter authors suggested that the histidine residue is one of the ligands to the calcium ion that is required for phospholipase A activity. In the case of notexin the His-48 derivative (PBP-notexin) elutes well behind the native molecule in elution chromatography on Bio-Rex 70 at pH 6.5 in ammonium acetate buffer, which enabled us to show that the reaction goes to completion and that the product contains no unmodified notexin. The PBP-notexin does not kill mice at a dose level of 100 μg, corresponding to 200 lethal doses of the native molecule. However, the PBP-notexin still blocks the nerve terminals in isolated nerve-muscle preparations and the dystrophic action on muscle is not greatly diminished, which means that the enzyme activity has no essential role in either function. The loss of lethality attending the chemical modification might therefore reflect a loss of specificity which causes the injected PBP-notexin to be diluted out by adsorption to membranes other than that of the motor nerve terminal. Also, it seems very likely that the PBP-notexin no longer binds calcium ion, and the structural consequences of this might seriously diminish the affinity of the molecule for its target in the nerve terminal. At any rate, we feel that the phospholipase activity *per se* has at most a secondary role in the toxic and dystrophic actions of notexin.

Taipoxin, isolated in Uppsala from the venom of the Australian taipan, has a mouse LD_{50} of 0.04 μg per mouse and is thus the most lethal substance yet isolated from

any snake venom. Taipoxin has a molecular weight of about 47,000 (ultracentrifuge) and is a glycoprotein, containing 5 residues of N-acetylglucosamine, 1 residue of fucose, 2 of mannose, 4 of galactose, and 4 of N-acetylneuraminic acid. Taipoxin is moderately acidic, the isoelectric point being about pH 5, and migrates as a homogeneous substance in electrophoresis at pH 7-9. At acid pH taipoxin dissociates to varying degrees depending on the conditions, and by the scheme of operations illustrated in Figure 4, we have shown it to be a 1:1:1 complex of three rather different subunits which are apparently held together by non-covalent interactions. The β-fraction which can be separated off by gel filtration at pH 3 (0.1 *M* acetic acid) in the absence of salt accounts for one-third of the total protein and is a mixture of two nearly neutral "iso-subunits," β_1 and β_2, which are separable by ion exchange chromatography on Sulfopropyl-Sephadex (SP-Sephadex). The strong interaction between the γ and the α subunit seems to be partly electrostatic, since the α-component dissociates off, with concommitant formation of the γ-dimer which permits separation by gel filtration, when salt is included in the medium. The α-component contains 12 or 13 residues of arginine and has an isoelectric point about pH 10, while the γ-component, which contains only 2 residues of arginine and all of the carbohydrate, is isoelectric at about pH 2.5. The enormous difference in electrophoretic mobility accounts for the fact that the α- and γ-components separate electrophoretically at acid pH even in the absence of salt.

Whole taipoxin has phospholipase A activity, but we have not yet assayed the purified subunits. Amino acid composition and amino-terminal sequence data indicate that the subunits contain about 120 amino acids and 7 disulfides and are thus homologs of the 6 phospholipases listed in *Figure 3*. The separated β- and α-components are much less lethal than the whole taipoxin, and the γ-component hardly toxic. However, the α-fraction is nearly as potent as whole taipoxin in blocking the nerve terminal *in vitro*. The complex might be required in order for the toxic subunit(s) to reach the target *in vivo*.

Probably all of the snake venom neurotoxins that suppress transmitter release are homologs of phospholipase A, and since the phospholipase A activity seems not to be essential we might eventually discover a potent homolog that lacks the catalytic activity altogether. The six sequences shown in *Figure 3* do not permit anything more than wild guesses regarding which amino acids might be responsible for the neurotoxic contra the phospholipase A activity

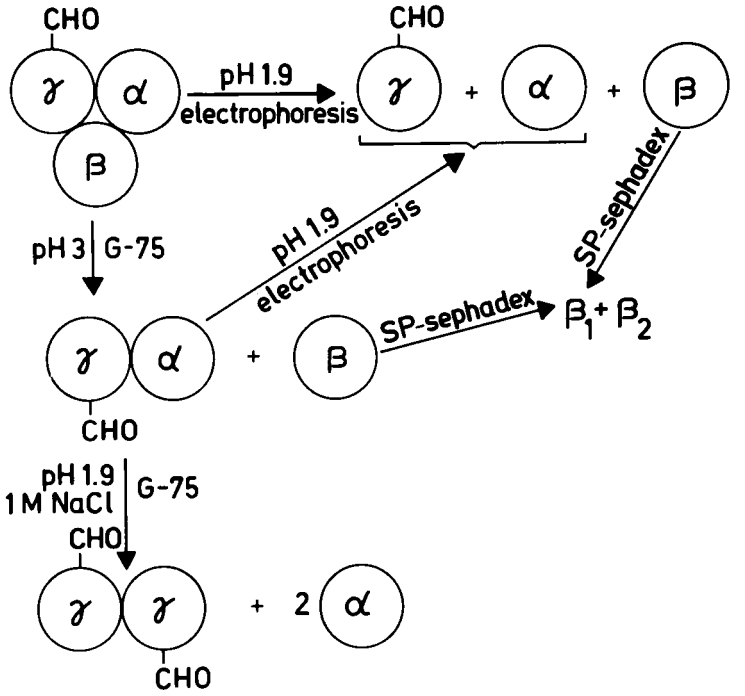

Figure 4: Schematic illustration of the dissociation and separation of the subunits of the pre-synaptic neurotoxin taipoxin, from taipan venom. The γ-subunit bears a large carbohydrate moiety and is isoelectric at about pH 2.5. The α-component has an isoelectric point above pH 10, while the β-components are approximately neutral. The intact complex is 10 to 100-fold more lethal than any of the subunits. SP-Sephadex = sulfopropyl-Sephadex.

because the molecules are too different. However, we are now beginning sequence work on a notexin homolog from Tiger Snake venom which has the same phospholipase A activity as notexin but is 20-fold less toxic. Amino acid composition data and tryptic peptide maps suggest that this poorly toxic homolog might differ from notexin by only four discrete substitutions, for example, Ser/Gly, Arg/Ala, Arg/Lys, and Ser/Phe.

Notexin crystallizes readily and beautifully from ammonium sulfate, and K. K. Kannan in Uppsala has obtained diffraction patterns with reflections out to 2 Å or better.

Although the pre-synaptic neurotoxins are twice the size of the post-synaptic ones, the three-dimensional structure might be much easier to solve. In the meantime we shall try to prepare suitable radioactive and matrix-bound derivatives of notexin in order to locate and, hopefully, to isolate the target molecule(s) in the nerve terminal and the muscle cell.

Acknowledgments

I am grateful to Professor Roderich Walter for the invitation to give this paper and to President Seitz for making me a guest of the Rockefeller University during my stay in New York. Most of all I am grateful to the late Lyman Craig for excellent training in the separation and analysis of proteins, which has been the foundation of all our accomplishments with the neurotoxins. The investigations described herein were supported by the Swedish Natural Science Research Council, dnr 2859-005 and 2859-008.

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SUBTILIN AND NISIN: THE CHEMISTRY AND BIOLOGY OF PEPTIDES WITH α,β-UNSATURATED AMINO ACIDS

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IT IS WITH ADMIRATION AND GRATITUDE that I stand here today to speak in honor of Dr. Lyman C. Craig. It was soon after my arrival in this country when I had the great privilege to join Dr. Craig's laboratory. This splendid affiliation with Dr. Craig was of the most profound influence on my subsequent career.

While in Dr. Craig's laboratory, we were concerned primarily with the possible chemical fragmentation of peptides resistant to the action of proteolytic enzymes and the separation, purification and characterization of the resulting fragments.

We addressed ourselves to the numerous peptide constituents of Bacillus brevis—among them the various tyrocidins[1] and the gramicidins A, B, and C[2]—and to subtilin from Bacillus subtilis.[3] It is with pride, when I say that the collaboration on subtilin lasted for many years. It was as recently as 1973 when we published with Dr. Craig our last paper[4] on aspects contributing to the structural elucidation of subtilin.[5]

The Gramicidins A, B, and C

The pioneering work in the course of which Dr. Craig showed us the way to the separation and purification of the gramicidins A, B, and C subsequently led to their structural elucidation. Today, we know that this family of gramicidins exists in nature in at least six analogs (Figure 1), three of which are also available synthetically.[7,8]

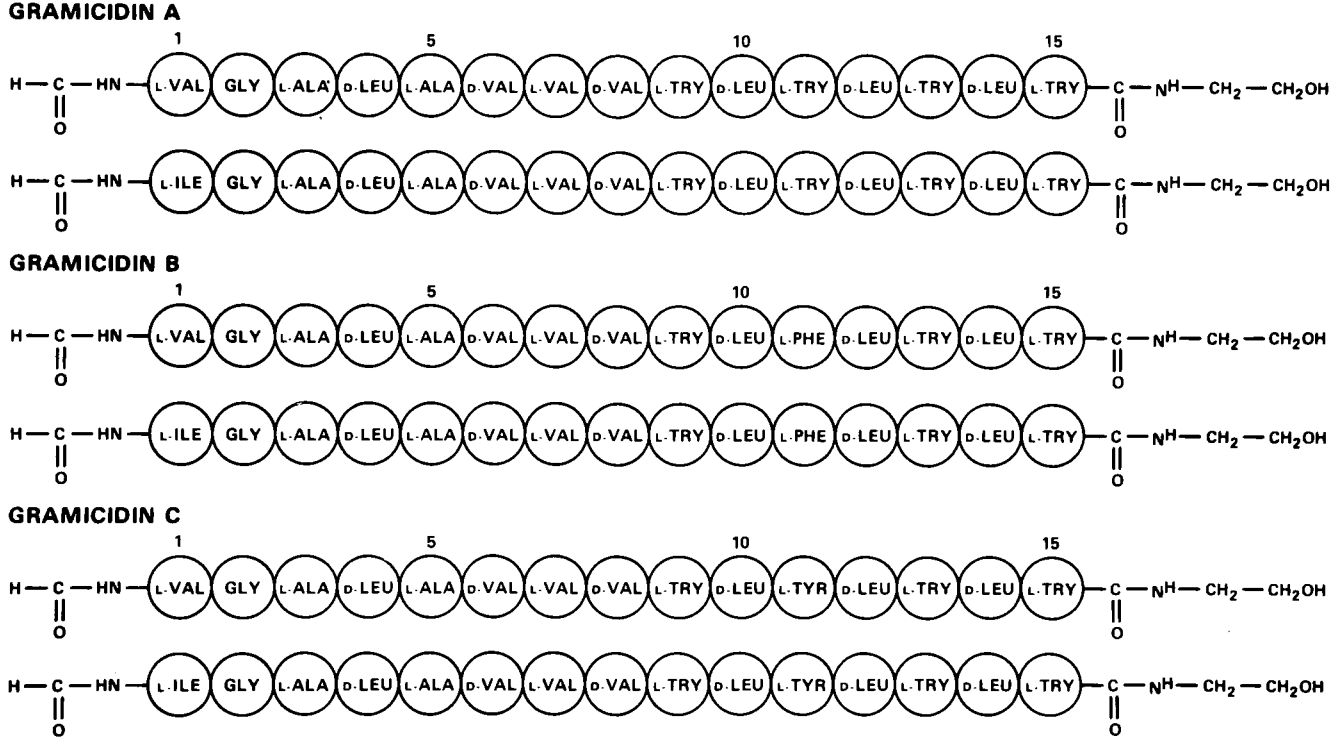

Figure 1: The structures of valine- and isoleucine gramicidins A, B, and C. H_2N-termini are formylated. COOH-Termini are hydroxyethylamidated. Amino acid residues show alternating L- and D-configurations.

In the face of their being perhaps the most *hydrophobic* peptides we know, *countercurrent distribution*[9] proved immensely valuable for the purification of the natural as well as the synthetic products (Figure 2). It is the high degree of *hydrophobicity* that places the gramicidins A, B, and C so prominently among ion transport mediating peptides. A unique helix,[10] possible only on the grounds of an alternating pattern of L and D configurations in all analogs enables the gramicidins A, B, and C to form transmembrane channels.[11]

As far as they are accessible today—valine-gramicidin A, B, and C—the synthetic analogs display in every respect the biological properties[7,8] of their natural counterparts. This may also be said with regard to the most recently explored (biological) property, namely the ability of the

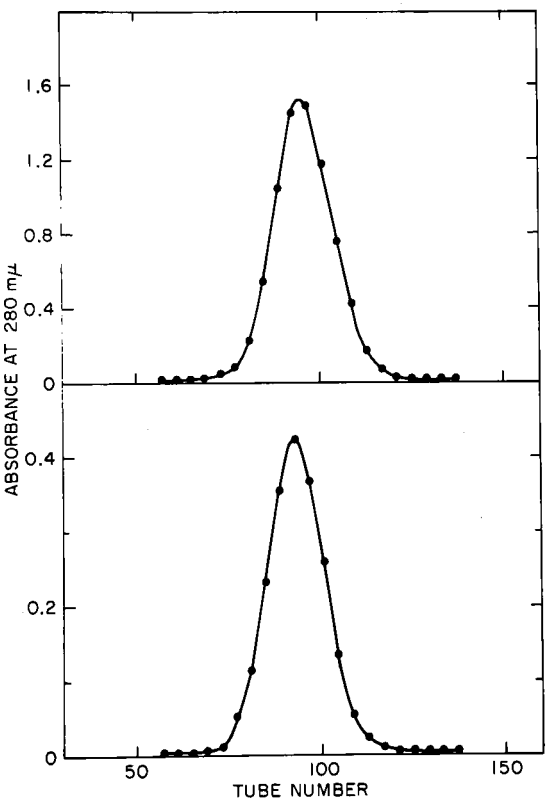

Figure 2: Countercurrent distribution of synthetic gramicidin A.[7] Upper panel: first redistribution. Lower panel: 1:1 mixture of natural and synthetic gramicidin A. Solvent system: benzene:chloroform:methanol:water = 15:15:23:7.

gramicidins A, B, and C to form transmembrane channels, thus promoting ion transport. In artificial membranes (1% glycerol monooleate in n-decane; 1 *M* NaCl) the single channel conductance of natural and synthetic gramicidin B agrees very well[12] (Figure 3).

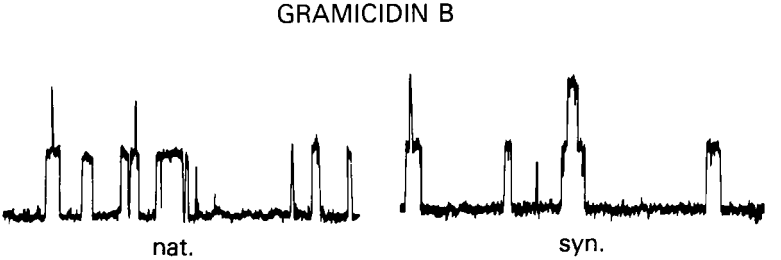

Figure 3: Single-channel conductance of natural (nat) and synthetic (syn) gramicidin B in an artificial membrane (1% glycerol monooleate in n-decane; 1 *M* NaCl). Ordinate: current; abscissa: time.

Subtilin and Nisin

Identical COOH-terminal sequences, dehydroalanyllysine, were established for subtilin and nisin[13] in 1969. At that time we knew that both peptides had in common the presence of lanthionine and β-methyllanthionine (Figure 4). We asked this question: if one of the two molecules contains these thioether amino acids and α,β-unsaturated amino acids are present in it,[14] will the latter also be seen in the other? The answer is yes, as we saw already in part. Moreover, the number of α,β-unsaturated amino acids is the same for subtilin and nisin, each molecule containing three, namely two residues each of dehydroalanine and one residue each of dehydrobutyrine.[15]

The dehydroalanine residue in the penultimate positions was first seen in nisin,[16] fortunately on the account of pursuing a major constituent observed in efforts aimed at the purification of the peptide.[16] This minor component in the preparation of nisin isolated from Streptococcus lactis[17] is pyruvyllysine, the product derived from the COOH-terminal sequence dehydroalanyllysine subsequent to the addition of water across the double bond of dehydroalanine and the concomitant formation of amide[16] (Figure 5).

The presence of α,β-unsaturated amino acids in subtilin and nisin imparts unique physical properties on the two molecules. These features are reflected to some extent in

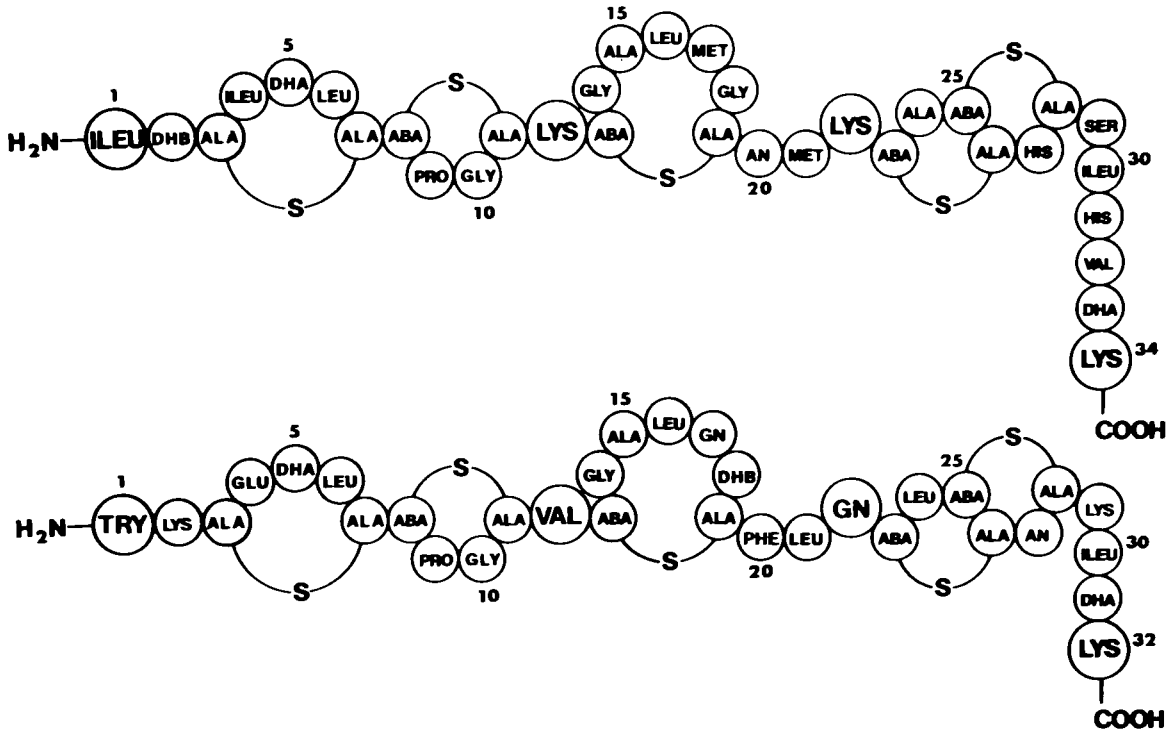

Figure 4: The structures of nisin and subtilin. ABA = aminobutyric acid; DHA = dehydroalanine; DHB = dehydrobutyrine (β-methyldehydroalanine); ALA-S-ALA = lanthionine; ABA-S-ALA = β-methyllanthionine.

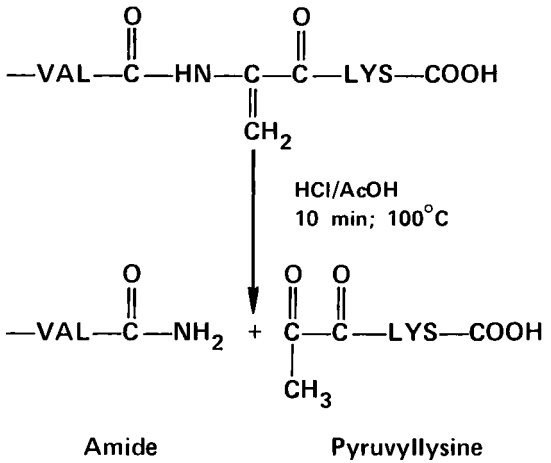

Figure 5: The release of pyruvyllysine from nisin. The dehydroalanine residue in the penultimate position of the molecule, upon treatment under acidic conditions, is converted to amide and the keto acylfunction.

the countercurrent distribution patterns (Figure 6). We believe that what we see here is the result of constantly shifting equilibria between α,β-unsaturated amino acids on the one hand and amides and keto acids on the other (Figure 7).

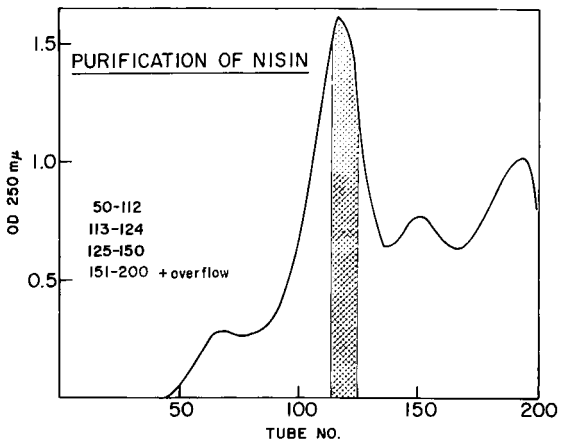

Figure 6: Countercurrent distribution of nisin. Solvent system: water:n-butanol:glacial acetic acid = 4:3:0.5.

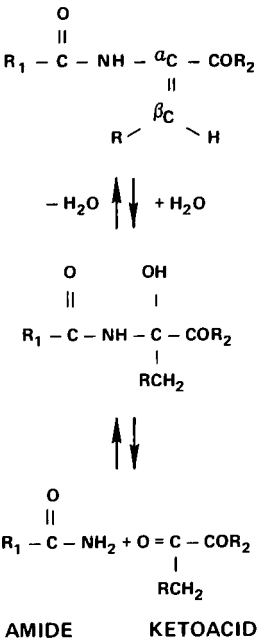

Figure 7: The interaction between α,β-unsaturated amino acids and amides and keto acids.

One may redistribute the material represented by the area shaded (Figure 6) and still continue to observe the discrepancy between the experimental and theoretical curve (Figure 8). Frankly, this problem is going to stay with us, as long as we allow for the presence of α,β-unsaturated amino acids in nisin. As long as countercurrent distribution[9] reveals these facts to us, we are by far much better off, knowing the chemically dynamic molecule than believing in something that bypasses reality.

These dynamics of chemistry, seen for instance in nisin, gave rise, however, to a somewhat more aggravating situation in the past. The molecular weight of nisin kept ranging from 3000 to 12,000[18] and more.

One day when Dr. Craig visited NIH and came, as he used to do frequently, to our laboratory, we discussed this question about the molecular weight. In his typical manner, he said: "Why don't you do...?" "Why don't you do what?" "Do a partial substitution!" This technique of molecular weight determination had proved its utility in the case of bacitracin.[19]

This suggestion was quickly put to work and within a very brief period of time a monodinitrophenylated derivative of nisin was purified by countercurrent distribution[9] (Figure 9) and a molecular weight of 3500 calculated for nisin.

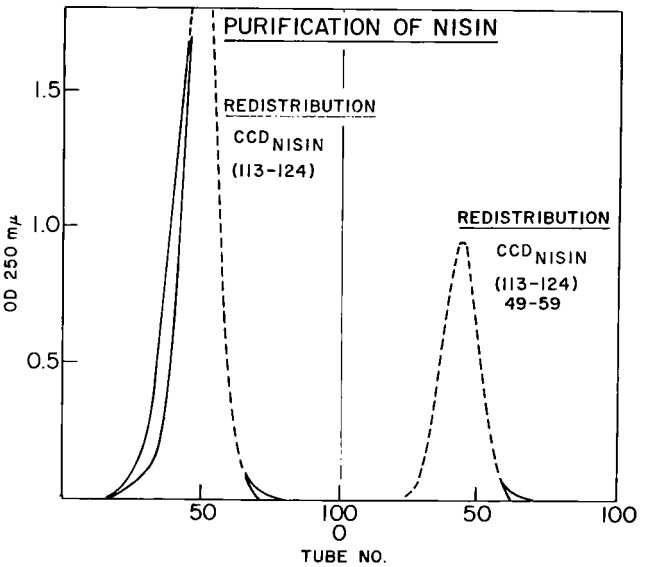

Figure 8: Countercurrent redistribution of nisin. Solvent system: water:n-butanol:glacial acetic acid = 4:3:0.5. Cf. the discrepancy between the experimental and theoretical curve (pattern to the left); multiples of the quantity represented by the curve in the right-hand pattern upon redistribution gave patterns reminiscent of that seen in Figure 6.

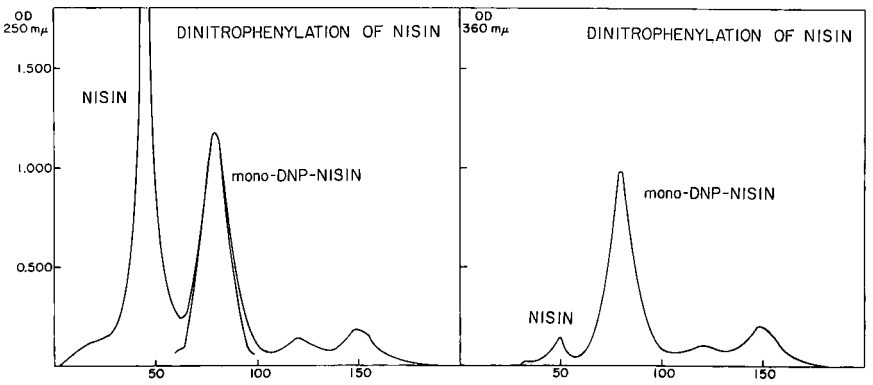

Figure 9: Countercurrent distribution of monodinitrophenylated nisin. Solvent system: water:n-butanol:glacial acetic acid = 4:3:0.5.

The higher molecular weights determined earlier for nisin[18] were the reflections of the chemistry intrinsic to the molecule (*vide supra*). Dimers, trimers, and higher polymers had been seen as the result of the formation of amide and keto acid from the α,β-unsaturated amino acids. Reversing this reaction and with the loss of water, α,β-unsaturated amino acids are regenerated, now, however, intermolecularly and linking two or more molecules of nisin.

The Biological Action of Peptides with α,β-Unsaturated Amino Acids

One working hypothesis for the biological action of peptides with α,β-unsaturated amino acids calls for the addition of essential sulfhydryl groups across the double bond in α,β-unsaturated amino acids. When this hypothesis was tested, it was found that nisin and nisin fragments show biological activities such as these: (a) they display antimalarial activity,[16] presumably by depriving the parasite of the necessary supply with coenzyme A through the host organism; (b) they cause the *in vitro* release of lysosomal enzymes, presumably by interacting with sulfhydryl group-dependent proteins and/or enzymes located in the organellar membrane; (c) they induce *fetal resorption* and/or inhibit implantation of the ovum[20] via mechanisms as yet not adequately studied.

At present, there are known to occur naturally the α,β-unsaturated analogs of approximately ten of the commonly found amino acids. The majority of these α,β-unsaturated amino acids have been isolated from microbial sources.[21] In a few instances, however, α,β-unsaturated amino acids have also been seen in plants[22] and, in at least one case, in a mammalian organism.[23]

α,β-Unsaturated amino acids must be considered to be rather cryptic when it comes to revealing their presence in peptides and/or proteins. This makes their detection sooner difficult than easy. In the course of the widely employed acid hydrolysis of peptides and proteins, α,β-unsaturated amino acids are converted to amides (with the subsequent release of ammonia) and keto acids (*vide supra*). Chemically, the α,β-unsaturated amino acids are most readily apprehended in the form of their mercaptan addition products,[16] *e.g.*, those of benzyl mercaptan,[24] which are easily identified and quantified in amino acid analysis.

α,β-Unsaturated amino acids may well be of greater physiological significance than is presently appreciated. The chemistry intrinsic to this class of compounds estab-

lishes links to a number of physiologically significant molecules and thus to vital metabolic pathways. The conversion to amides and keto acids lays open, via the latter ones, the route to and participation in transamination reactions. The earlier reaction is reversible, thus opening avenues to the reconstitution of α,β-unsaturated amino acids and/or access to α,β-unsaturated amino acids *per se* (*i.e., via* the *de novo* synthesis from amide and keto acid). In the latter reaction we ought not fail to recognize the potential for the biological formation of the peptide bond.

Another type of interaction central to which we see the α,β-unsaturated amino acids are the elimination reactions of β-substituted amino acids. Serine, threonine, and cysteine (or cystine), once suitably modified for β-elimination, are likely precursors of dehydroalanine and dehydrobutyrine, the α,β-unsaturated amino acids that are of concern to us today.

The elimination reactions indicated are reversible in principle. When we observe the reverse reactions, such as those between cysteine and dehydroalanine and/or dehydrobutyrine, we witness the formation of lanthionine and β-methyllanthionine. These events are steps in the proposed biosynthesis of nisin and subtilin.[25] The addition of the cysteine sulfhydryl group across the double bonds of dehydroalanine and/or dehydrobutyrine appears to be stereospecific, as we observe the D-configuration at all α-carbon atoms of one of the alanine moieties in the lanthionine residues and the α-aminobutyric acid moieties in the β-methyllanthionines[5,26] (Figure 4).

With this consideration, we may conclude this discussion and state that different classes of peptides with α,β-unsaturated amino acids have already emerged.

In one class we find the isolated occurrence of α,β-unsaturated amino acids and no obvious relationship between other existing structural features and α,β-unsaturated amino acids (cf. ref 21 for a list of naturally occurring peptides with α,β-unsaturated amino acids). Into a second class we must place peptides, such as nisin and subtilin, in which we observe the presence of dehydroalanine and dehydrobutyrine—however, also in the form of lanthionine and β-methyllanthionine, the addition products of cysteine to the unsaturated amino acids.

Recently, peptides have been seen with the products of different nucleophilic additions across the double bond of dehydroalanine. In two peptides, known as cinnamycin and duramycin and isolated from Streptomyces strains,[27,28] *lysinoalanine* has been found for the first time to occur

naturally.[29,30] The addition of the $^{\epsilon}NH_2$-group of a lysine residue across the double bond of dehydroalanine is believed to be the reaction leading to the formation of lysinoalanine.

The exposure to the chemistry of α,β-unsaturated amino acids has been most rewarding. The experience gained in the course of the *analysis* of peptides with dehydroalanine and dehydrobutyrine has been successfully applied to peptide *synthesis*, where dehydroalanine resins[31] function as convenient intermediates for many of the biologically important peptide amides.

Much of the work reported here was profoundly influenced by Dr. Lyman C. Craig. You have also seen the lasting impact of Dr. Craig's technique of countercurrent distribution[9] on this work. He will always be remembered for this—Dr. Lyman C. Craig, a great scholar and mentor and a very fine friend.

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CHEMICAL INVESTIGATIONS OF THE FUNCTION OF HEMOGLOBIN FROM A STRUCTURAL VIEW

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THE WORK I WILL DESCRIBE HERE was initiated as an attempt to use chemical methods to determine some of the conformational features of hemoglobin in solution. The plan was to react hemoglobin with various reagents and determine the relative reactivity of groups that were near the surface of the molecule. The rate constants for the reaction of reagents such as FDNB with specific amino acid residues would be compared to the rate of reaction with small model compounds. I reasoned that those groups which were freely accessible to the solvent would have reaction rates comparable to the rates for similar groups on small molecules, while those that were partially buried would react with a slower rate.

From some initial experiments using FDNB it soon was apparent that the most reactive group on hemoglobin using this reagent was the α-amino group of the α chain. Rhinesmith *et al.*[1] had shown previously that when Dnp-hemoglobin was treated for a brief period with HCl at 100° Dnp-Val-Leu was released from the amino terminus of the α chain. This fact was used to develop a quantitative assay for the extent of reaction of this group with FDNB. Since FDNB only reacts with uncharged amino groups the apparent rate constant for the reaction of an amino group with this reagent at a particular pH, k_H, is a function of the rate constant towards the uncharged group, k_o, the acid dissociation constant for the group, K, and the hydrogen-ion activity, a_H.

$$k_H = k_o\left[K/(K+a_H)\right]$$

By determining k_H at a series of pH values, I have calculated that the pK of this group in CO-hemoglobin is 6.72 and that the pH-independent rate constant, k_o, is 0.236 $sec^{-1}M^{-1}$. Surprisingly, the pK of the α-amino group of the model compound Val-Leu-NH_2 was one pH unit higher than this, 7.65, and k_o for the model was 0.062 $sec^{-1}M^{-1}$, one fourth the value for the amino terminus of the α chain.[2] Thus, my expectation that the pH-independent rate constant could be used to judge the accessibility of a group on the surface of a protein proved to be invalid; but I became curious as to why the pK of this group should be abnormally low.

It has been known for a long time that there are four groups on hemoglobin whose pK changes from 6.84 to 7.84 when hemoglobin is deoxygenated.[3] Since the titration curves of CO-hemoglobin and O_2-hemoglobin are identical[4] this same shift must occur when the CO is removed from CO-hemoglobin. It seemed possible that the α-amino groups of the α chain could account for two of these four oxygen-linked groups. If this is the case, the pK of these α-amino groups should shift from the abnormal value of 6.72 in CO-hemoglobin to a value similar to that of the amino group of Val-Leu-NH_2 when the CO is removed. To test this I repeated the experiments described above with deoxy-hemoglobin and found that the pK of the α-amino groups of the α chains had shifted to a value of 7.71±.02. Thus half of the Bohr effect can be attributed to these α-amino groups. Kilmartin and Rossi-Bernardi[5] have arrived at this same conclusion using a different method.

This phenomenon--the shift in pK of the amino groups linked to ligand binding to the hemes--could be accounted for if a positively charged group were located near the amino terminal valine in CO-hemoglobin and then moved 5 to 10 Å away as a result of the conformation change that occurs when the ligand is removed from the heme. From X-ray diffraction studies, Pertuz[6] has shown that in crystalline hemoglobin the amino terminal valine of the α chain is very near the carboxyl terminal arginine of its partner α chain. If this is the case, then removing the carboxyl-terminal arginine should restore the pK of the α-amino group to a normal value even when the heme group is liganded. In support of this idea, Antonini *et al.*[7] have shown that the Bohr effect of hemoglobin treated with carboxypeptidase-B in such a way as to remove not only the carboxyl-terminal arginine but the penultimate tyrosine and other residues as well, possessed a greatly reduced Bohr effect. In order to determine the effect of the carboxyl terminal arginine, chromatographically purified CO-hemoglobin was digested with

carboxypeptidase-B under conditions in which only trace amounts of residues other than arginine were removed and then the resulting dearginine-CO-hemoglobin was separated from the undigested hemoglobin by ion-exchange chromatography on SE-Sephadex using a 0.01 *M*, pH 7.7, phosphate buffer. The 0.1 *M* pH 6.46 phosphate buffer system normally used to chromatographically purify the hemoglobin used in these studies would not separate dearginine-hemoglobin from hemoglobin. When the pK of the α-amino group of the α chain of dearginine-CO-hemoglobin was determined in the same way as described above, it was found to be 7.42±.04, a value close to the normal pK for an α-amino group.

Although these data are consistent with a mechanism for the Bohr effect in which the guanido group of the carboxyl-terminal arginine of one α chain influences the pK of its partner's α-amino group during oxygenation, Perutz *et al.*[8] have not observed a shift in this direction in the positions of these groups in crystals of oxy- and deoxy-hemoglobin. Possibly the conformation of this region of hemoglobin is arranged differently in solution than it is in the crystal. The crystallographic results[6] indicate that the amino terminal residues of the two α chains are approximately 12 Å apart. This is the distance between the reactive groups of the cross-linking reagent p,p'-difluoro-m,m'-dinitrodiphenylsulfone. In order to determine if this relationship between the amino groups is maintained when hemoglobin is in solution, the product of the reaction of CO-hemoglobin with this reagent was studied.[9] Molecules that were cross-linked between subunits were separated from unreacted and noncross-linked molecules by gel filtration on Sephadex G-100 in the presence of 1 *M* $MgCl_2$, a reagent known to dissociate hemoglobin into αβ subunits.[10] The cross-linked hemoglobin was then hydrolyzed briefly with HCl to release p,p'-di(Val-Leu)-m,m'-dinitrodiphenylsulfone. This is the reaction product expected if the α-amino groups of the α chains had been cross-linked. Since 70% of the reaction of the cross-linking reagent with hemoglobin was found to occur at this site, it was clear that a 12 Å distance between the amino-terminal ends of the α chains could be accommodated in solution. One possibility of obtaining this result is that the distance between the amino-terminal ends is not rigid but that they are free to move in solution. If this is the case, mechanisms that required them to be in a fixed position would have to be discarded.

In order to better define the spatial relationship between the α-amino groups, hemoglobin was reacted with

1,5-difluoro-2,4-dinitrobenzene, a cross-linking reagent with a distance of only 6 Å between its reactive centers. Gel filtration on Sephadex G-100 in the presence of 1 *M* $MgCl_2$ revealed that the reaction of this reagent with CO-hemoglobin also produced a product that could not be dissociated into αβ subunits in 1 *M* $MgCl_2$.[11] However, when the reacted hemoglobin was hydrolyzed with HCl and the reaction product isolated it was found to be identical with synthetic FDnp-VAL-Leu rather than the expected cross-linked product 1,5-di(Val-Leu)-2,4-dinitrobenzene. Thus, although this reagent can react readily with the α-amino groups, the two amino-terminal residues are not free to move as close together as 6 Å. Apparently, the reason the noncross-linked reaction product will not dissociate in 1 *M* $MgCl_2$ is that to do so would require that the phenyl ring of the reagent would have to be exposed to the solvent. Neer and Konigsberg[12] have reported that Dnp-hemoglobin, in which the α-amino groups have been reacted, also does not dissociate in $MgCl_2$ and other solvents which readily dissociate unreacted hemoglobin.

These studies, using a chemical approach, suggest that the conformation in solution around the amino- and carboxyl-terminal ends of the α chains is the same as is seen in the crystal. They have also yielded information that cannot be obtained from crystals about how the acid dissociation properties of the α-amino groups change on ligand binding to the heme. However, the conclusion drawn over ten years ago by Guidotti and Konigsberg is still true: "Only a compilation of endless empirical observations of this kind involving many different residues can provide a fragmentary and tentative outline of the tertiary structure of hemoglobin in solution as compared with the molecule in the crystalline state."[13]

Acknowledgments

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PROTEIN-NUCLEIC ACID INTERACTIONS

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THE PROSPECT OF WORKING WITH DR. CRAIG 20 years ago was a source of great excitement to me. After being in an organic chemistry lab at Columbia as a graduate student, the luxurient facilities at Rockefeller inspired me with awe and energy.

As a mentor, Dr. Craig inspired enthusiasm and dedication by his own intense interest and participation in the experimental work of the laboratory. He channeled his energy and knowledge into solving problems that I sometimes found frustrating or intractable. He taught by example, rather than by exhortation. His intuitive approach to a problem, and his ability to design simple, practical experiments to test alternative hypotheses, served as a model which helped mold my outlook on research.

As a man, Dr. Craig was understanding and sensitive to feelings of his students and colleagues. He displayed the kind of wisdom that comes only from successful integration of the rational and emotional components of a complete human being.

Principal emphasis in his lab at that time was on developing separation methods which proved invaluable in our efforts to determine the structure of human hemoglobin. Interest in protein structure and function has continued to provide the underlying theme of much of my research since then, and I will tell you today about our attempts to understand certain features of protein-nucleic acid interactions. In particular I will talk about our efforts to produce covalent linkages between single stranded DNA and proteins that are known to bind specifically to single, rather than double stranded forms of DNA. The system chosen for study

was derived from the filamentous bacteriophage fd, which was originally isolated in 1961 by Leob and Zinder.[1] During the course of infection of *E. coli* by this phage, a small virus-specific protein, called the gene 5 protein, of molecular weight 10,000 is synthesized and binds to the single stranded form of fd DNA prior to its being packaged within the intact virus particle. The precise role of the gene 5 protein in the life cycle of the virus is not fully understood. Since it behaves like a DNA unwinding protein, in that it has a high affinity for single stranded DNA and binds to DNA in a cooperative fashion, it may serve an important function in the replicative process that generates the viral strand. It could prevent the single strand from being used as a template for the formation of the RF double-stranded intermediate. Gene 5 protein could also protect the single viral strand from digestion by nucleases which are present in the cell.[2] When *E. coli* is infected with the wild-type fd phage, there are about 100,000 copies of gene 5 protein produced per cell.[2] When complexes of gene 5 protein are formed with single stranded DNA, one molecule of gene 5 protein covers 4 DNA bases.[3] This means that a saturated complex containing single stranded DNA and gene 5 protein would have 1500 protein molecules per DNA circle.

Electron microscopy of fd DNA-gene 5 protein complexes isolated from the infected cell or formed *in vitro* are quite similar, in that they both appear as rods indicating a collapsed circle thick enough to accomodate two strands of DNA each covered by protein.[4] In this respect they differ from complexes of other DNA binding proteins such as the *E. coli* unwinding protein or the gene 32 protein from T4, where the DNA remains in the open circular form in the complex.[5]

We have determined the amino acid sequence of the gene 5 protein. There are 11 basic residues which are clustered near the amino and carboxyl ends of the molecule. There is no tryptophan but there are five tyrosine residues and one cysteine residue. Chemical modification, performed by Richard Anderson in Dr. Coleman's laboratory, has shown that all the lysine residues are accessible to reaction with acetyl imidazole and that the acetylation of lysines prevents complex formation with DNA. When the protein was treated with tetranitromethane, three of the five tyrosine residues were modified; however if gene 5 protein was first complexed with the DNA, none of the tyrosines were nitrated.[5] The nitrated tyrosines were located at positions 26, 41 and 50 in the protein which has a total of 87 residues.[6] Anderson and Coleman also prepared gene 5 protein that contained ^{19}F

fluorotyrosine residues. NMR spectra of this material when complexed with tetranucleotides showed upfield shifts in the ^{19}F resonances of the three surface tyrosines, a result compatible with intercalalation of the aromatic rings between the stacked nucleotides.[6]

With this background about the gene 5 protein, I would like to turn now to our studies on the UV-induced cross-linking of gene 5 protein and fd DNA.

The reason for attempting to use a photo-induced reaction to link the gene 5 protein to DNA was to try to get more information about the regions of the protein that were in close proximity to the DNA, the assumption being that only those residues in contact with the DNA bases would have a chance of being linked. Clearly, X-ray crystallography would provide the most detailed information about the nucleic acid protein interactions but crystallization of a gene 5 protein polynucleotide complex has not been successful as yet. The possibility that UV light could be used to link proteins and nucleic acid was hinted at a long time ago when it was observed that the recovery of DNA-protein complexes, after phenol extraction, was markedly reduced after irradiation of the complex with UV light.[7] Recently, UV-induced cross-linking has been used to generate covalent bonds between the *E. coli* lac repressor and BrdU-substituted lac operator DNA but the nature of the linkage has not been determined.[8] Encouraged by these observations we were tempted to see if we could use the same technique to link the gene 5 protein to DNA.

Accordingly, we irradiated a saturated, *in vitro* complex of gene 5 protein and ^{32}P labeled fd DNA, using an ordinary 15 W GE germicidal lamp with its main energy output at 253 nm, providing a dosage of 476 erg/mm^2/sec. The solution was exposed to the UV light for various times, aliquots taken, extracted with phenol and the aqueous phase counted for ^{32}P to see how much uncross-linked DNA remained. As shown in Figure 1, the rate of reaction is rapid, as measured by the loss of water soluble, ^{32}P labeled DNA. The rate of reaction was independent of the absolute concentrations of fd DNA and gene 5 protein, providing that enough gene 5 protein was used to form a saturated complex. On the other hand, when photolysis was carried under dissociating conditions such as 1 *M* NaCl, the amount of DNA remaining in the aqueous phase was dependent on the absolute concentration of protein and DNA. Thus, in the first instance, where there is a tight complex formed, the rate is zero order, while in the second case the reaction is second order. Various controls were run. For instance, photolysis of ^{32}P

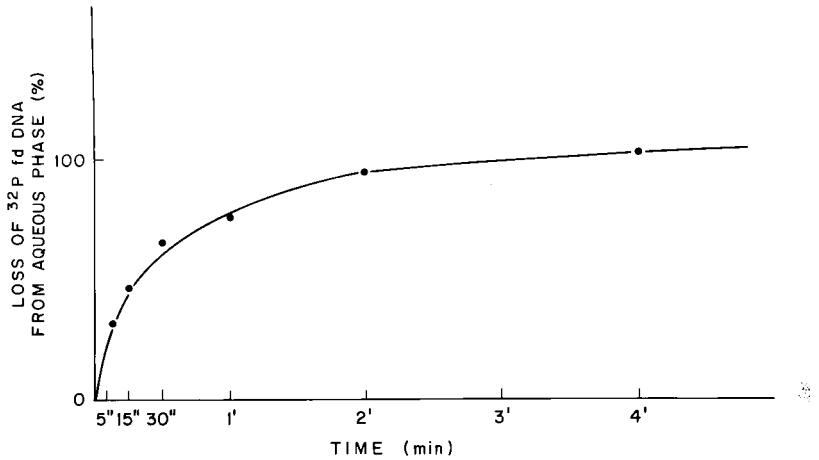

Figure 1: Time course for the reaction of gene 5 protein and ^{32}P-labeled fd DNA. The concentrations of the reactants were: gene 5 protein, 0.2 µg/ml; ^{32}P-labeled fd DNA, 0.7 µg/ml. Samples for irradiation (0.1 ml) were placed 6 cm from the center of a 15 W (GE) germicidal lamp, main output 253 nm. The dosage of UV light was 476 erg/mm^2/sec. For details of the phenol extraction procedure, see reference 9.

labelled fd phage, fd DNA alone or with bovine serum albumin did not result in any significant loss of ^{32}P DNA from the aqueous phase. We also irradiated ^{32}P-labeled Qβ and R17 bacteriophage but no cross-linking could be observed. The remarkable fact about the reaction of gene 5 protein and fd DNA was that it occurred without the usual requirement for the presence of BrDU in the DNA. In the case of the UV-induced cross-linking of the lac repressor and the lac operator, no reaction occurred unless BrDU was present in the DNA.[8]

In the gene 5 protein-fd DNA system we decided to get more information about the photolytic reaction using several approaches, our objective being to find out if a covalent bond was actually formed between the gene 5 protein and the fd DNA. We wanted to establish the stoichiometry of the reaction. We also wanted to determine which amino acid residue and which of the DNA bases had reacted. We started out by mixing ^{14}C-labeled gene 5 protein and ^{32}P-labeled fd DNA, using more protein than required to form a saturated complex. Half of the sample was irradiated for 15 min and

the other half was not. Both portions were then sedimented on a 5-20% linear sucrose gradient in 0.6 *M* NaCl, conditions sufficient to dissociate most of the non-covalently bound DNA from the protein.

The results in Figure 2c show that about 20% of the gene 5 protein cosediments with the fd DNA after photolysis. At this dosage of UV light some of the phosphodiester linkages are broken as shown by the sedimentation pattern of

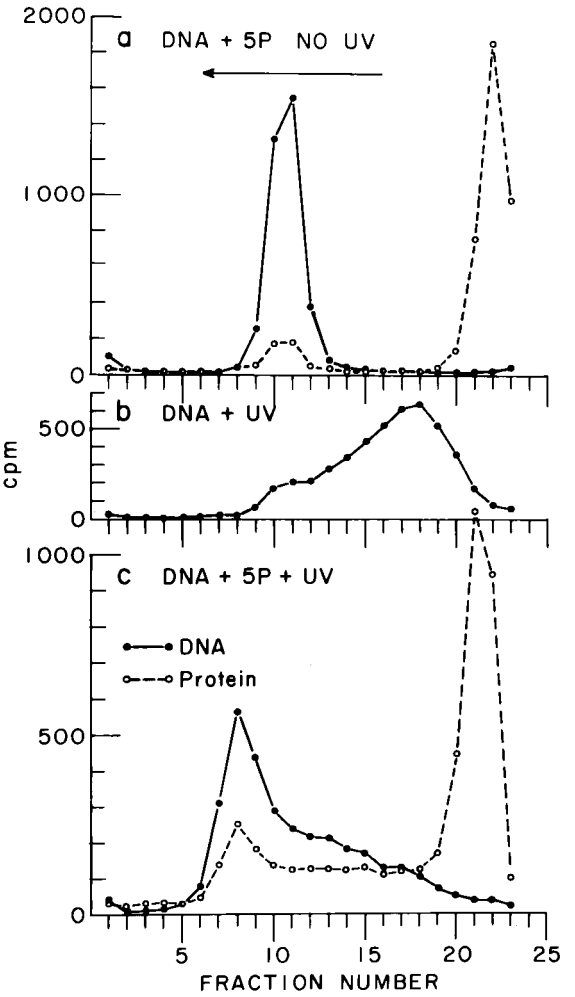

Figure 2: Sucrose density gradient sedimentation of the gene 5 protein-fd DNA complex and fd alone, with and without irradiation. For details of the experimental procedure, see reference 9.

fd DNA alone (Figure 2b). This accounts for the trailing observed in Figure 2c. Having demonstrated that the protein would cosediment with DNA, we wanted to see whether any DNA fragments would have the same mobility as the gene 5 protein in SDS polyacylamide gel electrophoresis. For this purpose we used ^{3}H-thymidine-labeled fd DNA, excess cold gene 5 protein, exposed the complexes to UV light, added excess cold fd DNA to allow exchange of the non-covalent bound protein, digested with DNAse to produce small fragments, then submitted the mixture to electrophoresis in polyacrylamide gels in SDS. As a control, ^{3}H-fd DNA was irradiated in the presence of bovine serum albumin and then submitted to the same treatment. The results (Figure 3)

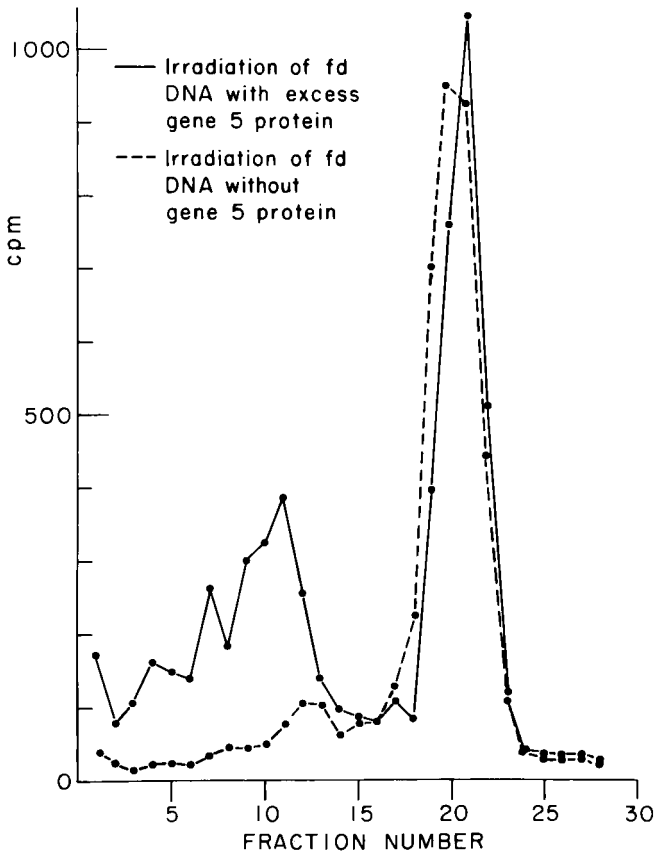

Figure 3: SDS acrylamide gel electrophoresis of the gene 5 protein ^{3}H-fd DNA complex after UV irradiation and treatment with deoxyribonuclease. For details of the experimental procedure, see reference 9.

show that 50% of the ^{3}H-thymidine-labeled DNA was retarded in the gel at positions approximately corresponding to the position of the gene 5 protein itself.

Finally, we have attempted to determine the chemical nature of the cross-link. To determine which amino acid residues were involved, we used the approach shown in Figures 4 and 5. The saturated gene 5 protein, ^{32}P-labeled fd DNA complex was irradiated for 2 minutes, treated with SDS, and filtered through Sephadex to remove unattached protein. The material emerging at the front of the column was treated with DNAse 1 and micrococcal nuclease to split the DNA into small pieces. Perchloric acid was used to precipitate the protein attached to DNA. The precipitate was then digested with a mixture of trypsin and chymotrypsin, filtered through Sephadex G-50 and the radioactive fraction submitted to paper electrophoresis at pH 1.9. A ^{32}P-containing band associated with a hexapeptide was eluted, treated with aminopeptidase M and pronase, then subjected once more to paper electrophoresis. This time, only cysteine could be found in ^{32}P-containing bands. Since the association of cysteine and the DNA fragment persisted, when electrophoresis was carried out at several different pH's, we concluded that cross-linking occurred via the

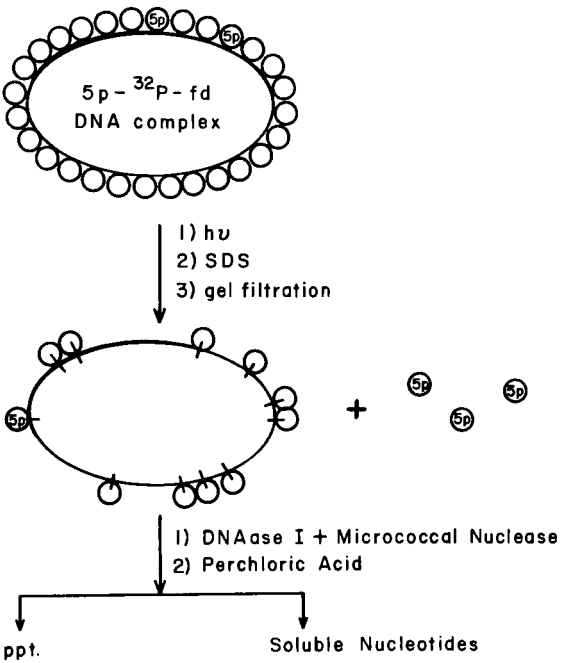

Figure 4: Scheme for the cross-linking of gene 5 protein and fd DNA.

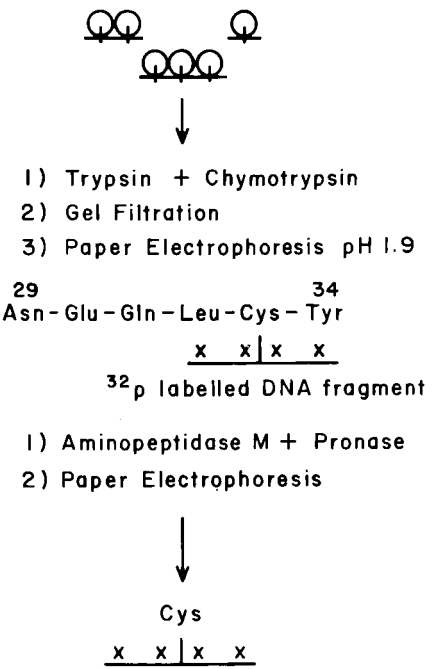

Figure 5: Scheme for the isolation of the amino acid residue linked to the DNA.

SH group of cysteine. In a preliminary attempt to determine which base or bases could react, we irradiated four deoxynucleotide homopolymers in the presence of ^{14}C-labeled gene 5 protein as a model for what might happen in the real case. We found that 20% of the input protein was linked to poly dT, 5% to poly dA, 5% to poly dG and none to poly dC, using labels in each of the four bases in four separate experiments to see if one or more are involved in the reaction. To do this we have to digest the complex to the point where only one base is attached to the protein. Since we are reasonably sure that UV-induced cross-linking, in this system, can occur *in vitro*, we wanted to find out if the reactions could also occur *in vivo*, so we irradiated fd-infected *E. coli* cells and showed that we could isolate the cross-linked complexes. This suggests that we could use this photolysis procedure to look for single stranded regions of DNA in fd-infected cells, under various conditions. It might be possible, for instance, to stabilize and isolate recombination intermediates in cells infected

first with phage λ followed by fd. This method of photolysis might also permit the isolation of single stranded regions of coli or plasmid DNA during replication or transcription.

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SECTION II

CONFORMATIONAL STUDIES

THE STATE OF THE ART OF X-RAY CRYSTALLOGRAPHY OF PEPTIDES

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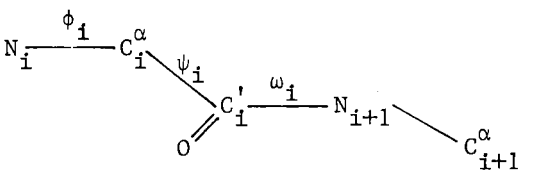
THE GEOMETRY OF THE PEPTIDE UNIT, C_i^α to C_{i+1}^α in



that is, the bond lengths, bond angles and the value of ω_i (near 0°, *cis*, or near 180°, *trans*), is known to a good approximation. When several peptide units are joined to each other, the molecules have the property of being very flexible since rotations can occur about each $N_i—C_i^\alpha$ bond and the adjacent $C_i^\alpha—C_i'$ bond where the torsional angles ϕ_i and ψ_i are allowed to assume a range of values. Several questions arise: Is there one conformation that is more stable than any other? Are there a number of equivalently stable conformations? How is the conformation influenced by solvent molecules and polar ions? Are there conformational features that are common from one substance to another?

X-ray diffraction analysis of single crystals can provide the answer to a number of these questions. The advantage of this method of analysis is that each atom is located accurately. From the coordinates of the atoms, the exact values of the conformational angles can be calculated, as well as bond lengths, bond angles, hydrogen-bond

separations, and nearest approaches between nonbonded atoms, so that the exact geometry of the molecule in the solid state is established. Peptides, however, are chemically or biologically active in solution. It has been shown that some peptides in solution assume a number of conformations. Other means of analyses, particularly spectroscopic, coupled with energy calculations, are necessary complementary tools. Nevertheless, a knowledge of the conformation in the solid state is an excellent starting point.

What is the scope of analysis by x-ray diffraction and to what kinds of problems can it be applied?

A primary requirement is a good single crystal. Many peptides crystallize with the solvent and the crystal must be enclosed in a thin-walled, sealed capillary with some mother liquor to keep the crystal from drying and, consequently, disintegrating during the collection of x-ray data. Typical complications which must be avoided are intimate twinning and gross disorder. A minor twin or minor disorders, usually in the conformation of side groups such as the pyrrolidine ring or the isopropyl groups, can be tolerated if they do not limit severely the number of coherent diffraction intensities that can be measured. The solution of a crystal structure is dependent upon a large enough set of diffraction data.

Ease of solution depends upon several factors. For example, a heavy atom in the crystal, such as a K^+ or heavier, acts as a marker and greatly simplifies the problem. Molecules that contain a center of symmetry or other substances than can crystallize in centrosymmetric space groups, such as many macrotetrolides and polyethers, can be solved automatically with existing computer programs, principally because the phase values in the electron density expression are either 0 or π for the centrosymmetric space groups. For acentric space groups, the phases take on all values between 0 and 2π. Thus, the most difficult problems are those crystals that contain only light atoms and are non-centrosymmetric, a class to which peptides belong. The difficulty generally increases with the number of atoms in the asymmetric unit of the unit cell, since the crystals of larger molecules proportionally do not scatter x-rays as well as the crystals of smaller molecules.

The theory for determining phases directly from x-ray intensities, without using any structural information, was established by J. Karle and Hauptman[1,2,3] and the practical procedure for applying the phase formulas to experimental data is described by Karle and Karle.[4] The

largest light atom, non-centrosymmetric structure solved so far is that of valinomycin crystallizing with two independent molecules in a triclinic cell and containing 156 C, N and O atoms.[5]

Multiple Conformations

Curiously, one of the first applications of the direct method of crystal structure analysis was to the structure cyclic-hexaglycyl.[6] This structure demonstrated very graphically the flexibility of polypeptides. Among the eight molecules in the triclinic cell, there were four different conformers crystallized side-by-side in the same cell. Obviously, the energy of each conformation must be very similar. The four conformers occur in the ratio of 4:2:1:1 for forms (a), (b), (c) and (d), respectively.

A recent example in which more than one polypeptide conformer occurs in the same crystal is that of cyclic triproline. Details of this structure will appear in another section of this book.[7]

The conformations for cyclic-hexaglycyl are not unique to that substance. The compound, cyclic(Gly-Gly-Gly-Gly-D-Ala-D-Ala), has a conformation[8,9] very similar to the most populous form (a) of cyclic-hexaglycyl, the one that contains two intramolecular hydrogen bonds. In the c-4Gly-2D-Ala molecule, the two Ala residues are at the corners of one of the β-turns. In addition to the intramolecular bonds, there are hydrogen bonds to three H_2O molecules cocrystallized with the peptide.

Conformer (b) of c-$(gly)_6$ is mimicked by a tetrapeptide containing an 18-membered ring, cyclic(L-Leu-L-Tyr-δ-Avaler-δ-Avaler),[9] as shown in Figure 1. This compound has been reported to be an effective and unusual inhibitor of α-chymotrypsin, competetive with linear peptides like Ac-L-Leu-L-Tyr-OMe. The three additional CH_2 moieties in each δ-Avaler residue occupy the same space as another peptide unit, and thus the 18-membered ring has the same size and conformation as a hexapeptide. Conformer (b) does not have any intramolecular hydrogen bonds and in this molecule there is no internal hydrogen bond. In fact, the NH moiety that could be expected to participate in a H-bond across a β-turn, does not participate in any hydrogen bonding at all. All the other NH and CO moieties enter into hydrogen bonds with neighboring peptide molecules or the solvent molecules, $(CH_3)_2SO$ and H_2O, cocrystallized with the peptide,[10] Figure 2.

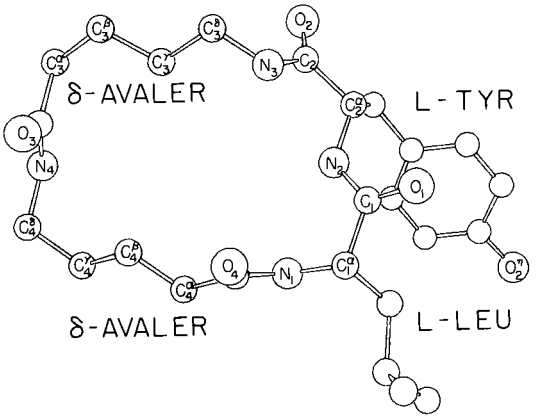


Figure 1: Conformation of cyclic(L-Leu-L-Tyr-δ-Avaler-δ-Avaler).

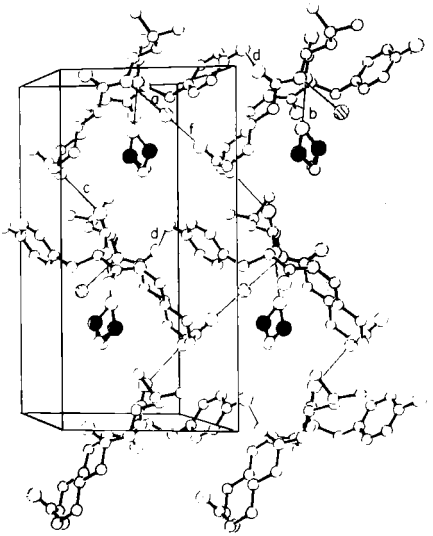


Figure 2: The crystalline packing of cyclic(L-Leu-L-Tyr-δ-Avaler-δ-Avaler) with solvent molecules $(CH_3)_2SO$ (the S atom, represented by ●, is disordered among two positions) and H_2O, represented by ⊛ . Intermolecular hydrogen bonds, a-f, are indicated by thin lines.[10]

The structures of only a few 18-membered ring peptides have been determined and there has not yet been an opportunity to find analogues of conformers (c) and (d). Ferrichrome A contains an 18-membered polypeptide ring of still another conformation[11] with only one intramolecular hydrogen bond. The backbone of the ring in ferrichrome A is compared to form (a) of cyclic-hexaglycyl in Figure 3.

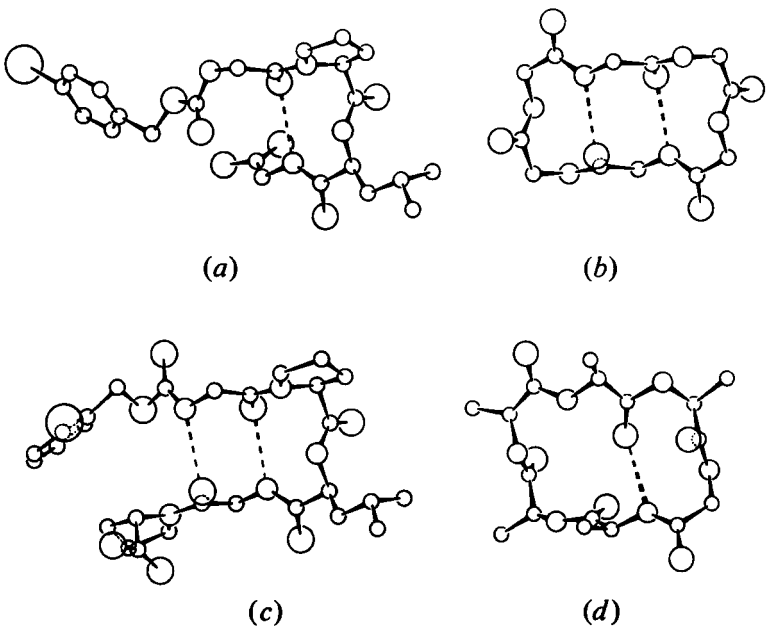


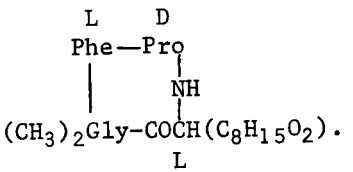
Figure 3: Computer drawings of molecules oriented to compare similar features. (a) p-Br carbobenzoxy-Gly-L-Pro-L-Leu-Gly·OH; (b) Form *a* of cyclic hexaglycyl; (c) o-Br carbobenzoxy-Gly-L-Pro-L-Leu-Gly-L-Pro·OH; (d) Backbone of the ring in Ferrichrome A. (From reference 12)

Also shown in Figure 3 are two linear peptides, p-bromocarbobenzoxy-Gly-L-Pro-L-Leu-Gly·OH[12] and o-bromocarbobenzoxy-Gly-L-Pro-L-Leu-Gly-Pro·OH,[13] a substrate of collagenase. Ueki *et al.*[12,13] have demonstrated that these linear peptides form a U-shaped bend and contain intramolecular hydrogen bonds in a conformation very similar to form (a) of cyclic hexaglycyl.

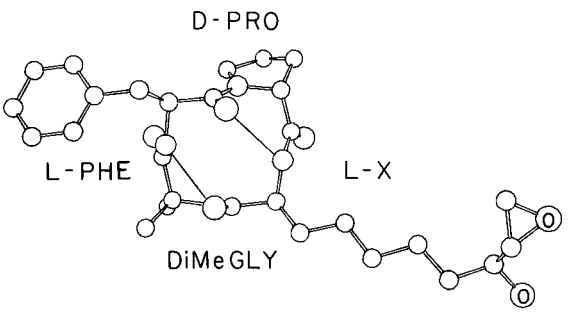
Intramolecular Hydrogen Bonds

Five different types of intramolecular hydrogen bond conformations have been observed and characterized in peptides in the solid state. They include seven-, ten- and thirteen-membered turns between NH and O=C, labeled 3⟶1, 4⟶1 and 5⟶1.

The 3⟶1 bond has been demonstrated in a crystal only recently in the natural tetrapeptide dihydrochlamydocin.[14]



The conformation of this cyclic tetrapeptide,[15] shown in Figure 4, is all *trans*, also a new observation for a cyclic



DIHYDROCHLAMYDOCIN

Figure 4: Conformation of dihydrochlamydocin. Two 3⟶1 hydrogen bonds are indicated by the light lines. The four peptide units assume the *trans* form.[15]

tetrapeptide. An earlier crystal structure investigation of the cylcic tetradepsipeptide, cyclic(D-HyIv-L-MeIleu-D-HyIv-L-MeLeu), showed that the two peptide groups assumed the *cis* conformation while the two ester groups

were in the *trans* form.[16] Similarly, in cyclictetrasarcosyl[17] the conformations of the peptide units alternate between *cis* and *trans*. One of the consequences of the different conformations, *i.e. trans cis trans cis* as compared to all *trans*, is that in the former all the carbonyl oxygen atoms are on the same side of the average plane of the ring and the side chains, non-polar in this example, are on the other side, while in the all *trans* conformation the carbonyl oxygens alternate above and below the average plane of the ring.

The all *trans* conformation in dihydrochlamydocin contains peptide units that are considerably less planar than have been observed in other peptides. The ω angles, which would be 180° for a planar structure, have the following values: $(CH_3)_2$Gly, 162°; L-Phe, -166°; D-Pro, 156°; L-X, -164°. The details of one of the two 3⟶1 bonds are illustrated in Figure 5 and the geometric parameters for both are listed in Table 1, Section A.

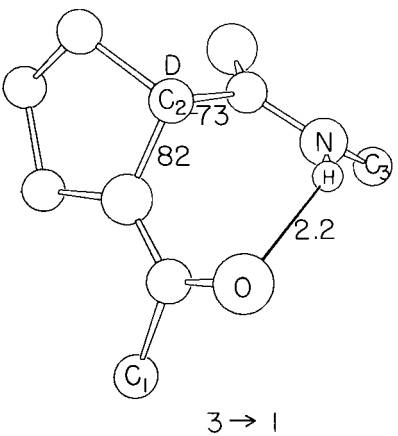


Figure 5: Details for one of the two 3⟶1 hydrogen bonds in dihydrochlamydocin.

Three different kinds of 4⟶1 hydrogen bonds have been found in crystalline peptides, namely *trans* I, *trans* II, and *cis* as illustrated in Figure 6 where the conformations have been drawn by computer[18] using the experimentally determined coordinates. Section B of Table 1 shows the substances in which the *trans* type I bond has been

Table I

Intramolecular NH···O Bonds

Substance	2	3	4	ϕ_2	ψ_2	ϕ_3	ψ_3	ϕ_4	ψ_4	N...O,Å	Ref.
A. 3⟶1 TRANS											
Dihydrochlamydocin	$(CH_3)_2$Gly			+72	-65					2.82[a]	15
	D-Pro			+82	-73					2.94[b]	
B. 4⟶1 TRANS, Type I											
c-hexaglycyl	Gly	Gly		-69	-30	-94	+11			2.96	6
	Gly	Gly		+69	+33	+92	-7			2.96	
c-4gly-2D-Ala	D-Ala	D-Ala		+66	+15	+131	-31			3.16	8
Li^+ antamanide	L-Ala	L-Phe		-69	-13	-84	-6			3.06	19
	L-Phe			-79	-13	-90	+8			3.00	
S-benzyl-L-Cys-L-Pro-L-Leu-Gly·NH_2	L-Pro	L-Leu		-66	-29	-115	+13				20
p-BrCarBz-Gly-L-Pro-L-Leu-Gly·OH	L-Pro	L-Leu		-58	-27	-104	+8			2.96	12
o-BrCarBz-Gly-L-Pro-L-Leu-Gly-L-Pro·OH	L-Pro	L-Leu		-65	-27	-105	+8			3.00	13
N-Ac-L-Pro-L-Lac-$NHCH_3$	L-Pro	L-Lac*		-55	-22	-81	-11			2.89	21
Tuberactinomycin O	L-Ser	=CHNHCONH$_2$								2.95	22
Viomycin	L-Ser	=CHNHCONH$_2$									23
C. 4⟶1 TRANS, Type II											
Ferrichrome A	L-Ser	Gly		-57	+132	+82	-1			2.98	11
L-Pro-L-Leu-Gly·NH_2	L-Leu	Gly		-61	+128	+72				3.04	24
N-Ac-L-Pro-D-Lac-$NHCH_3$	L-Pro	D-Lac*		-62	+140	+91	-8			2.97	25
valinomycin (Pl)	L-Val	D-Hyv*		-63	+129	+96	-3			3.07	5
	L-Val	D-Hyv*		-67	+130	+82	+3			2.90	
	D-Val	L-Lac*		+63	-134	-74	-6			2.86	
	D-Val	L-Lac*		+60	-135	-98	+14			2.99	
D. 4⟶1 CIS											
Ilamycin B_1	L-Try	L-MeLeu		-86	+117	-128	+99			2.80	26
	L-Ala	L-MeLeu		-61	+126	-121	+38			2.98	
E. 5⟶1 TRANS											
valinomycin (Pl)	L-Lac*	L-Val	D-Hyv*	-74	-6	-108	+78	+146	-11	3.13[c]	5
	D-Hyv*	D-Val	L-Lac*	+82	+3	+108	-69	-164	+23	2.99[d]	

Notes: [a] H··O = 2.1 Å, NH··O = 138°
[b] H··O = 2.2 Å, NH··O = 144°
[c] H··O = 2.3 Å, NH··O = 125°
*O replaces NH

Section B. Both L or both D or both Gly.
Section C. L,D or D,L or L, Gly.
Section D. Residue 3 N-substituted.

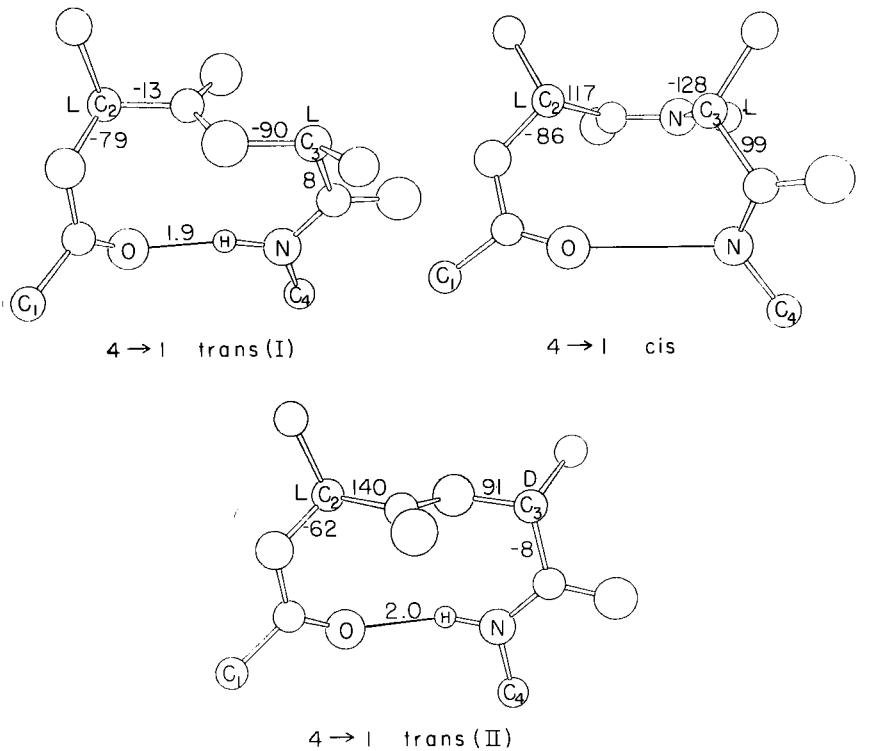


Figure 6: Examples of three types of 4⟶1 hydrogen bonds found in crystalline peptides. The figures were drawn by computer[18] using experimentally determined coordinates.

found thus far. Also are listed the peptide units at each corner and the values for the conformational angles (ϕ_2, ψ_2) and (ϕ_3, ψ_3) at each corner. It should be noted that each of the conformations shown in Figure 6 can have a mirror image if L peptide units are replaced with D, and vice versa. The effect of such a replacement is to change the signs on the values of the conformational angles. The numerical values for these angles are quite similar for all the substances, virtually independent of the side groups on C_2^{α} and C_3^{α}. For this type of bond, the peptide units at positions 2 and 3 are both L or both D or both Gly, except in viomycin[23] and its analogue, tuberactinomycin O[22], where C_3^{α} is a planar atom with a double bond to the side chain.

The type II *trans* 4⟶1 bond has occurred in substances with L, D or D, L or L, Gly groups at positions 2 and 3. The values for the conformational angles for these substances are even more consistent than for type I as illustrated in Table 1, section C. For both types I and II, the replacement of O for NH in residue 3 does not make any significant difference in the values of the conformational angles.

A third type of 4⟶1 hydrogen bond has a *cis* conformation between C_2^{α} and C_3^{α}. Two such bonds occur in ilamycin B_1 [26] where in each *cis* unit, the N atom has a CH_3 substituent.

Possible 5⟶1 hydrogen bonding, with a 13-membered loop, takes place in valinomycin crystallized from n-octane. A statement to that effect was published in a preliminary article on valinomycin crystallizing in space group $P2_1$ by Duax *et al.*[27]

A subsequent investigation of valinomycin[5] in space group P1,* crystallized from n-octane or from acetone, has shown that the two crystallographically independent molecules contained in the triclinic cell have very similar conformations. One of these molecules is illustrated in the stereodiagram in Figure 7. Except for three side

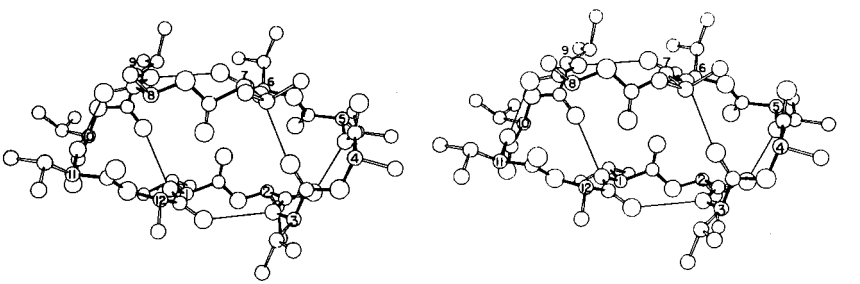


Figure 7: Stereodiagram of valinomycin crystallized from n-octane or acetone.[5]

groups which are methyl, rather than isopropyl, the molecule very nearly has a center of symmetry. Of the six NH groups available for hydrogen bonding, four of them participate in *trans* 4⟶1 bonds of type II. The

* Coordinates, bond lengths and angles and all the conformational angles are contained in ref. 5.

conformational angles and NH...O distances for one of the molecules are shown in Table I, section C. The remaining two NH moieties appear to form 5⟶1 hydrogen bonds as shown in Figure 8. A question arises about the strength

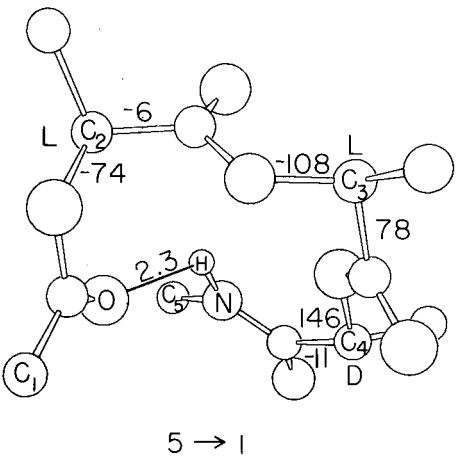


Figure 8: Details for a possible 5⟶1 hydrogen bond in valinomycin.[5]

of these bonds. Although the N...O distances, 2.99A and 3.13A, are only somewhat larger than the average N...O listed for all the other types of hydrogen bonds, the orientation of the NH bond with respect to the O atom is unfavorable. The H...O distance is 2.3A and the NH...O angle is only ∿125°, as compared to H...O distances of 1.8-2.0A and NH...O angles near 168° in 4⟶1 bonds[21,25], see Figure 6. Even in the 3⟶1 bonds, the H...O distances are between 2.1 and 2.2A and the NH...O angles are 138° and 144°. If Pauling's values for van der Waals' radii[28] are accepted, then unbonded H and O atoms can approach each other within 2.6A. Strong hydrogen bonds have H...O separations in the range of 1.6 to 2.1A, as shown in neutron diffraction experiments of amino acids and dipeptides.[29]

Representative conformational angles for the five types of intramolecular hydrogen bonds are plotted on the (ϕ, ψ) map in Figure 9. Soon after the appearance of the hexaglycyl[6] and ferrichrome A[11] structures, in which structural parameters were determined for the two types

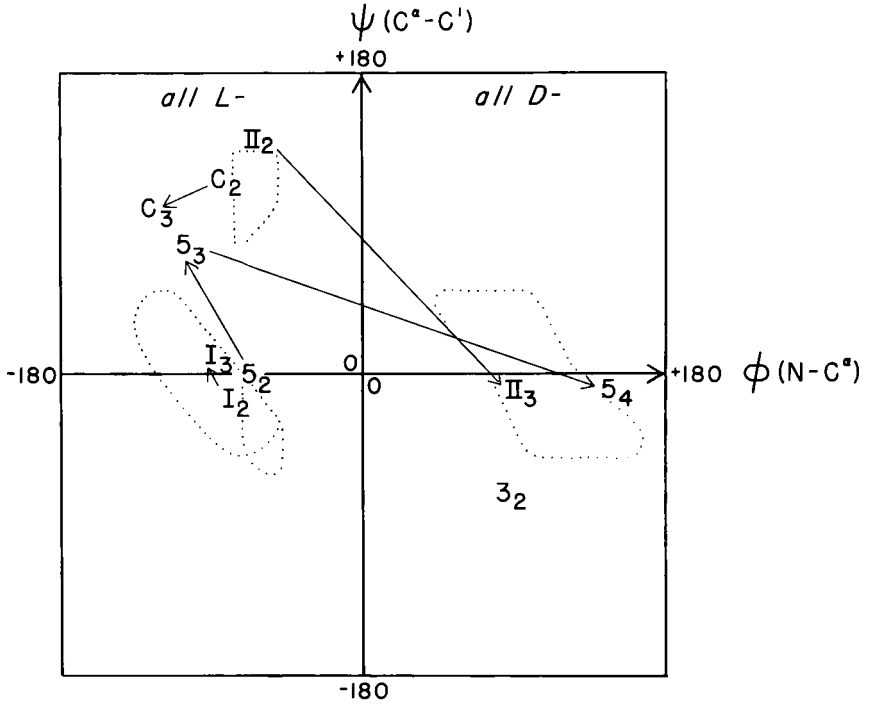


Figure 9: A (ϕ, ψ) conformational map for representative intramolecular hydrogen bonds. Points 3_2 for 3⟶1 bond; I_2 and I_3 for type I *trans* 4⟶1; II_2 and II_3 for type II *trans* 4⟶1; C_2 and C_3 for *cis* 4⟶1; 5_2, 5_3 and 5_4 for 5⟶1. The dotted lines represent the allowable regions for types I and II as calculated by Venkatachalam.[30]

of *trans* 4⟶1 bonds, Venkatachalam[30] calculated conformations for three linked peptide units which contain an NH...O bond based on contact distance criteria and potential functions. The dotted areas in Figure 9 show the calculated allowable regions for type I and type II bonds. These areas correspond reasonably well to the experimentally determined values. Recently, these calculations have been refined by Chandrasekaran *et al.*[31]

Non-Participation in Hydrogen Bonds

In some cyclic peptides, NH moieties occur that do not participate either in intramolecular or intermolecular hydrogen bonding. One such occurrence for the cyclic(L-Leu-L-Tyr-δ-Avaler-δ-Avaler), Figures 1 and 2, has already been discussed. Other examples are Li^+ antamanide[19] and Na^+ [Phe^4Val^6] antamanide[32], cyclic decapeptides, in which two of the six available NH groups are effectively blocked by neighboring $-CH_2C_6H_5$ groups. The antamanide structures will be discussed later.

A third example is in the cyclic(L-Ser(O-t-Bu)-β-Ala-Gly-L-β-Asp(OMe)). This synthetic tetrapeptide containing alternate α-amino and β-amino peptide groups, similar to the naturally occurring depsipeptide serratamolide, represents a preliminary step in the proposed synthesis of a cylindrical polypeptide.[33] In the proposed cylindrical peptide, there are covalent bonds between each pair of stacked rings. Fourteen-membered rings were chosen because the assumed conformation allowed the greatest number of hydrogen bonds in the cylinder, four between each pair of rings. The 14-membered ring "monomer" that was synthesized does have a conformation similar to the proposed one; *i.e.* there are four planar segments in the *trans* conformation.[34] In the crystal, the molecules of the monomer are stacked over each other. However, they are tilted so that only two NH...O bonds form between the rings and the other two NH moieties remain unbonded, Figure 10. They simply are too far away from any C=O groups.

Cis Peptide Units

The peptide unit $C_i^\alpha(C'O)(NH)C_{i+1}^\alpha$ usually assumes the *trans* conformation and is essentially planar. The ω_i angle rarely deviates by more than 5° to 10° from 180°. The *cis* conformation with $\omega_i \approx 0°$ is forced in cyclic dipeptides*. However, the *cis* conformation also occurs in much larger rings. In Table II, some of the structural features for the cyclic polypeptides analyzed by x-ray diffraction are listed. The cyclic tetrapeptide can occur in an all *trans* conformation, therefore if we examine all the cyclic peptides with four or more units, we see the *cis* conformation occurring in tetra, penta, hepta, octa and decapeptides. In each case, the peptide units assuming the *cis*

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* A survey of the structural and conformational parameters in cyclic dipeptides has been published elsewhere.[35]

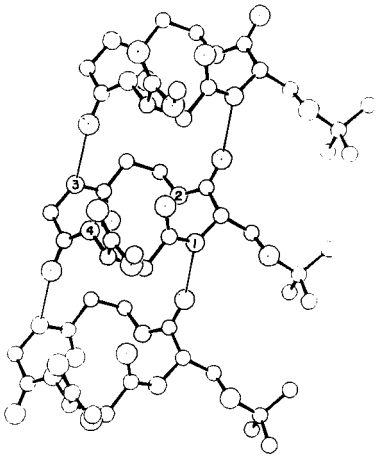

Figure 10: Stacked molecules of cyclic(L-Ser(O-t-Bu)-β-ala-Gly-L-β-Asp(OMe)) in the crystal. Two NH...O bonds are formed between each pair of molecules. Two other NH moieties in each molecule do not participate in nydrogen bonding.

conformation are only those with a substitution on the N atom, *i.e.* only prolyl or N-CH_3 residues. The occurrence of a N-substituted residue in a cyclic polypeptide does not guarantee a *cis* conformation; but the occurrence of a *cis* conformation has involved an N-substituted peptide unit in all the known structures.

Crystal structure results on *linear* oligopeptides containing prolyl units show *trans* conformations, as for example, in L-Leu-L-Pro-Gly,[40] Tos-L-Pro-L-HyPro,[41] and t-Amyloxycarbonyl-L-Pro-L-Pro-L-Pro[42] as well as those linear peptides included in Table I. However, in t-butylcarbonyl-L-Pro-L-Pro-L-Pro-L-Pro benzyl ester, the N-terminal peptide bond takes on the *cis* conformation.[43] It is interesting to note that this linear tetraproline compound forms one turn of a poly-L-Pro II helix.

Disorder

The prolyl residue has been commonly involved in disorders in the solid state. The pyrrolidine ring can assume a number of different conformations, usually an envelope form with four atoms in a plane. The out-of-plane atom has been found to be either C^{β}, C^{γ}, or N.[44]

Table II

Occurrence of *cis* Peptide Units

Substance	*No. of residues in ring*	*No. of atoms in ring*	*No. of internal H-bonds*	*Conformation*	*cis Peptide Units*	*Ref.*
c-dipeptides	2	6	0	cc	All (forced)	35
c-di β-Ala	2	8	0	cc	All (forced)	36
c-tri L-Pro	3	9	0	ccc	Pro,Pro,Pro	7,37
c-D-HyIv-L-MeIleu-D-HyIv-L-MeLeu	4	12	0	tctc	N-MeIleu,N-MeLeu	16
c-tetra Sar	4	12	0	tctc	N-MeGly,N-MeGly	17
c-DiMe Gly-L-Phe-D-Pro-LX	4	12	2	tttt		15
c-L-Ser(O-t-Bu)-β-Ala-Gly-L-β-Asp(OMe)	4	14	0	tttt		34
c-L-Leu-L-Tyr-δ-Avaler-δ-Avaler	4	18	0	tttt		10
Actinomycin	5	16	0	ccttt	N-MeGly,Pro	38
*Tuberactinomycin O	5	16	1	ttttt		22
*Viomycin	5	16	1	ttttt		23
c-hexa Gly	6	18	0,2	tttttt		6
c-Gly-Gly-Gly-Gly-D-Ala-D-Ala	6	18	2	tttttt		8
Ferrichrome A	6	18	1	tttttt		11
Ilamycin B_1	7	22	2	tctttct	N-MeLeu,N-MeLeu	26
c-octa Sar	8	24	0	ccttcctt	N-MeGly (four)	39
Li^+-Antamanide	10	30	2	tctttttttt	Pro,Pro	19
Na^+[Phe^4Val^6] Antamanide	10	30	2 (?)	tctttttttt	Pro,Pro	32
Valinomycin ($P2_1$)	12	36	6	all t		27
Valinomycin (P1)	12	36	6	all t		5

* Viomycin differs from Tuberactinomycin by one OH group.

The out-of-plane atom can be either *endo* or *exo* with respect to the adjacent C' atom. In crystals of such compounds as L-Leu-L-Pro-Gly,[40] N-Ac-L-Pro-L-Lac-$NHCH_3$,[21] and the M^+-antamanides,[19,32] the out-of-plane atoms in the pyrrolidine rings have been in both the *endo* and *exo* conformations, at random throughout the crystal, usually in a 1:1 ratio.

Another type of disorder that has occurred concerns the isopropyl side chains of some of the Val and Hyv residues in valinomycin.[5] A rotational disorder about the C^{α}-C^{β} bond places the H on C^{β} *gauche* with respect to the H on C^{α}, as well as in the more usual *trans* position.

Solvent Molecules and Complexing Ions

Investigations of cyclic polypeptides in solution and in the solid state by spectroscopic means and other physical methods have demonstrated that the conformation often undergoes a change in going from a non-polar to a polar solvent. The conformation appears to be different also for the uncomplexed and metal-complexed forms of such ion carriers as antamanide[45,47] and valinomycin.[47,48,49] A sufficient number of crystal structure analyses for the various possibilities are not yet completed and therefore, a total picture of the different conformations that a polypeptide can assume in the solid is not yet available. The conformational analyses that have been obtained by single crystal x-ray diffraction on octa to dodeca cyclic polypeptides are summarized below.

Cyclic octasarcosyl,[39] having all the N atoms methyl substituted, cannot contain any intramolecular NH...O bonds. The 24-membered ring has the *cis cis trans trans cis cis trans trans* conformation, as shown in Table II. The large ring is open enough to accommodate four molecules of H_2O that form OH...O bridges between themselves as well as to two carbonyl oxygens of the peptide ring surrounding the H_2O molecules and to additional carbonyl oxygens of neighboring peptide molecules, Figure 11.

Uncomplexed valinomycin[5] crystallized from n-octane or acetone is not associated with any solvent molecules in the lattice. The 36-membered ring has three loops up and three loops down, rigidly held by six intramolecular hydrogen bonds. The gross shape of the molecule is that of a short, flattened oval tube with parallel ridges on the outer surface containing the hydrocarbon side chains and an interior surface lined with polar groups, Figure 7. The top rim of the tube differs from the bottom rim in

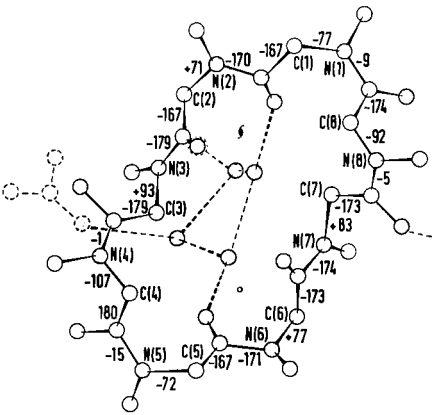

Figure 11: Cyclic-octa Sar with a ring open enough to accommodate four H_2O molecules[39].

that the three side chains consisting only of a CH_3 group are on the top whereas the equivalent sites on the bottom rim are occupied by isopropyl groups. The packing of the molecules in the crystal lattice is shown in Figure 12,

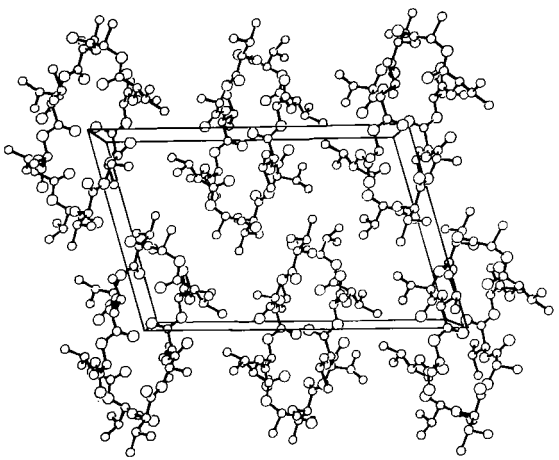

Figure 12: Packing of valinomycin molecules in the triclinic cell. The molecules on the corners are crystallographically unrelated to the molecules down the middle of the cell, although the conformations of each set of molecules is very similar to each other.

where the molecules in the center are crystallographically independent (*i.e.* not related by symmetry) from the molecules on the corners of the cell. The only forces between the molecules are relatively weak van der Waal's attractions, accounting for the soft, waxy character of the crystals and for the ease of disorder and twinning in the crystal lattice.

Crystals of valinomycin obtained from $(CH_3)_2SO$ have a different space group than those obtained from n-octane or acetone.[5] The volume available for each molecule is 1974 A^3 in the crystals from $(CH_3)_2SO$ as compared to 1615 A^3 in the crystals from n-octane. The implication is that either the molecule has unwound to occupy 20% more space, or that the polar solvent has cocrystallized with the valinomycin, or both. Since the crystals are not stable if allowed to dry, it appears that solvent must be included in the cell. The structure analysis of this form is in progress. Therefore it is premature to speculate upon the mechanism of changes in conformation, if any, upon exposure to different solvents and during complexation with metallic ions.

A number of molecules containing large rings complex with metallic ions and transport them across biological membranes. The manner of enfolding the metallic ion so that the charged moiety is in the interior of the molecular complex while the exterior of the molecule presents a lipophilic surface has shown considerable variation, depending upon the specific substance involved. A few examples of the conformations of complexes in the solid state are illustrated in Figure 13. The K^+ and Na^+ complexes of enniatin B, a cyclic hexadepsipeptide, are disordered in the crystal. A reasonable model to fit the data, Figure 13 (a), is a disc-shaped molecule with octahedral coordination of the six carbonyl oxygens to the K^+ ion.[50] Valinomycin, a cyclic dodecadepsipeptide, complexing preferentially with K^+, forms a ring around the K^+ and also makes octahedral coordination to the K^+ with six carbonyl oxygen atoms,[51] Figure 13 (b). The six K—O distances in both these complexes are estimated to be 2.6 - 2.8 A.

Nonactin, although not a polypeptide but a cyclic ester containing a 32-membered ring, enfolds a K^+ ion into the interior and makes eight K—O ligands in a cubic arrangement, four with carbonyl oxygens and four with ester oxygens,[52] Figure 13 (c). The Na^+-nonactin complex[53] is very similar to the K^+-nonactin complex, except that four of the Na—O distances are 2.4 A and four are 2.8 A while all eight of

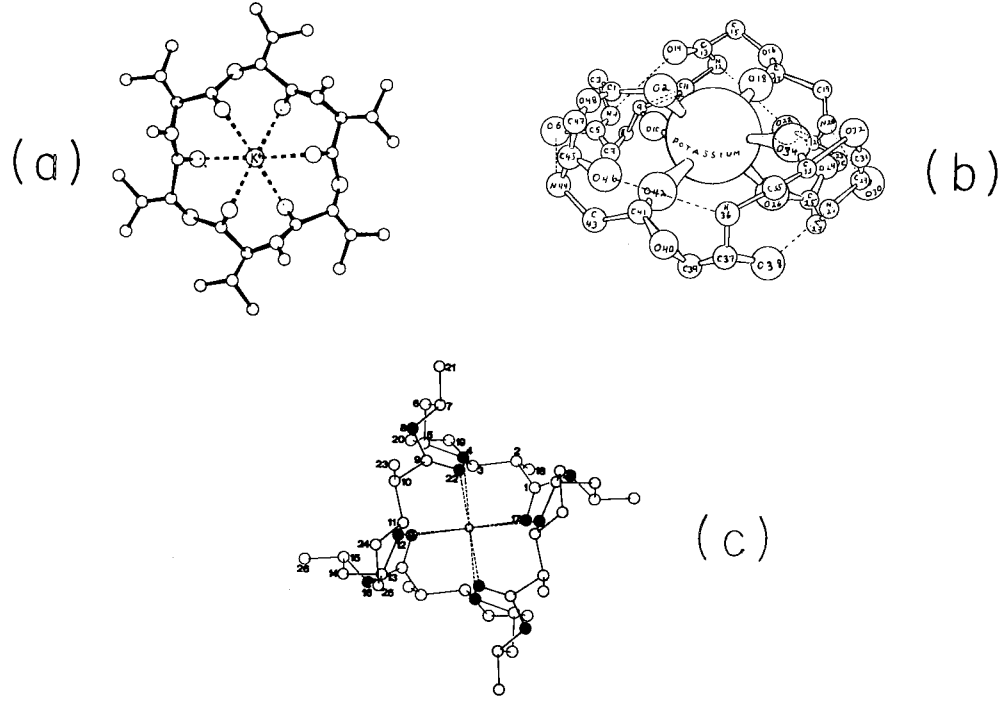

Figure 13: (a) K^+ enniatin B complex[50]; (b) K^+-valinomycin complex[51]; (c) K^+-nonactin complex[52].

the K—O distances are between 2.7 - 2.8 A. Even the uncomplexed nonactin has nearly the same conformation[54]. Tetranactin, a homologous compound of nonactin, has the same conformation as nonactin while complexed, but the uncomplexed tetranactin changes its conformation to an elongated ring[55].

Antamanide, a cyclic dodecapeptide with the sequence [-Val-Pro-Pro-Ala-Phe-Phe-Pro-Pro-Phe-Phe-], and a biologically active synthetic analogue, with Phe in position 4 and Val in position 6 giving the formula a 2-fold symmetry, preferentially complex with Na^+ and Li^+ rather than K^+[45]. The structures of the complexes Li^+ antamanide·CH_3CN[19] and Na^+[Phe^4Val^6] antamanide·C_2H_5OH[32] are very similar even though the metallic ions are different, some of the side chains are different, the solvents are different and the crystal packing schemes are different. The coordination to the Li^+ and Na^+ is especially interesting since the metallic ion rests in a cup formed by the polypeptide, Figure 14, and is coordinated to four carbonyl oxygen

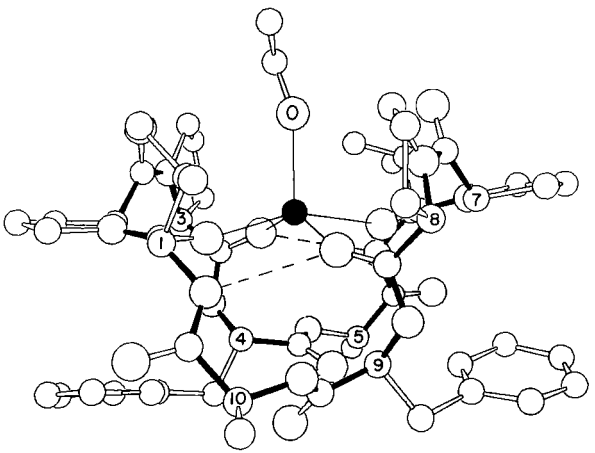

Figure 14: Na^+ [Phe^4Val^6] antamanide·C_2H_5OH complex[32]. The phenyl groups on Phe^5 and Phe^{10} have been omitted for clarity. The metal ion is represented by the black dot, the numbers refer to the C^α atoms, and the dashed lines indicate intramolecular NH...O bonds.

atoms at 2.0-2.2 A for the Li—O ligands and 2.25-2.36 A for the Na—O ligands. The cup is rimmed with hydrogen atoms of the lipophylic side chains of residues 1, 2, 3 and 6, 7, 8. The cavity is plugged with a solvent molecule and the nonpolar end of the solvent molecule completes the lipophilic exterior of the complex. A fifth ligand to the metallic ion is formed with the polar end of the solvent molecule, CH_3CN or C_2H_5OH in these examples. The five-fold coordination to the Na^+ is shown in more detail in Figure 15.

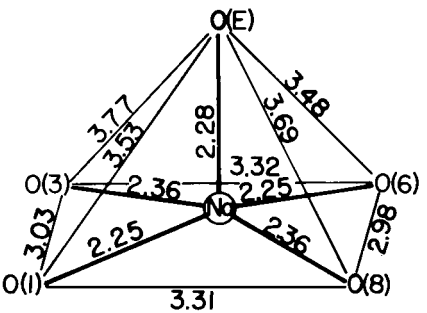

Figure 15: The coordination polyhedron for the Na^+ in the $Na^+[Phe^4Val^6]$antamanide·C_2H_5OH complex showing lengths for the Na—O ligands.

Thus, the examples in Figures 13 and 14 illustrate a variety of ways in which metallic ions are enfolded in ion carriers. In general, the complexes have a globular shape with a charged interior and a lipophilic exterior. Moreover, the M^+ complexes take advantage of all possible symmetry; the enniatin B and valinomycin have a 3-fold rotation axis, the nonactin and tetranactin have 4 symmetry, while the antamanides have an approximate 2-fold rotation axis.

Concluding Remarks

Crystal structure analyses of small polypeptides, thus far up to a dodecapeptide, have demonstrated that similar conformations do occur in different molecules, while a particular molecule can have a number of different conformations in the same unit cell of a crystal; that conformational parameters for internal hydrogen bonds are

remarkedly constant, regardless of whether some of the residues contain an amide or ester, while five different kinds of hydrogen bonds, 3⟶1, 4⟶1 and 5⟶1, can be formed; that there are a number of examples in which the NH moiety does not participate in any hydrogen bonding; that *cis* conformations for peptide units are fairly common and appear to occur only for peptide units with N-substitution, although N-substituted peptide units do not necessarily assume the *cis* conformation; that the folding of a cyclic polypeptide around a metallic ion in complex formation occurs in a number of different ways, including the incorporation of solvent molecules; and that the conformation of the uncomplexed and complexed form of a particular substance may or may not be the same. Thus, much more information needs to be gathered before more specific generalizations concerning conformation can be made.

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A CRYSTALLOGRAPHIC STUDY OF CYCLIC(TRIPROLYL): FLEXIBILITY OF PROLYL RESIDUES

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CYCLO(tri-L-Prolyl) CONSISTS OF THREE PROLYL RESIDUES joined by *cis* peptide bonds. Preliminary crystal structure studies[1] of cyclo(tri-L-prolyl) and of cyclo(L-prolyl-L-hydroxyprolyl) revealed several different pyrrolidine ring conformations, suggesting less rigidity than anticipated. We have carried out a detailed crystal and molecular structure analysis on cyclo(tri-L-prolyl) to investigate allowed conformations.

Large crystals of cyclo(tri-L-prolyl) were generously provided by Dr. Roderich Walter. The crystal data are given in Table I. Intensities for 6412 reflections were collected on a Picker FACS-I diffractometer with Mo K_α radiation to 2 θ_{max} = 55°. After application of absorption, Lorentz, and polarization corrections, symmetry related reflections were averaged to give 3547 unique observations.

Kartha *et al.*[1] supplied the coordinates from their preliminary study. The higher quality of data obtainable from our crystal enabled us to place and refine hydrogen atom positions as well as the heavier atom parameters. The heavier atoms were refined by least squares analysis with anisotropic thermal parameters, and the positions of the hydrogen atoms were refined using a fixed isotropic

Table I

Crystal Data

Space Group	$P2_12_12_1$
a	15.94 Å
b	19.10 Å
c	9.23 Å
Z	8
Molecules per asymmetric unit	2
Wavelength	0.71069 Å
R	4.8%

temperature factor of 5 $Å^2$ to a final R value of 4.8%. The asymmetric unit is shown in Figure 1.

PMR spectra from cyclo(tri-L-prolyl) have a single doublet assignable to the alpha protons. The crystal structure data support the suggestion[2] that one of the H_α-H_β coupling constants is near zero because of a 90° dihedral angle; for the six prolyl residues studied here, the H_α-C_α-C_β-$H_{\beta 1}$ dihedral angle ranges from 88°-92°. Analysis of the geometry of the two independent molecules of cyclo(tri-L-prolyl), the molecular geometry of cyclo-(L-prolyl-L-prolyl-L-hydroxyprolyl) reported by Kartha and Ambady,[3] and the geometry of other prolyl rings indicates a fair degree of mobility of pyrrolidine rings and that the peptide bonds in such compounds are frequently nonplanar. Similar conclusions concerning the mobility of pyrrolidine rings were independently drawn from carbon-13 NMR studies by Deslauriers *et al.*[4,5].

The occurrence of the single doublet implies an equivalent fixed conformational environment for each of the three alpha protons[2] or alternatively, may reflect a rapid equilibrium (on the NMR time scale) among various conformations. The molecules in the crystal do not exhibit three-fold symmetry as can be seen from the data in Tables II and III. The pyrrolidine rings exist in both the envelope and twist conformations as seen in Figure 2. The number of discrete conformations observed in this and other[6] crystallographic studies is striking and suggests considerable flexibility for the pyrrolidine rings which

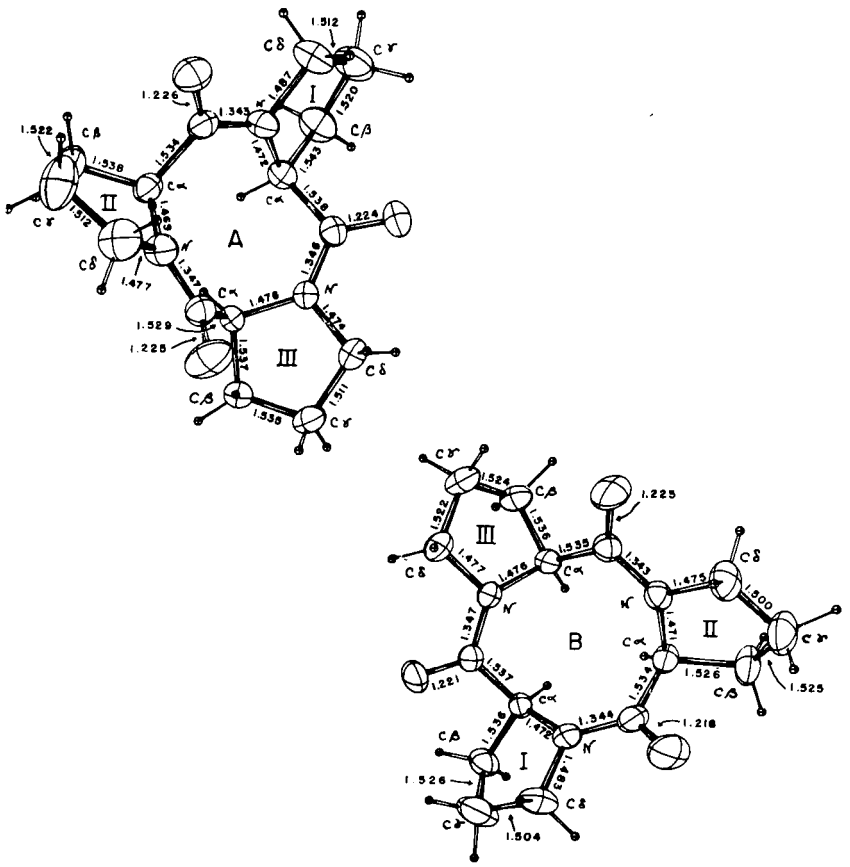

Figure 1: The crystallographic asymmetric unit.

could result in time-averaged three-fold symmetry in solution. We have taken PMR spectra from 30°C to -60°C and see no significant broadening of the H_α doublet (Lavallee, D., unpublished data). This suggests that energy barriers between conformations are low.

Our study and that of Deslauriers *et al.*[5] suggest that cyclo(tri-L-prolyl) is more flexible than expected. It is therefore not a suitable compound for discriminating possible conformations from spectroscopic data.

Table II

Cyclo(tri-L-prolyl) Main Chain Dihedral Angles

Angle	*Molecule A*	*Molecule B*
$O=C'-C_\alpha-C_\beta$	27.9°	19.7°
	27.7	29.2
	29.0	20.3
$\phi-C_\alpha-N$	-97.2	-94.8
	-94.9	-97.6
	-95.1	-106.0
$\omega-N-C'$	0.9	-2.5
	-2.7	5.7
	-1.2	12.6
$\psi-C'-C_\alpha$	93.6	97.4
	96.8	88.7
	94.7	87.3

Table III

Peptide Least-Squares Planes

Peptide	*Mean Deviation (A)*	*Mean Deviation (B)*
I	0.0036 Å	0.0067 Å
II	0.009	0.025
III	0.0066	0.049

Angles Between Planes:

A: 71.9°, 71.2°, 69.9°

B: 65.9°, 62.9°, 71.1°

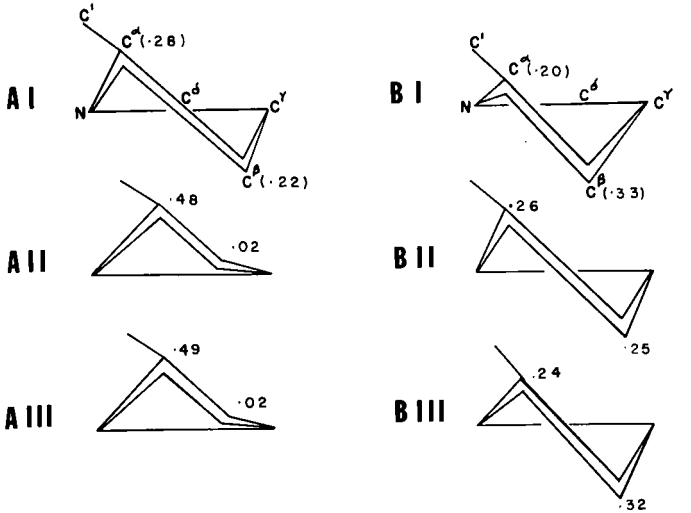

Figure 2: Puckering of the pyrrolidine rings for cyclo (tri-L-prolyl). The numbering of the rings corresponds to the convention of Figure 1.

Acknowledgment

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CONFORMATIONAL FLEXIBILITY OF *CYCLO*(TRIPROLYL) IN SOLUTION - A CARBON-13 NMR INVESTIGATION

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CARBON-13 NMR SPECTROSCOPY is becoming increasingly useful in the study of time-averaged peptide conformations in solution.[1] One particular application of this form of spectroscopy has been in the study of prolyl residues. *Cis* and *trans* isomers about the X-Pro bond give rise to distinct, non-overlapping, resonances for the γ-carbon of the pyrrolidine ring. The chemical shifts of the γ-carbons can be used to monitor the presence of either *cis* or *trans* peptide bonds or an equilibrium between *cis* and *trans* peptide bonds in a single compound. Furthermore, ^{13}C spin-lattice relaxation times (T_1) reflect conformational flexibility *within* the pyrrolidine ring of proline in peptides, peptide hormones[2] and polymers[3,4].

Cyclo(Pro_3) has been the subject of analysis both by ^1H NMR spectroscopy[5] and X-ray diffraction[6]. These studies have involved conformational analysis of the cyclic peptide backbone and the pyrrolidine ring. The conformations reported for the prolyl ring from X-ray and ^1H NMR studies

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differ; this is not surprising in view of the different time scales and solvent conditions in both experiments. We have used ^{13}C NMR to investigate the dynamic behaviour of *cyclo*(Pro$_3$) in solution.

Spin-lattice relaxation times (T_1) were measured on a Varian XL-100 spectrometer using the inversion-recovery method of Freeman and Hill[7]. T_1 values were calculated by a least squares fit to the best straight line on a semilogarithmic plot. *Cyclo*(Pro$_3$) was prepared as described in reference [8].

Chemical Shifts

^{13}C chemical shifts of *cyclo*(Pro$_3$) dissolved in $CDCl_3$ and $(CD_3)_2SO$ are given in Table I. The assignments are based on those of the free amino acid in D_2O and $(CD_3)_2SO$. Only one resonance arises for each of the carbons in proline. This implies either that only one conformation exists *or* that there is a rapid interconversion between various conformers. The latter inplies that intercoversion must be rapid on the time scale of ^{13}C chemical shifts, *i.e.* $> 10^3$ sec^{-1}. The chemical shift of the γ carbon of proline ($\simeq$23.4 ppm) is indicative of a *cis* peptide bond. Dorman and Bovey,[9] in a study of proline-containing peptides, found that in the *trans* conformer about the X-Pro bond the γ carbon chemical shift occurred 25.1 $\pm$ 0.5 ppm downfield from external $(CH_3)_4Si$ and the *cis* conformer at 23.4 $\pm$ 0.3 ppm. Similar results were obtained by Bystrov[10] in an analysis of all available literature on ^{13}C chemical shifts of proline. Furthermore, Bystrov reports that the *difference* between the β and γ chemical shifts is characteristic of the isomer about the proline bond: 1.3 to 6.0 ppm for a *trans* X-Pro bond and 8.3 to 10.0 ppm for a *cis* X-Pro bond (excluding diketopiperazines). In *cyclo*(Pro$_3$) we find a difference of 7.5 ppm in $CDCl_3$ and 7.3 ppm in $(CD_3)_2SO$. Thus, the *difference* between the chemical shifts of the β and γ carbons in *cyclo*(Pro$_3$) is characteristic of neither a *cis* nor a *trans* X-Pro peptide bond. This is undoubtedly due to an anomalous chemical shift for the β carbon resonance in *cyclo*(Pro$_3$) because the γ resonance, which is the most reliable in detecting *cis-trans* isomerism, corresponds to that expected for the *cis* isomer. Indeed, Dorman and Bovey[9] found that the chemical shift of the β carbon of proline is sensitive to conformational effects other than those arising from the *cis* and *trans* nature of the peptide

Table I

^{13}C Chemical Shifts (ppm)[+] and Spin-Lattice Relaxation Times (sec)[‡] of *Cyclo*(Pro_3)

Solvent	$CDCl_3$[+a]	$CDCl_3$[+b]	$(CD_3)_2SO$[+a]	$(CD_3)_2SO$[+b]	$CDCl_3$[‡] *110 mgs/1.5 ml*	$(CD_3)_2SO$[‡] *90 mgs/1.5 ml*
Pro αCH	58.48	57.38	57.76	56.46	0.62	0.61
βCH$_2$	31.05	29.95	30.61	29.31	1.01	0.75
γCH$_2$	23.50	22.40	23.34	22.04	1.17	0.86
δCH$_2$	46.81	45.71	46.62	45.32	1.09	0.74
C=O	168.83	167.73	169.79	168.49		

+ Chemical shifts are reported in parts per million downfield from external[a] or internal[b] Me_4Si, with an accuracy of ± 0.05 ppm.

‡ Multiplied by N, the number of contiguous hydrogens, accuracy ± 10%.

bond. The β carbon chemical shift may in effect be more sensitive to ring conformation than is the γ carbon. Torchia and co-workers[11] showed that in imino acids both the α and δ carbon resonances are affected by the nature of the substituent on the carbonyl group. Thus we see that the chemical shifts of carbons in proline are subject to at least three effects: 1) the *cis* and *trans* nature of peptide bond, 2) the conformation of the pyrrolidine ring itself and 3) the nature of the substituent attached to the nitrogen.

^{13}C *Spin-Lattice Relaxation Times*

The ^{13}C spin-lattice relaxation times (T_1) of *cyclo*-(Pro$_3$) dissolved in $CDCl_3$ and $(CD_3)_2SO$ are given in Table I. For the conditions used in this study, the longer the NT_1 value, the greater is the mobility of that carbon atom.

Cyclo(Pro$_3$) provides an interesting model to study the molecular mobility of *cis* isomers of proline. We see from Table I that the β, γ and δ carbons have longer NT_1 values than the α carbon. This is a consequence of greater mobility of these carbon atoms within the pyrrolidine ring. On the time scale of ^{13}C T_1 measurements, rapid interconversion between ring-puckered conformers implies motion which occurs at a rate greater than *ca.* 10^{11} sec^{-1}. Furthermore, the NT_1 values of the β, γ and δ carbons are similar. This contrasts with data obtained for large peptides and polypeptides, where the δ carbon shows an NT_1 value similar to that of the α carbon[2,3], and reflects the steric freedom of the δ carbon of proline in a *cis* peptide bond. Thus, the T_1 values of proline in *cyclo*-(Pro$_3$) resemble more closely those for free proline than they do those for peptides and polypeptides containing *trans* peptide bonds. The longer NT_1 values for the carbons in *cyclo*(Pro$_3$) may also be attributed in part to the closing of the cyclic peptide backbone which minimizes steric hindrance at the δ carbon.

In $CDCl_3$ and $(CD_3)_2SO$ the α carbons of *cyclo*(Pro$_3$) show similar NT_1 values. In contrast, the NT_1 values of the β, γ and δ carbons are greater in $CDCl_3$. The latter observation indicates a smaller effective correlation time for intracyclic motion in the prolyl residue in $CDCl_3$, *i.e.* the degree of intracyclic motion of the β, γ and δ carbons of proline is greater in $CDCl_3$ than in $(CD_3)_2SO$. One must point out however that T_1 values give indications

of the rates of both overall and intramolecular motion and therefore the greater NT_1 values in $CDCl_3$ can also reflect enhanced overall molecular motion. T_1 values are known to depend on the microviscosity of the solution. In some cases, the rate of rotational diffusion can also be correlated with the macroscopic viscosity of the solution. However, the correlation is usually better with rates of translational diffusion. The macroscopic viscosities of $CDCl_3$ and $(CD_3)_2SO$ at 32° are 0.005[14] and 0.02[15] poise, respectively.

The T_1 values of the α carbons of proline in *cyclo*-(Pro_3) dissolved in $CDCl_3$ and $(CD_3)_2SO$ are quite similar. This could indicate that for *cyclo*(Pro_3) the rate of overall molecular reorientation is not dependent on the macroscopic viscosity of the solution or that the α carbon resonances are also subject to intracyclic motion, which occurs to a lesser extent than for the β, γ and δ carbons. The latter explanation is more plausible because it is unlikely that motion at the β, γ and δ carbons can proceed completely independently of the α carbon. Therefore, we must conclude that *cyclo*(Pro_3) in solution undergoes intracyclic motion at all carbon atoms of the pyrrolidine ring. The degree of motion is most restricted at the α carbon. The β, γ and δ carbons undergo equal relatively large degrees of motion.

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CONFORMATIONAL MOBILITY OF PEPTIDES AS EVALUATED FROM SPIN-LATTICE RELAXATION TIMES OF ^{13}C AND ^{2}H

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CARBON-13 NUCLEAR MAGNETIC RESONANCE has almost become a routine technique for study of the composition and conformation of peptides[1]. The ^{13}C chemical shifts provide useful indicators of the state of ionization of a residue[2], the preferred tautomeric form of the imidazole ring in histidine[3], and the relative populations of *cis* and *trans* isomers of proline about the X-Pro bond[4-7]. However, more subtle conformational features such as hydrogen-bonding, backbone turns, and hindered rotation about C-C bonds are not easily determined from the chemical shifts. Increasing use is being made of the spin-lattice relaxation times (T_1) of ^{13}C to explore these latter aspects[8-10]. Interpretation of the T_1 values in terms of correlation times for individual modes of motion is quite complex, and must be done with great care[11]. However, before this interpretation can be done, it is essential to establish the mechanism(s) of the ^{13}C spin-lattice relaxation for the

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compound of interest. Usually one assumes that dipole-dipole interaction with the directly-attached ^1H is the dominant mechanism, and often it is assumed that overall motion of the peptide is isotropic and rapid on the ^{13}C NMR time scale. None of these assumptions is necessarily true, so we have tested them critically for some compounds of particular interest to us[12]. We report here an examination of the contribution of the dipole-dipole mechanism to ^{13}C spin-lattice relaxation in proline, and give an overview of the usefulness of ^{13}C T_1 values for conformational studies of peptide hormones.

^{13}C spin-lattice relaxation times of proton-bearing carbons are related to molecular motion according to[11],

$$\frac{1}{T_1^{DD}} = \left\langle \frac{1}{r^6} \right\rangle N\hbar^2\gamma_C^2\gamma_H^2 \{f(\omega_H-\omega_C) + 3f(\omega_C) + 6f(\omega_H+\omega_C)\} \qquad [1]$$

where $f(\omega) = \tau_{eff}/(1 + \omega^2\tau_{eff}^2)$, ω_H and ω_C are the resonance frequencies of ^1H and ^{13}C, $\hbar$ is Planck's constant divided by 2π, $\langle r^{-6} \rangle$ is the vibrationally-averaged inverse sixth power of the ^{13}C-^1H internuclear distance, T_1^{DD} is the spin-lattice relaxation contribution from dipole-dipole interactions, τ_{eff} is the effective correlation time for reorientation of the internuclear C-H vector, and N is the number of hydrogens directly-bonded to the carbon undergoing relaxation. However, if dipole-dipole interactions do not provide the sole relaxation mechanism, the observed T_1 is given by,

$$\frac{1}{T_1} = \frac{1}{T_1^{DD}} + \frac{1}{T_1^{other}} \qquad [2]$$

where T_1^{other} comprises such mechanisms as spin-rotation, chemical shift anisotropy, and scalar relaxation. Furthermore T_1^{DD} can arise not only from interaction with ^1H nuclei but also by interaction with unpaired electron spins. The magnetic moment of the electron is greater than that of the proton and this leads to very efficient relaxation. For this reason traces of paramagnetic impurities or dissolved oxygen can drastically affect observed T_1 values.

The relative extent of the ^{13}C-^1H dipole-dipole mechanism can be estimated by measurement of the nuclear Overhauser enhancement of intensity of a ^{13}C resonance under conditions of high power irradiation (decoupling) of

^1H at its resonance frequency. The theoretical maximum nuclear Overhauser enhancement (NOE) is 1.998; NOE = {integrated intensity (decoupled)/ integrated intensity (coupled)}-1.[13] If mechanisms other than dipole-dipole between ^{13}C and ^1H are present, the NOE will be reduced. The NOE yields an estimate of the contribution of *all* ^1H to the relaxation of the ^{13}C nuclei. In general, however, for carbons directly bonded to hydrogen, only the directly-bonded hydrogens are considered important in the relaxation of ^{13}C because of the r^{-6} dependence of the dipole-dipole interaction.

One can evaluate the importance of the dipole-dipole relaxation mechanism between protons and directly-bonded carbon nuclei by measuring deuterium relaxation times. The only significant contribution to the relaxation of deuterium is the interaction of its quadrupole moment with the fluctuating electric field gradient at the nucleus. Thus, for rapid molecular reorientation, the relaxation rate of a deuteron can be related to the correlation time, τ, for the motion by

$$\frac{1}{T_1(^2H)} = 3(e^2qQ/\hbar)^2 \tau/8 \qquad [3]$$

where $e^2q\,Q/\hbar$ is the deuterium quadrupole coupling constant. This relationship is valid if the asymmetry parameter for the quadrupole coupling tensor is zero or negligible.

Both ^2H and ^{13}C relaxation of a carbon-hydrogen moiety arise from intramolecular interactions with the same angular- and time-dependence. Therefore by measuring the ratio of T_1 for a proton-bearing ^{13}C nucleus to the T_1 of a deuteron bonded to the same carbon in an isotopically-substituted compound, we can deduce the contribution of dipole-dipole relaxation to the total relaxation rate of a given carbon[14]. In the case of rapid isotropic diffusion and dipole-dipole interaction, the sole relaxation mechanism for ^{13}C, equations [1] and [3] yield

$$\frac{T_1\ (^{13}C)}{T_1\ (^2H)} = \frac{19.9}{N} \qquad [4]$$

assuming a C-H bond distance of 1.09 Å and a quadrupole coupling constant of 170 KHz for the sp^3 C-^2H bond.

Equation [1] relates the observed T_1 value for dipole-dipole relaxation of a ^{13}C nucleus to an effective correlation time for reorientation of the carbon nucleus under investigation. It must be emphasized that such an observed effective correlation time can be the result of internal motion as well as overall molecular motion in non-rigid molecules. In some cases it is possible to estimate rates of overall molecular motion from carbon nuclei which are held rigidly within the molecular framework. Using this value of the correlation time for overall molecular motion it is then possible to deduce the contribution of internal motion to the observed effective correlation time[9,11,12].

Methods

^{13}C and ^{2}H NMR spectra were obtained on a Varian XL-100-15 spectrometer operating in the pulsed Fourier transform mode with a Varian 620-L computer of 16K memory. The ^{13}C spectra were obtained with complete proton noise-decoupling at a probe temperature of 32° C in tubes of o.d. 12 mm. Chemical shifts are referenced to tetramethylsilane contained in a concentric tube of o.d. 5 mm within the sample tube.

Spin-lattice relaxation times (T_1) were measured using the inversion-recovery method with a $(180°-\tau-90°-t)_n$ pulse sequence, or by progressive saturation with a pulse sequence $(90°-\tau-90°-AT)_n$, where τ is a variable delay time, and t is at least five times longer than the longest T_1 value to be measured. The width of a 90° pulse is 14 μsec for ^{13}C and 28 μsec for ^{2}H. T_1 values were determined using a non-linear two parameter regression analysis in M_o and T_1, where M_o is the thermal equilibrium value of the magnetization.

Material

L-Proline was obtained from Seikagaku Kyogo Co., Tokyo, Japan. The sample was run at a concentration of 100 mg/ml in H_2O or D_2O. pH was adjusted using DCL or NaOD. Deuteriated L-proline (99%-d_7, Lot # MD-1239) was a product of Merck, Sharp and Dohme, Canada, Ltd. "pH" values are the pH-meter readings obtained in D_2O and are uncorrected for the deuterium isotope effect on the glass electrode. The compounds α, δ, δ, trideuterioproline, β, β, γ, γ, tetradeuterioproline and α, β, γ, γ, δ, δ hexadeuterioproline were prepared as described elsewhere[15].

Results

Table I shows the NT_1 values obtained for proline dissolved in D_2O at various "pH" values. A rigid molecule which undergoes isotropic molecular motion and is relaxed by a purely dipolar mechanism with directly-bonded protons should show equal NT_1 values for all carbons. However, the inequalities seen in Table I are a general property of proline in peptides, the particular nature of the inequality depending upon the neighbouring amino acids[16]. Possible sources of the inequalities are: the presence of slightly different relaxation mechanisms for individual carbons; anisotropic overall molecular motion which affects some carbon atoms more than others; and intramolecular motion of varying degrees for the various carbons. These points have been discussed previously[16], and it was concluded that intramolecular motion was the source of the variation in NT_1 values around the ring. However, this conclusion was based on the implicit assumption that only dipole-dipole relaxation from the directly-attached hydrogen need be considered. We substantiate this assumption by consideration of the data in Tables II-IV and Figure 1.

Figure 1 shows the method of measurement of the nuclear Overhauser effect; the results are given in Table II. The NOE values of proline are maximal, indicating that dipole-dipole interaction with ^1H is the only significant relaxation mechanism. However, such measurements yield only the NOE from all possible protons, not just from those directly-bonded. We have verified that the NOE arises from the directly-bonded protons. Table IIb gives the NOE for α, β, γ, γ, δ, δ-hexadeuterioproline in D_2O. Only the β-carbon has an attached proton. Nonetheless, its NOE value is also the maximum possible, confirming the assumption that the T_1 values of its proton-bearing carbons are determined by dipole-dipole interaction with attached protons. Thus, effective correlation times for molecular reorientation can be obtained from equation [1].

Further verification of the above assumptions comes from the measurement of the deuterium T_1 values and comparison with the corresponding ^{13}C T_1 values. The data for β, β, γ, γ-proline-d_4 and α, δ, δ-proline-d_3 in Table I show that substitution of deuterium does not substantially affect the T_1 values of the adjacent proton-bearing carbons. However one would expect a significant effect for neighbouring non-protonated carbons. Deuterium substitution could

Table I

NT_1 Values for ^{13}C in Proline and Partially-Deuterated Derivatives

	*L-proline**				β,β,γ,γ-*proline-*d_4*	α,δ,δ-*proline-*d_3*
	*"pH" 1.0**	*"pH" 6.4**	*"pH 11.0**	*H_2O*†		
αCH	4.3 ± 0.2	4.2 ± 0.2	4.5 ± 0.2	4.7 ± 0.1 (4.1)	5.1 ± 0.2	
βCH_2	7.4 ± 0.2	7.2 ± 0.6	7.4 ± 0.2	7.8 ± 0.2 (6.8)		7.3 ± 0.3
γCH_2	9.0 ± 0.4	8.8 ± 0.4	8.9 ± 0.6	10.0 ± 0.4 (8.8)		8.7 ± 0.4
δCH_2	6.4 ± 0.2	6.8 ± 0.6	6.6 ± 0.4	7.0 ± 0.3 (6.1)	7.2 ± 0.8	
C=O	45.1 ± 8.9	42.8 ± 11.8	35.9 ± 8.6			

* Determined in ^2H_2O, 100 mg/ml

† 100 mg/ml in H_2O, values in parentheses correspond to values expected in ^2H_2O after correction for viscosity

Table II

Nuclear Overhauser Enhancements of Proline

Integrated Intensity (arbitrary units)

	Decoupler "on" all the time (I)	*Decoupler "on" during data acquisition only (I_o)*	I/I_o	$(I/I_o)-1$
a. Proline				
αCH	4.8	1.6	3.00	2.0
βCH_2	5.0	1.7	2.94	1.9
γCH_2	5.1	1.7	3.00	2.0
δCH_2	4.9	1.7	2.88	1.9
α,β,γ,γ,δ,δ-deuterioproline				
βCHD	2.7	0.9	3.0	2.0

Table III

^2H T_1 Values (sec) of Deuterated Prolines[+]

Position Deuterated	α, β, β, γ, γ, δ, δ	α, β, γ, γ, δ, δ	β, β, γ, γ	α, δ, δ
α	0.27	0.25	-	0.25
β	0.42	-	0.40	-
βγγ[±]	0.44	0.42	0.43	-
δ	0.40	0.39	-	0.38

^{13}C NT_1 Values Predicted From Above ^2H Relaxation Times

	α, β, β, γ, γ, δ, δ	α, β, γ, γ, δ, δ	β, β, γ, γ	α, δ, δ
αCH	5.37	4.97	-	4.97
βCH_2	8.36	-	7.96	-
γCH_2	8.76	8.16	8.55	-
δCH_2	7.96	7.76	-	7.56

[+] Determined by the inversion-recovery method, 100 mg/ml H_2O (glass distilled), values ± 10%.

± The resonance of one β-deuteron overlaps that of the two γ-deuterons.

Table IV

Correlation Between ^2H Relaxation and ^{13}C Relaxation. Prediction of ^{13}C NT_1 Values (sec) for Proline from ^2H T_1 Data*

	Predicted		*Observed*	
	H_2O	*D_2O*	*H_2O*	*D_2O*
αCH	5.4 ± 0.5	4.7 ± 0.5	4.7 ± 0.1	4.2 ± 0.2
βCH_2	8.4 ± 0.8	7.4 ± 0.7	7.8 ± 0.2	7.2 ± 0.6
γCH_2	8.8 ± 0.9	7.7 ± 0.8	10.0 ± 0.4	8.8 ± 0.4
δCH_2	8.0 ± 0.8	7.0 ± 0.7	7.0 ± 0.3	6.8 ± 0.6

* Predicted values obtained from deuterium relaxation times of deuterioproline α, β, β, γ, γ, δ, δ.

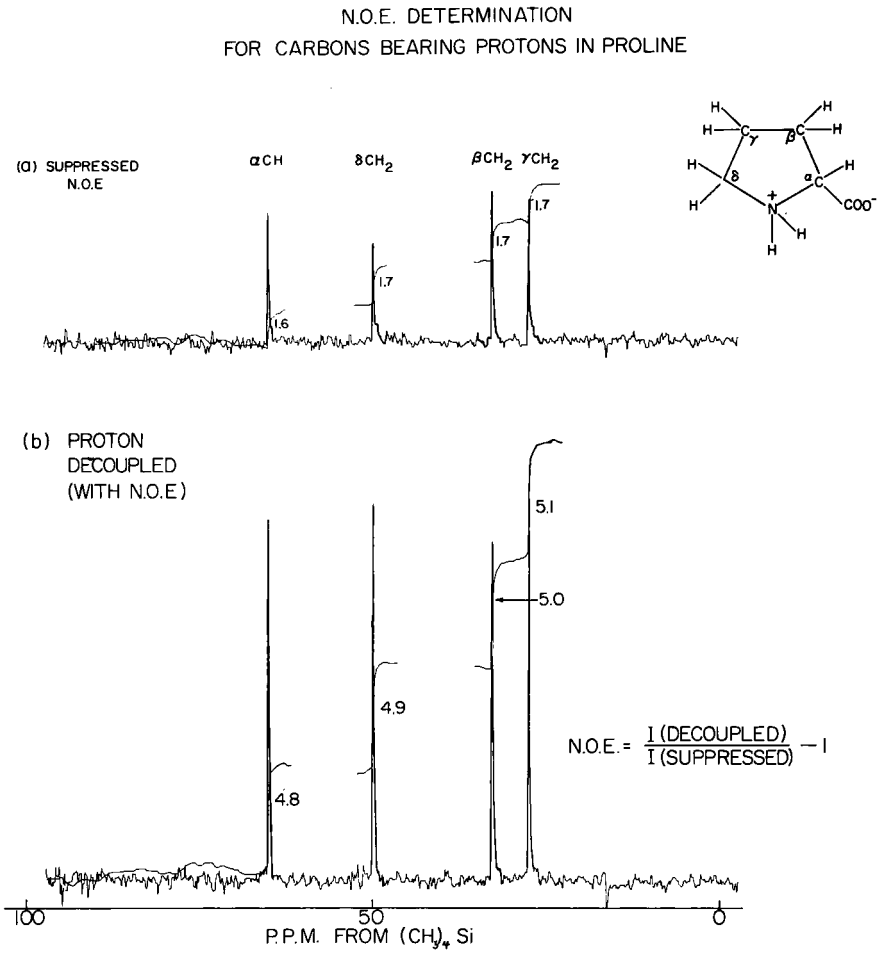

Figure 1: ^{13}C NMR spectra of proline in D_2O, "pH" 6.4, 100 mg/ml, with and without nuclear Overhauser enhancement. In the latter case the couplings to ^{1}H were removed by activating the decoupler at the start of acquisition of the free induction decay, inactivating it at the end of the acquisition and during a delay greater than four times the longest T_1 value. Operating parameters were: number of accumulated transients, 600; acquisition time, 0.5 sec; delay, 50 sec; pulse angle, 90°; Varian CFT-20 spectrometer.

thus be used to map the geometry of fairly rigid peptides by following the effect of such a change on the T_1 values of the non-protonated carbons.

Table III shows the ^2H T_1 values of various deuterium-substituted prolines. The ^2H T_1 values are independent of the degree of deuterium substitution. Table III also shows the NT_1 values predicted for the carbons in a protonated analog. It should be noted that these values were measured for proline in H_2O. D_2O and H_2O differ in viscosity by ≃14% at 23° and a correction for differing overall molecular correlation times needs to be made when calculating values expected in D_2O. Table IV compares NT_1 values for protonated carbons, predicted from the deuterium relaxation times with the appropriate viscosity correction, to the values observed in D_2O. The agreement between the predicted and observed values confirms the assumption that dipole-dipole relaxation to directly attached hydrogen dominates the ^{13}C relaxation times.

The experimental NT_1 values obtained for proline in D_2O and H_2O differ on the average by ca. 11%. This is almost within experimental error; however, it should be noted that the NT_1 values observed in H_2O are all larger than those observed in D_2O, as expected if the rate of overall molecular reorientation were dependent on the macroscopic viscosity of the solution. We have determined the T_1 values of proline in D_2O-glycerol mixtures (R. Deslauriers, I. C. P. Smith, unpublished) in order to study the influence of the macroscopic viscosity of the solvent on the rates of overall molecular motion (monitored mainly at the α carbon) and intramolecular motion (monitored mainly at the γ carbon). We have found a linear relation between $1/T_1$, the relaxation rate, and the glycerol concentration (% v/v). The change in $1/T_1$ is greatest for the α carbon and least for the γ carbon. It should be noted that the viscosity of the D_2O-glycerol mixture does not vary linearly with glycerol concentration. The linear relationship between $1/T_1$ and % glycerol implies some type of preferential solvation of proline in an aqueous microscopic environment.

It is worth noting that no particular precautions were taken when measured NOE or T_1 values on the prolines and deuterium-substituted prolines studied here. Thus, paramagnetic impurities in the solvent, such as metal ions from containers or dissolved O_2, do not affect the NOE values of the proton-bearing carbons. Their effect can be pronounced on quaternary carbons[17].

An alternate method to estimate the contribution of dipole-dipole relaxation from hydrogens not directly attached to a carbon atom would be to compare the ^{13}C T_1 values for the C-^{1}H and C-^{2}H moieties. They are simply related if the only relaxation mechanism is dipole-dipole coupling to the attached hydrogen. This method is much less desirable than those we have used above, as failure to deuterate a ^{13}C completely can lead to erroneous T_1 values for the ^{13}C-^{2}H moieties.

^{13}C SPIN-LATTICE RELAXATION TIMES OF PEPTIDE HORMONES

Carbon-13 T_1 measurements are proving to be very useful indicators of the conformations of peptide hormones[1]. With conventional 25 MHz spectrometers they can be conveniently measured for most carbons of oligopeptides containing up to ten amino acids. For more complex peptides insufficiency of both sensitivity and dispersion becomes serious. This can be overcome by the use of spectrometers operating at higher frequency[18].

Measurement of the nuclear Overhauser enhancements for ^{13}C, and comparison of line widths (or spin-spin relaxation times, T_2) with T_1 Values[19], will indicate whether or not a given peptide has sufficiently rapid motions to justify use of the simplified form of equation [1],

$$\frac{1}{T_1} = \left\langle \frac{1}{r^6} \right\rangle N\hbar^2\gamma_C^2\gamma_H^2 \tau_{eff} \qquad [5]$$

A rough description of equation [5] is that the NT_1 value is proportional to the mobility of the carbon atom. When molecular motion is anisotropic, τ_{eff} must be broken down into components descriptive of motion about each of the principal axes[20]. Also, independent motion of some parts of the molecule relative to the overall motion of the molecule can take place. In this case separate correlation times for the two types of motion must be introduced[9]. In the general case more than two correlation times may be necessary to describe correctly the motion of a given ^{13}C-^{1}H vector. As the number of parameters required for the analysis increases, it becomes important to measure the T_1 values at more than one resonance frequency to ensure that the derived parameters constitute a unique solution.

Cyclic dipeptides provide one of the simplest systems for analysis via the ^{13}C T_1 method[21]. Cyclization decreases the tendency for segmental motion of the backbone,

and the molecular shape in most cases leads one to expect isotropic rotational motion. Usually, after correction for microscopic viscosity effects, the α-carbon T_1 values are those expected from the molecular size. Exceptions are found in the case of cyclic dipeptides containing glycine - the T_1 value of the α-carbon of glycine is always longer than that of the other amino acyl residue. This indicates rapid segmental motion of the glycine α-carbon relative to the rest of the peptide backbone, presumably due to the decreased steric hindrance in glycine arising from the lack of a side chain.

The neurohypophyseal hormones oxytocin and vasopressin provide a more complex example of the usefulness of ^{13}C T_1 values as indicators of molecular dynamics[22]. They are composed of a cyclic portion of six amino acids and an exocyclic tripeptide tail. The data for 8-lysine vasopressin are shown in Figure 2. The T_1 values of the

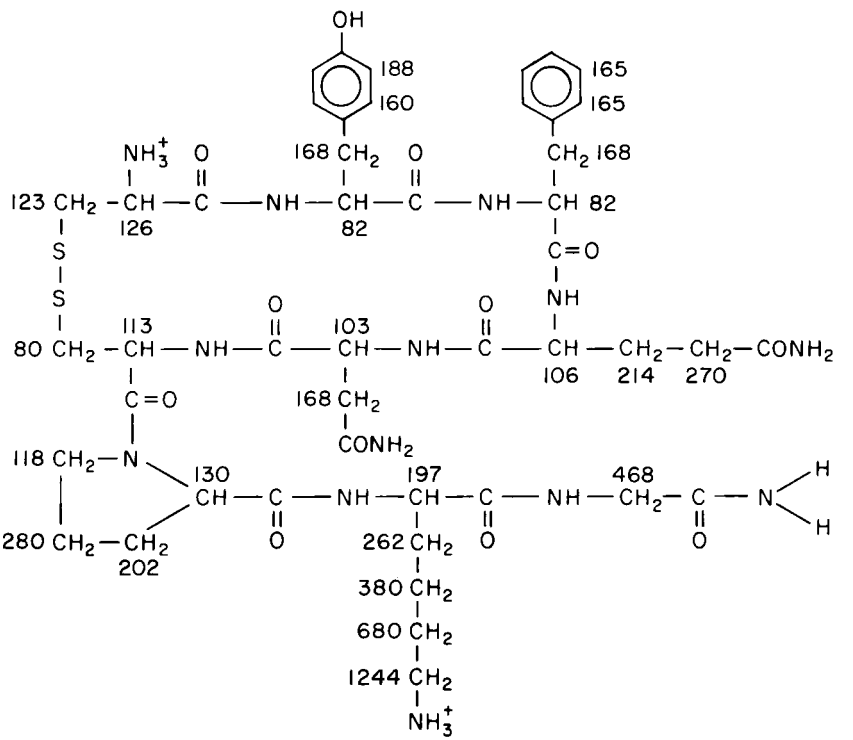

Figure 2: ^{13}C NT_1 values (msec) determined at 25 MHz and 30° for the carbon atoms of lysine vasopressin in D_2O, 100 mg/ml, "pH" 4.2[22].

α-carbons in the cyclic portion are essentially equal, whereas those in the tripeptide tail increase rapidly with distance from the cyclic portion. This is the behaviour expected if there is no rapid segmental motion within the cyclic backbone, and no restriction of the glycineamide moiety. The latter implies that a conformer with binding of the glycineamide to the cyclic portion can have only minor significance. The successively increasing NT_1 values for the lysine β- to ε-carbons are characteristic of segmental motion in this residue[2], implying no involvement in secondary structure for the ε-amino group. Notice that the NT_1 values of the β- and γ-carbons of the prolyl residue are considerably longer than those of the α- and δ-carbons. This demonstrates once again the phenomenon of rapid intracyclic motion of some carbon atoms in proline[16].

Angiotensin is a linear octapeptide of molecular weight similar to those of oxytocin and vasopressin. The absence of a covalent ring structure allows this peptide a much larger number of degrees of conformational freedom. It could exist as an extended chain, in which case the backbone NT_1 values should increase sequentially from the center of the peptide due to segmental motion, or as a folded structure held together by hydrogen bonds, in which case the NT_1 values of all the α-carbons would be very similar. The latter was found to be true for [5-isoleucine]-angiotensin II in aqueous solution[12]. The NT_1 values have an average value of 90 msec, much the same as found in the rigid cyclic moiety of lysine vasopressin. A slight increase was noticeable at both the amino and carboxamide ends suggestive of a loosening of the folded structure near the ends.

Luteinizing hormone-releasing hormone is a decapeptide. The ^{13}C NT_1 values determined for it in aqueous solution are shown in Figure 3. Notice that despite its larger size, the NT_1 values of the α-carbons are considerably longer than those of lysine vasopressin. A correction for the higher magnetic field used in Figure 3 must be made; doing so, a T_1 value of 170 msec at 68 MHz is equivalent to 130 msec at 25 MHz. Even after this correction, the larger NT_1 values for luteinizing hormone-releasing hormone imply that it is conformationally much less rigid than lysine vasopressin. The increase in NT_1 values of the α-carbons from the center of the peptide to the ends is characteristic of segmental motion. A detailed analysis of the 25 MHz and 68 MHz data will appear shortly[18].

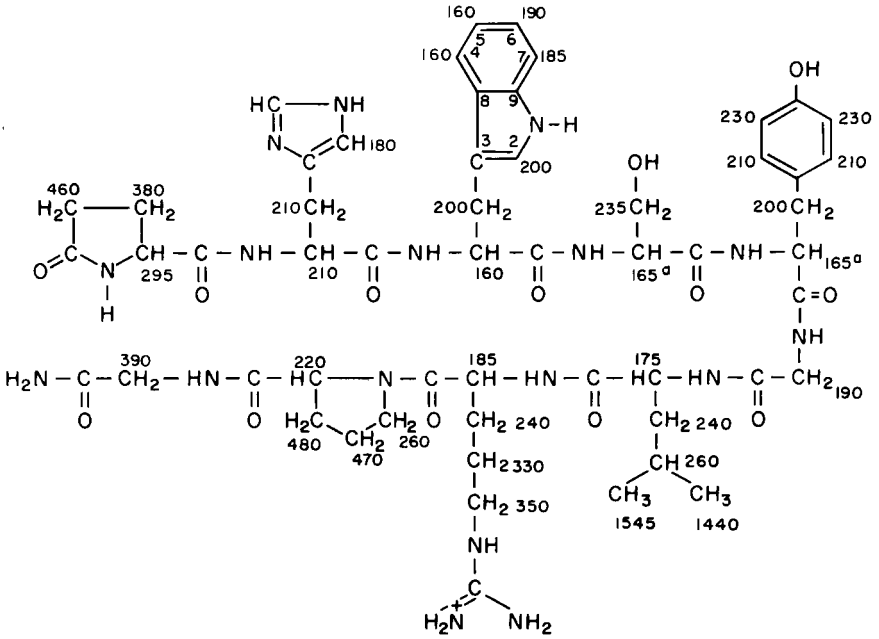

Figure 3: ^{13}C NT_1 values (msec) determined at 68 MHz and 35° for the carbon atoms of luteinizing hormone-releasing hormone in D_2O, 200 mg/ml, "pH" 5.5. The superscript *a* indicates NT_1 values derived from a composite resonance[18].

Conclusion

The dominance of the ^{13}C-^{1}H dipole-dipole mechanism for ^{13}C spin-lattice relaxation in proline has been established. Using this mechanism, the T_1 values for ^{13}C in a variety of peptides can be interpreted in terms of conformational mobility. More detailed methods for decomposition of effective correlation times into values characteristic of the various types of possible motion are required, but much contemporary interest in this subject is apparent. The future for this approach to the conformational dynamics of peptides looks bright indeed.

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UTILIZATION OF PROTON AND CARBON-13 MAGNETIC RESONANCE IN THE EVALUATION OF POLYPEPTIDE SECONDARY STRUCTURE IN SOLUTION

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Introduction

IN THE PROCESS OF STUDYING BIOLOGICAL MOLECULES beginning with the isolation of a polypeptide or protein and characterization of its biological activity and ending with the knowledge of its three dimensional structure and the relationship of structure to function, a current barrier is the step from knowledge of explicit primary structure to details of secondary structure. Proton and carbon-13 magnetic resonance spectroscopy can be utilized to assess secondary structure in solution. For several years proton magnetic resonance (pmr) has been employed to identify those peptide NH moieties which are involved in intramolecular hydrogen bonding.[1-10] More recently it has been proposed that carbon-13 magnetic resonance (cmr) can be utilized in a similar manner to identify those peptide C-O moieties which are the second component of each secondary structural feature.[11-13] The pmr approach uses temperature and solvent dependence of peptide *NH* chemical shifts of assigned resonances coupled with αCH-NH coupling constant data. At this stage the cmr approach has effectively utilized solvent dependence of peptide *C*-O chemical shifts of assigned carbonyl carbon resonances in combination with information from the pmr approach.

In general nmr studies of polypeptide secondary structure are complicated by conformational changes associated with temperature and solvent perturbation studies and by side chains that can shield peptide NH's and carbonyls from solvent, that can catalyze peptide N*H* exchange and that can contribute local magnetic fields. Also it is necessary to know whether a specific peptide moiety formed of a gly-gly sequence, for example, might have inductive effects that cause the peptide carbonyl to respond significantly differently to solvent perturbations than that of another sequence such as val-pro. More specifically interpretation of temperature dependence of chemical shift data is complicated by temperature dependence of equilibria between conformations and of proton exchange processes. Such effects can, over substantial temperature ranges, be linear with temperature and give fortuitously high or low temperature coefficients of chemical shift. The solvent perturbation studies also suffer the complication of possible conformational changes (solvent induced) but in this case the curves in the absence of conformational change are rather characteristic for a given solvent pair and variation from this characteristic form is indicative of conformational changes with changes in mole fraction of solvent.

While the above complications, as well as others not discussed, cause significant limitation to the rote use of pmr and cmr for evaluation of secondary structure, the awareness of these limitations does not justify the viewpoint that it is not possible to detail intramolecular hydrogen bonding of polypeptides by nmr. It would be a pernicious loss of critical information if this capacity of nmr were not fully utilized. There are already many examples where secondary structures, deduced by nmr methods, have subsequently or independently been substantiated by x-ray diffraction data.[5,14-16] Our purpose here is not to review the literature on nmr and polypeptide secondary structure but to outline the information that the approach can provide by considering a relatively rigid model cyclic peptide and to assess the relative constancy of peptide carbonyl solvent shifts for a series of dipeptides.

Transitions in the State of Peptide Moieties During Perturbation Studies

The state of a peptide moiety may be changed either by a change in temperature or by a change in the solvent. Such a change of state may or may not involve a conformational change. During temperature and solvent perturbation studies there are four transitions, seen as changes in chemical shift, that may occur to a peptide carbonyl, $CO_{se} \rightarrow CO_{se}$, $CO_{se} \rightarrow CO_{ss}$, $CO_{ss} \rightarrow CO_{se}$ and $CO_{ss} \rightarrow CO_{ss}$ where se = solvent exposed and ss = solvent shielded. Our interest, of course, is in the case where solvent shielding is due to intramolecular hydrogen bonding. For each of these four transitions of the carbonyl group, the peptide NH portion of the peptide moiety may also undergo four transitions. The total of sixteen possible transitions are listed in Table I. A thorough understanding of this approach would entail the following:

Table I

Transitions in the State of Peptide Moieties During Perturbation Studies

A1	$CO_{se}\ NH_{se}$	$\longrightarrow CO_{se}\ NH_{se}$	B1	$CO_{se}\ NH_{se}$	$\longrightarrow CO_{ss}\ NH_{se}$
A2	$CO_{se}\ NH_{se}$	$\longrightarrow CO_{se}\ NH_{ss}$	B2	$CO_{se}\ NH_{se}$	$\longrightarrow CO_{ss}\ NH_{ss}$
A3	$CO_{se}\ NH_{ss}$	$\longrightarrow CO_{se}\ NH_{se}$	B3	$CO_{se}\ NH_{ss}$	$\longrightarrow CO_{ss}\ NH_{se}$
A4	$CO_{se}\ NH_{ss}$	$\longrightarrow CO_{se}\ NH_{ss}$	B4	$CO_{se}\ NH_{ss}$	$\longrightarrow CO_{ss}\ NH_{ss}$
C1	$CO_{ss}\ NH_{se}$	$\longrightarrow CO_{se}\ NH_{se}$	D1	$CO_{ss}\ NH_{se}$	$\longrightarrow CO_{ss}\ NH_{se}$
C2	$CO_{ss}\ NH_{se}$	$\longrightarrow CO_{se}\ NH_{ss}$	D2	$CO_{ss}\ NH_{se}$	$\longrightarrow CO_{ss}\ NH_{ss}$
C3	$CO_{ss}\ NH_{ss}$	$\longrightarrow CO_{se}\ NH_{se}$	D3	$CO_{ss}\ NH_{ss}$	$\longrightarrow CO_{ss}\ NH_{se}$
C4	$CO_{ss}\ NH_{ss}$	$\longrightarrow CO_{se}\ NH_{ss}$	D4	$CO_{ss}\ NH_{ss}$	$\longrightarrow CO_{ss}\ NH_{ss}$

Unique Transitions

A1	B1 = -C1	D1
A2 = -A3	B2 = -C3	D2 = -D3
A4	B3 = -C2	D4
	B4 = -C4	

se = solvent exposed ss = solvent shielded

a knowledge of the chemical shifts, both in pmr and cmr, resulting from each of these transitions whether elicited by temperature or solvent. A closer look at Table I shows that for a given solvent pair some transitions are the negative of others, that is A2 = -A3, D2 = -D3 and the set of B transitions have negative counterparts in the set of C transitions. This results in ten unique transitions.

Whenever conformational changes occur during the perturbation studies the set of transitions in Table I need consideration, but when there is evidence that the conformation of a peptide is the same in two solvents of interest or unchanged in this respect over a given temperature range then only transitions A1, A4, D1 and D4 need be considered. From the model system gramicidin S we have values for A4 and D1. Some of these data are presented below. A study of N-acetyl-O-methyl dipeptides provides information on A1 and the valinomycin-K^+ complex allows some limited consideration of D4.

Proton Magnetic Resonance Delineation of Polypeptide Secondary Structure

The primary and secondary structure of gramicidin S (GS) is considered to be as given in Figure 1.[17-19]

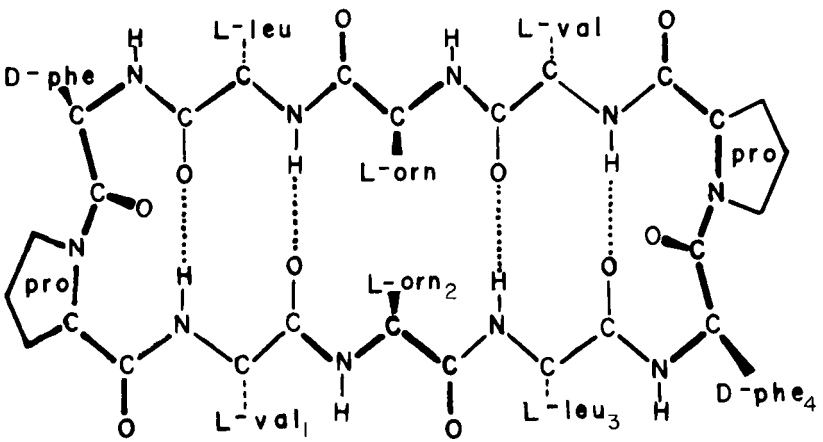

Figure 1: β-conformation of gramicidin S.

On hydrogenation of GS[20] D-phenylalanine is converted to D-β-cyclohexylalanine to give 4-4'-Di-β-cyclohexylalanyl gramicidin S (Cha GS). The hydrogenated derivative has the advantage of no ring current effects from side chains; it still suffers, however, as a model due to the ornithyl side chains which can partially shield the peptide moieties from solvent.

The temperature dependence of peptide N*H* chemical shift of Cha GS is given in Figure 2 in three solvents and the solvent shift for each pair is given in Table II. The slopes for the Orn N*H* and Cha N*H* are seen to be steeper than for GS[21] because of the presence of residual acetate from the reduction reaction. The steeper slope results from acetate catalyzed exchange of peptide N*H*'s with traces of water in the solvent. The steeper slope is because the water resonance is upfield of the peptide N*H* resonances. This is an important point to be aware of when attempting comparisons between studies; it is usually evident because of the broadening of the resonance. With increasing temperature the resonance continues to broaden and move upfield. In DMSO-d_6 the Orn N*H* resonance was too broad, even at low temperature, to follow. In all solvents the Val N*H* and Leu N*H* resonances exhibit closely parallel slopes which are similar to those of GS with the exception that the slopes are identical in DMSO-d_6 for Cha GS. The difference in these slopes for GS in DMSO-d_6 is likely due to ring current effects of the vicinal Phe residue on the Val N*H* in GS. The capacity to delineate between peptide N*H* moieties which are solvent exposed from those which are solvent shielded by intramolecular hydrogen bonding is most evident.

The solvent dependences of chemical shift of Cha GS peptide N*H*'s are given in Table III-A and in Figure 3 for three solvent pairs. The delineation between solvent exposed and solvent shielded peptide N*H*'s is perhaps more dramatic with solvent than with temperature perturbation and the delineation is most apparent when trifluoroethanol is one of the solvent pairs.

Carbon-13 Magnetic Resonance Delineation of Polypeptide Secondary Structure

The Cha GS peptide carbonyl carbon chemical shifts resulting from solvent transitions are given in Table III-B. These values were derived from the titrations given in Figure 4. The upper set of curves are those of GS

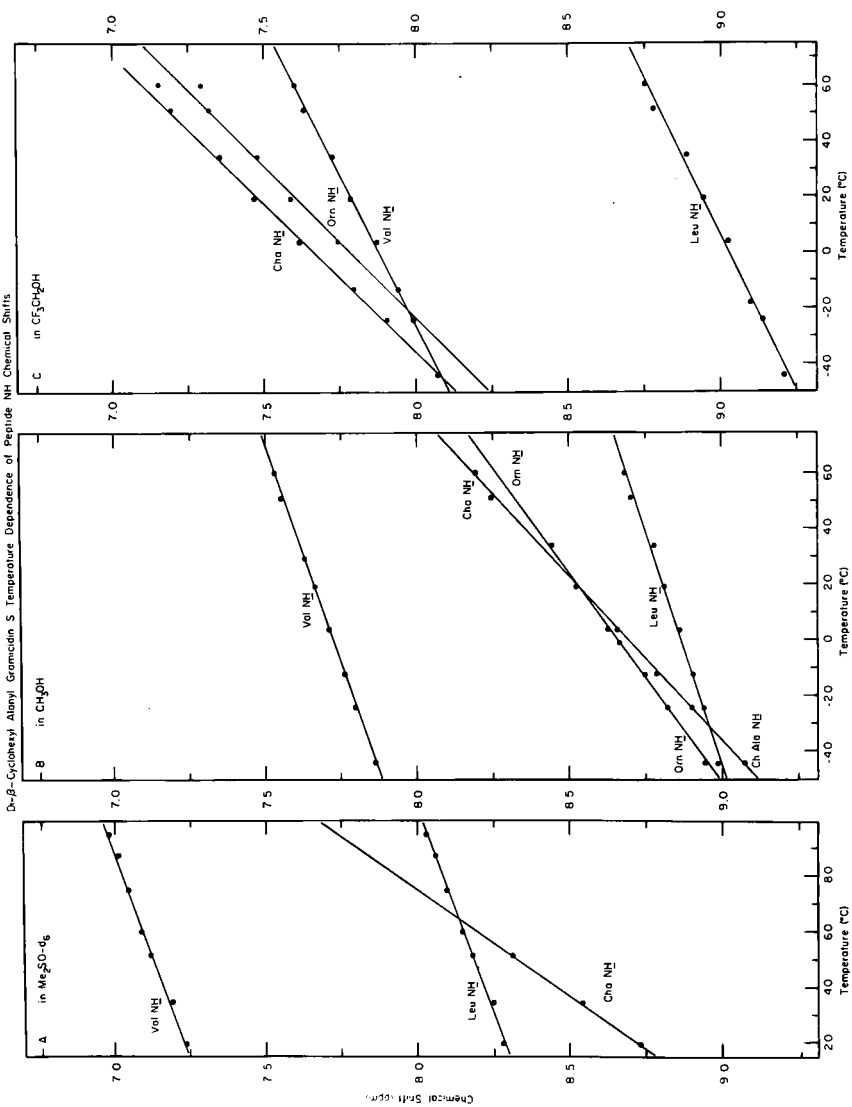

Figure 2: Temperature dependence of peptide NH chemical shifts.

Table II

Temperature Dependence of 4-4'-Di-β-Cyclohexylalanyl Gramicidin S Peptide *NH* Chemical Shifts in Several Solvents

PEPTIDE RESIDUE	DMSO-d_6		CH_3OH		CF_3CH_2OH	
	Slope ppm/°C	0°C Intercept ppm	Slope ppm/°C	0°C Intercept ppm	Slope ppm/°C	0°C Intercept ppm
Val_1 NH	-0.0034 (-7.4)[a]	7.3 (1605)[b]	-0.0031 (-6.9)[a]	7.7 (1699)[b]	-0.0046 (-10.1)[a]	7.9 (1734)[b]
Orn_2 NH			-0.0066 (-14.5)	8.7 (1906)	-0.0092 (-20.2)	7.8 (1714)
Leu_3 NH	-0.0034 (-7.5)	8.4 (1838)	-0.0029 (-6.5)	8.9 (1951)	-0.0045 (-9.9)	9.0 (1987)
Cha_4 NH	-0.0131 (-28.8)	9.0 1977	-0.0084 (-18.5)	8.7 (1914)	-0.0093 (-20.5)	7.7 (1687)

a. The values in brackets are the temperature coefficients in units of Hz/10°C.
b. The values in brackets are the intercepts in Hz.

Table III

Solvent Dependence of 4-4'-Di-β-Cyclohexylalanyl Gramidicin S

A. PEPTIDE NH CHEMICAL SHIFTS[a]

PEPTIDE RESIDUE	Chemical Shift, δ[b]			Solvent Shifts, Δδ[c]		
	1 $DMSO\text{-}d_6$	2 CH_3OH	3 CF_3CH_2OH	1 → 2	1 → 3	2 → 3
Val_1 NH	7.22	7.65	7.80	0.43 (0.00)	0.58 (0.00)	0.15 (0.00)
Orn_2 NH	8.57	8.52	7.61	-0.05 (-0.48)	-0.96 (-1.54)	-0.91 (-1.06)
Leu_3 NH	8.28	8.83	8.95	0.55 (0.12)	0.67 (0.09)	0.12 (-0.03)
Cha_4 NH	8.75	8.54	7.50	-0.21 (-0.64)	-1.25 (-1.83)	-1.04 (-1.19)

B. PEPTIDE CARBONYL CARBON CHEMICAL SHIFTS

PEPTIDE[d] RESIDUE	Resonance	Chemical Shift, δ[b]			Solvent Shifts, Δδ[e]		
		1 $DMSO\text{-}d_6$	2 CD_3OD	3 CF_3CD_2OD	1 → 2	1 → 3	2 → 3
Val_1 C-O	c	170.65	172.40	173.13	1.75 (-0.14)	2.48 (0.05)	0.73 (0.19)
Orn_2 C-O	d	170.52	173.13	174.01	2.61 (0.72)	3.49 (1.06)	0.88 (0.34)
Leu_3 C-O	a	171.97	173.86	174.40	1.89 (0.00)	2.43 (0.00)	0.54 (0.00)
Cha_4 C-O	e	170.37	173.47	175.51	3.10 (1.21)	5.14 (2.71)	2.04 (1.50)
Pro_5 C-O	b	171.34	173.86	174.93	2.52 (0.63)	3.59 (1.16)	1.07 (0.53)

a. These values are the average of several runs.
b. Chemical shifts are given in ppm with respect to an internal reference of Tetramethylsilane (TMS).
c. Solvent shifts in brackets are given with respect to Val NH.
d. With the exception of resonances b and e these assignments are derived from those of Sogn, J.A., Craig, L.C., Gibbons, W.A., JACS, 96, 3306, 1974 for Gramicidin S by comparison of titration data of Gramicidin S with that of the Cha derivative.
e. Solvent shifts in brackets are given with respect to the Leu C-O which is the carbonyl that shifts downfield least on addition of CF_3CD_2OD i.e. $\Delta\delta_j = \delta_j$ (C-O in proton donating solvent) $-\delta_j$ (C-O in Me_2SO) $-\Delta\delta$ (Leu C-O). Positive values indicate a greater downfield shift.

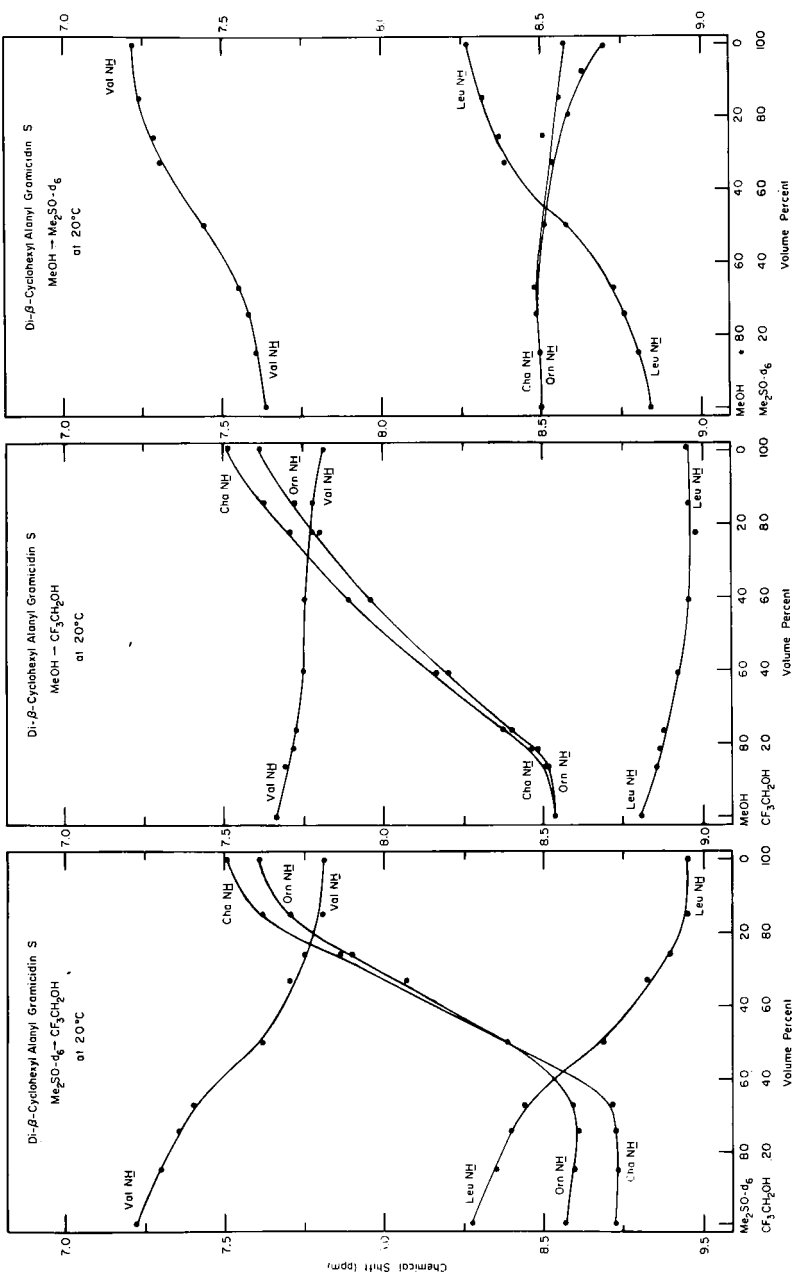

Figure 3: Solvent dependence of peptide *NH* chemical shifts of 4-4'-Di-β-cyclohexylalayl gramicidin S.

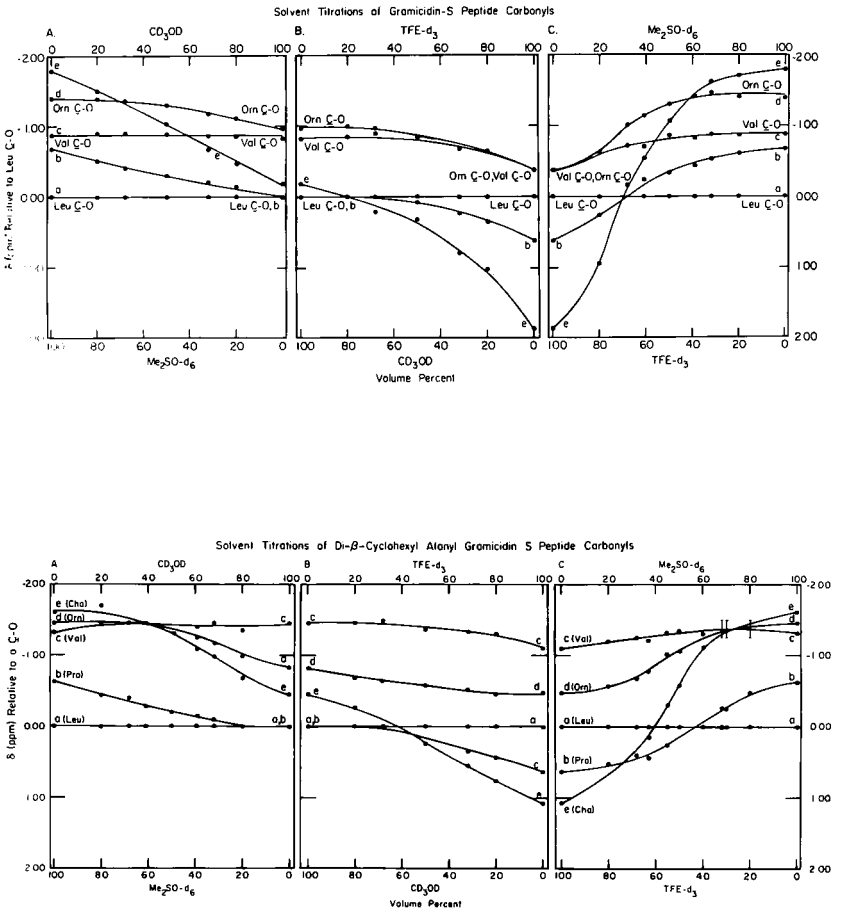

Figure 4: Solvent titrations (cmr) plotted with respect to the Leu C-O resonance (*a*) which shows the least solvent sensitivity. The upper curves are from reference 21.

and the labelled assignments are from Sogn *et al.*[22] It should be noted, however, that the delineation of the Leu *C*-O and Val *C*-O resonances as resonances *a* and *c* was achieved on the basis of identifying shielding due to intramolecular hydrogen bonding by titration studies[11] prior to the publication of the assignments based on enrichment studies.[22] The lower set of curves in Figure 4 are those of Cha GS and the assignments of resonances *a*, *c* and *d* are by comparison with the upper set of curves. The five resonances of GS and Cha GS tend to pair in their solvent perturbation behavior with resonance *e* as the odd resonance. Since *e* is the odd resonance, since the four peptide *NH* resonances pair in their solvent responses (See Figure 3), and since hydrogenation causes the largest shift in resonance *e* in trifluoroethanol, resonance *e* is assigned to the Cha residue. Resonance *d*, the Orn *C*-O resonance, pairs with resonance *b* which is taken to be the Pro *C*-O resonance. The very large shifts exhibited by resonance *e* are are most likely due to distortions of the Cha-Pro peptide linkage from a planar and trans configuration.

The emphasis, so far, has been on peptide moieties in which one or the other end is hydrogen bonded. Consideration of amino and carboxyl blocked dipeptides allows estimation of Al transitions and importantly provides an estimate of the effect of sequence on the response of peptide carbonyls to changing solvent. Table IV contains

Table IV

Chemical Shifts[a] of Central Dipeptide Carbonyl Carbon

DIPEPTIDE	SOLVENT[b]						
	7 $CDCl_3$	1 DMSO-d_6	5 DMA[c]	6 NMA-d_6	2 CD_3OD	3 CF_3CD_2OD	4 D_2O
NAc-Gly(1-C-13)-Pro-OMe	167.07	167.02	167.70	168.71	169.44	170.07	170.46
NAc-Gly(1-C-13)-Ala-OMe	168.23	168.71	169.40	170.56	171.24	171.92	172.40
NAc-Gly(1-C-13)-Gly-OMe	169.21	169.55	170.13	171.34	172.16	172.79	173.28
NAc-Val(1-C-13)-Pro-OMe	171.00	170.17	170.85	171.77	172.79	173.37	173.52
NAc-Val(1-C-13)-Ala-OMe	170.90	171.04	171.53	172.65	173.47	173.96	174.59
NAc-Val(1-C-13)-Gly-OMe	171.63	171.58	172.26	173.43	174.25	174.88	175.46

a. Resolution is 0.05 ppm.

b. Internal tetramethylsilane (TMS) as reference except for D_2O where dioxane at 67.4 ppm was used.

c. Dimethylacetamide (DMA) contained 3.8% DMSO-d_6 (by volume) for locking.

data on six dipeptides in seven solvents. The range of chemical shifts exhibited by the linkage peptide carbonyl of the six dipeptides in a single solvent is greater than 4 ppm, and the range of chemical shifts of the linkage peptide carbonyl of a single dipeptide resulting from solvent effects is about 4 ppm. Different sequences obviously cause large changes in chemical shifts. The important question is whether different sequences have different solvent shifts. The solvent shifts exhibited by the linkage peptide carbonyl of each of the six dipeptides for five solvent pairs are given in Table V.

Table V

Solvent Shifts

for the Central Carbonyl Carbon of Dipeptides

DIPEPTIDE	SOLVENT SHIFTS[a]				
	1 → 2[b]	1 → 3[b]	1 → 4[b]	2 → 3[b]	5 → 6[b]
NAc-Gly(1-C-13)-Pro-OMe	2.42	3.05	3.44	0.63	1.01
NAc-Gly(1-C-13)-Ala-OMe	2.53	3.21	3.69	0.68	1.16
NAc-Gly(1-C-13)-Gly-OMe	2.61	3.24	3.73	0.63	1.21
NAc-Val(1-C-13)-Pro-OMe	2.62	3.20	3.35	0.58	0.92
NAc-Val(1-C-13)-Ala-OMe	2.43	2.92	3.55	0.49	1.12
NAc-Val(1-C-13)-Gly-OMe	2.67	3.30	3.88	0.63	1.17
Mean Value (Al)	2.54±0.09	3.15±0.12	3.61±0.18	0.61±0.06	1.10±0.09

a. Resolution alone could be expected to give variations of 0.1 ppm. Problems of adequate locking, particularly with solvents 2, 3 and 5, contribute to variations over that of resolution.

b. For identification of solvent see Table IV.

The striking feature of Table V is the constancy of the solvent shifts exhibited by each of the six dipeptides for a given solvent pair. While the chemical shifts in Table IV cover a range of 8.44 ppm, the standard deviations in the solvent shifts of the six dipeptides to a given solvent pair is of the order of 0.1 ppm with a mean value for the solvent shift, for example of the DMSO-d_6-trifluoroethanol solvent pair, of 3.15 ppm. The solvent shift differences exhibited by the peptide carbonyls of Cha GS for

the DMSO-d_6 → trifluoroethanol solvent transition are an order of magnitude greater with a standard deviation of 1.0 ppm for a mean value of 3.43 ppm. The constancy of the solvent shifts, for a given solvent pair regardless of the sequence of the dipeptide and the order of magnitude difference exhibited by the carbonyls of Cha GS, indicate the validity of the cmr solvent shift approach to evaluate secondary structure.

Acknowledgments

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THE STUDY OF PEPTIDES BY INDOR, DIFFERENCE NMR AND TIME-RESOLVED DOUBLE RESONANCE TECHNIQUES

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Introduction

THERE HAVE BEEN SEVERAL REPORTS OF the use of difference NMR and double resonance to analyze peptide NMR spectra and consequently deduce structural and dynamic information.[1-8] Here we wish to report further uses of homonuclear internuclear double resonance (INDOR)[3,4] to simplify complex spectra and to detect *both* scalar decoupling and Nuclear Overhauser Effects (NOE's) between protons. A novel combination of prepulse gated decoupling[9-12] (time-resolved double resonance) and difference NMR* is also reported which enabled us to obtain spectra containing *only* the NOE's between individual gramicidin-S protons and protons in their immediate

*The pulse methods reported here are identical to those pioneered by Freeman and his co-workers[9,10,12] and Johnson.[11] These workers, however, did not apply difference methods, nor did they use INDOR to confirm or complement their NOE results.

spatial environment. This latter technique is therefore complementary to the INDOR-NOE method also described here which yielded both scalar decoupling and NOE effects and a previous technique[7] which yielded spectra containing predominantly scalar decoupling effects. The application of these techniques and the NOE to peptide conformation and sequence determination is described.

Experimental[9-13]

All reported spectra were taken on the Cameca 250 MHz instrument. The INDOR spectra were taken in the continuous-wave (CW) mode of operation using a proton lock and were the result of a single scan. The frequencies f_1 and f_2 correspond to the monitoring and perturbing frequencies, respectively.[13] The difference double-resonance spectra were obtained by applying the two-second pulse to saturate each proton resonance in turn. After a variable delay, a 90° sampling pulse was used to record the spectra. When the variable delay was greater than 2 seconds, the spectrum was indistinguishable from a non-decoupled spectrum. When the variable delay was in the range of 50 microseconds to 1 second, partial saturation of the resonance being irradiated could be observed (see Figure 1). To obtain *only* the NOE's the 50-microsecond delay spectra were subtracted from the 2-second delay spectrum. In this way the Bloch-Siegert Effect was eliminated. Two identical spectra could be subtracted with an accuracy of greater than 2%. Only effects that were greater than 10% are reported. All percentage figures refer to intensity changes relative to the corresponding ordinary single-resonance spectrum.

This method of obtaining NOE's, combining as it does time-resolved double resonance and difference NMR, should be contrasted with other pulse decoupling methods in which the decoupler is switched on during the data accumulation (albeit with a relevant duty cycle).[7] It was possible to separately obtain difference spectra showing only NOE's by virtue of the different times for both establishment and decay of multiplet collapse and the NOE. The time scale of the latter is of the order of T_1, whereas that of the former (because the mechanism of coupling involves chemical bonds) is by comparison instantaneous. A delay of 50 microseconds between pulses allows the multiplet to recover its structure, but it took about two seconds for the NOE to decay.

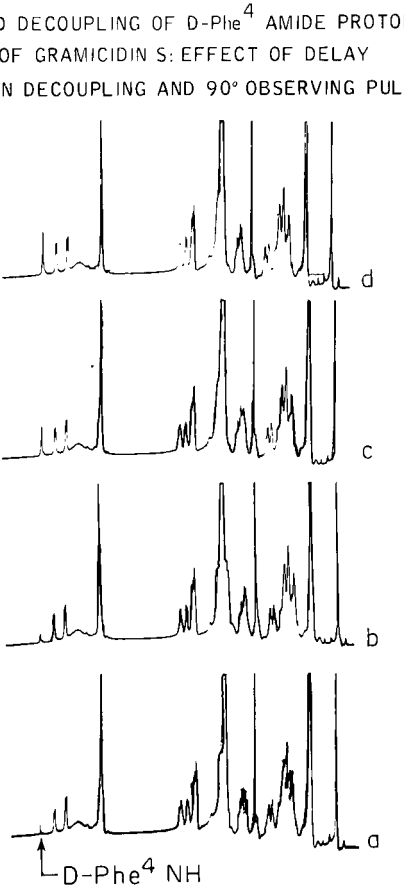

Figure 1: 250-MHz PMR spectra of gramicidin S in DMSO-d_6, taken with the following sequence: decoupling pulse, variable delay, 90° pulse, accumulation time.

a. Normal Fourier NMR spectrum.
b. Variable delay = 50 μsec.
c. Variable delay = 1 msec.
d. Variable delay = 100 msec.
e. Variable delay = 2 sec.

The decoupling frequency was at the center of the D-Phe^4 NH resonances.

Results and Discussion

I. INDOR-NOE Results for Gramicidin S

Expanded versions of the amide proton and the C^{α} proton region of the 250-MHz proton NMR spectrum of gramicidin S are shown in Figures 2a and 3a, respectively. The assignments are those originally determined by spin decoupling at several temperatures.[14]

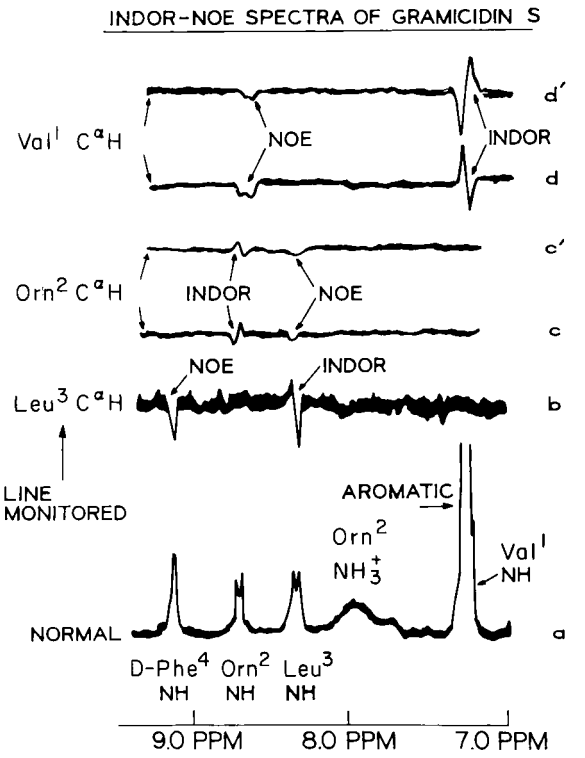

Figure 2: 250-MHz proton INDOR-NOE spectra of gramicidin S in DMSO-d_6.

a. The amide-aromatic proton region of gramicidin S in DMSO-d_6.
b. INDOR spectrum obtained by monitoring one of the Leu^3 $C^{\alpha}H$ resonances.
c and c'. INDOR spectrum obtained by monitoring one of the Orn^2 $C^{\alpha}H$ resonances.
d and d'. INDOR spectrum obtained by monitoring one

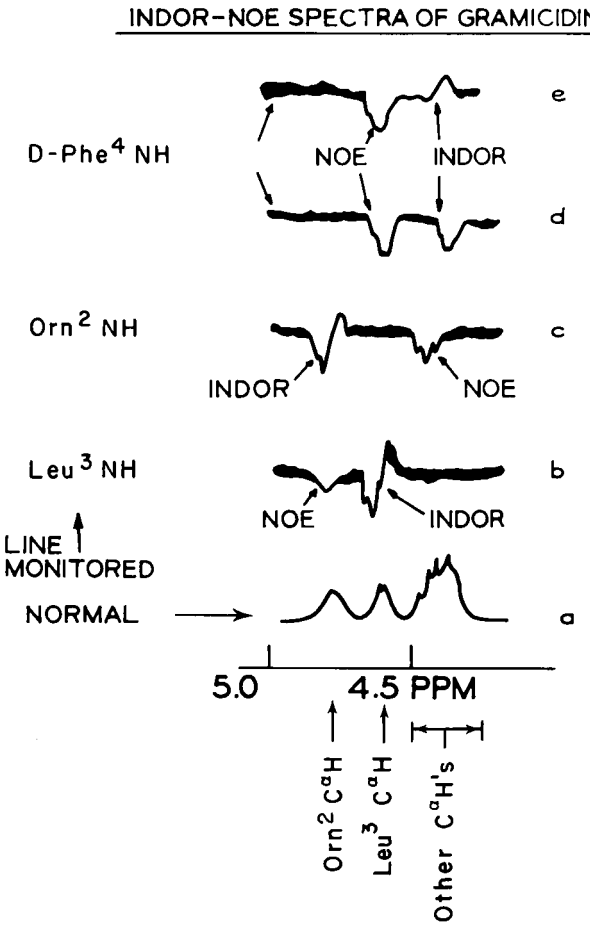

Figure 3: 250-MHz proton INDOR-NOE spectra of gramicidin S in DMSO-d_6.

a. Expanded version of the C^{α} proton region of the PMR spectrum of gramicidin S.
b. INDOR spectrum obtained by monitoring the one of the Leu^3 NH resonance lines.
c. INDOR spectrum obtained by monitoring the one of the Orn^2 NH resonance lines.
d&e. INDOR spectrum obtained by monitoring each of the D-Phe^4 NH resonance lines.

By monitoring the low-field line of the Orn^2_{α} amide proton doublet with f_1 and scanning the whole C^{α} proton region with f_2, the INDOR spectrum (shown in Figure 3c) was obtained. The low-field multiplet containing both positive and negative lines, corresponds to the $C^{\alpha}H$ multiplet of Orn^2. The high-field multiplet contains only negative lines and corresponds to the $C^{\alpha}H$ multiplet of Val^1. Care was taken to insure that monitoring of the high-field line of the Orn^2 NH doublet changed the sign of the INDOR lines of Figure 3c, while the Val^1 $C^{\alpha}H$ multiplet still exhibited a negative NOE.

The spectrum of Figure 3b was obtained by monitoring the low-field line of the Leu^3 amide proton doublet while perturbing the $C^{\alpha}H$ region. The expected result, if the $Val^1 \rightarrow Orn^2 \rightarrow Leu^3$ tripeptide forms a β-pleated sheet sequence, was obtained, viz., an Overhauser Effect for the Orn^2 C^{α} proton and the usual INDOR spectrum of the Leu^3 C^{α} proton itself. It is significant that again in this experiment the only C^{α} proton exhibiting the NOE effect was the Orn^2 C^{α} proton. These experiments were easily reciprocated, as seen in Figure 2, by monitoring the $C^{\alpha}H$ multiplets--*e.g.*, monitoring the Val^1 C^{α} proton gave NOE effects only in the Orn^2 amide proton doublet shown in Figure 2d and d' and not in the Leu^3 or D-Phe^4 amide proton doublets; note that in both spectra the Orn^2 NH doublet did not manifest biphasic behavior and only decreased in intensity, thereby proving that we are detecting an NOE as distinct from the indirect through-bond coupling reflected by the biphasic INDOR signals.

II. Detection of NOE's by Prepulse Gated Decoupling (Time-Resolved Double Resonance)

The actual spectra resulting from irradiation of the Orn^2, Leu^3, and D-Phe^4 amide proton doublets of gramicidin S are shown in Figure 4 and the difference spectra obtained by subtracting these from the reference spectrum with a 2-second delay are shown in Figure 5. Only the effects observed in the C^{α} proton region will be discussed here, although interactions between the three amide protons and the solvent (DMSO-d_5 impurity), the H_2O impurity, TMS, and certain of the side-chain protons can also be detected in the difference spectra shown in Figure 5. Particularly important features of these spectra (Figure 5) can be summarized as follows:

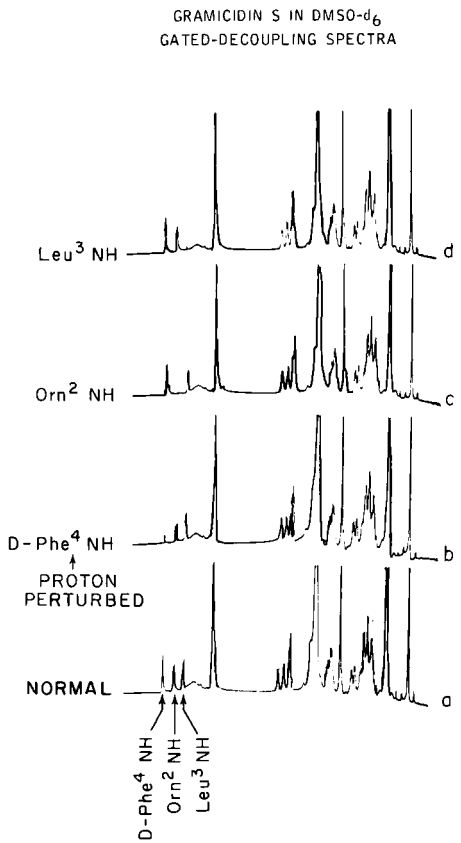

Figure 4: 250-MHz gated decoupling PMR spectra of gramicidin S in DMSO-d_6.

a. Reference spectrum taken with variable delay equal to 2 seconds and duration of decoupling pulse equal to 2 seconds.

b, c, and d. Spectra of gramicidin S in DMSO-d_6 obtained by prepulse gated decoupling with variable delay equal to 50 μsec and duration of decoupling pulse equal to 2 seconds: b, f_2 set on the D-Phe^4 NH doublet; c, f_2 set on the Orn^2 NH doublet; d, f_2 set on the Leu^3 NH doublet.

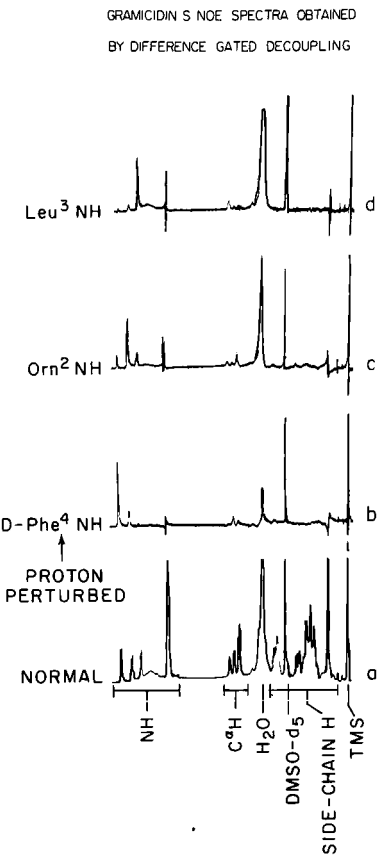

Figure 5: 250-MHz proton NOE spectra of gramicidin S in DMSO-d_6 obtained by difference gated decoupling.

a. Reference spectrum, as in Figure 4a.
b, c, and d. Difference spectra obtained by subtracting spectra of Figure 4b, c, and d, respectively, from their corresponding reference spectra.

1. Within experimental error (±5%) no detectable "decoupling" effects were observed between the amide and the C^{α} protons of D-Phe^4. NOE effects between backbone protons on neighboring residues were observed, however.
2. The NOE's detected agree qualitatively with those obtained by the complementary technique of INDOR (Figures 2 and 3).
3. No Bloch-Siegert Effect is detectable and errors due to incorrect cancellation of difference spectra are less than 4%.
4. Most of the very small "NOE's" in the C^{α} proton region are attributable to partial saturation, say, of the Leu^3 and D-Phe^4 amide protons while irradiating the Orn^2 amide protons. These effects, however, can easily be corrected by data processing.

Quantitative estimation of distances between the amide protons of one residue and the C^{α} proton of a neighboring residue can be extracted from these NOE's as can amide-water proton exchange rate constants. These analyses are currently under way, but it suffices to say here that the negative NOE's detected between amide and C^{α} protons are largely the result of exchange effects mediated by scalar coupling; otherwise, these NOE's would be mainly positive.[15]

III. The NOE and Peptide Structure

Conformational information, while still qualitative, can be extracted from the INDOR and difference double resonance spectra reported here--*e.g.*, (1) the detection of NOE's between the backbone protons of the Val^1, Orn^2, and Leu^3 residues is consistent with their being part of a β-pleated sheet structure, and (2) the NOE between the Leu^3 C^{α} and D-Phe^4 amide protons is consistent with the Leu^3 → D-Phe^4→ Pro^5→ Val^6 sequence being a β-turn. We have also detected an NOE between the C^{α} proton of D-Phe^4 and Pro^5, a finding which is consistent with a Type II' (but not Type I') β-turn for gramicidin S.[16,17]

Peptide sequencing is also possible by combining the various double resonance methods as is clearly shown by the sequences of NMR experiments: (1) Irradiation of Val^1 NH gives an NOE at the Orn^2 C^{α}H. The latter proton was then decoupled from the Orn^2 NH by INDOR or conventional Fourier techniques. (2) The Orn^2 NH when irradiated produced an NOE at the Leu^3 C^{α}H. (3) A connection between residues 3 and 4 was established by an NOE between the Leu^3 C^{α}H and D-Phe^4 NH. (4) The D-Phe^4 C^{α}H was decoupled from its own D-Phe^4 NH and exhibited an NOE with one of the Pro^5 C^{α}

protons. In this way double resonance confirmed the sequence $Val^1 \rightarrow Orn^2 \rightarrow Leu^3 \rightarrow D\text{-}Phe^4 \rightarrow Pro^5$! The advantages of this technique for unequivocally identifying individual resonances when a peptide contains more than one residue of the same species of amino acid is obvious--*e.g.*, this method could be used to assign unequivocally the various proton resonances of the four aromatic residues of tyrocidine A. The only alternative method of assignment is synthesis of isotopically-labeled analogs of such peptides.

Summary

Specifically, in this publication we have (1) demonstrated a new use for homonuclear INDOR, viz. the detection of NOE's between protons of different residues, (2) obtained difference NMR spectra of gramicidin S by time-resolved double resonance (these latter spectra contain *only* NOE's --indirect effects due *purely* to through-bond coupling are eliminated!), (3) demonstrated that double resonance can in some circumstances be used to sequence peptides, and (4) qualitatively confirmed (by the NOE) that gramicidin S contains an antiparallel β-pleated sheet and a Type II' β-turn.

Of particular general importance and explicitly contained herein are NOE criteria for antiparallel β-structures and Type I' and II' β-turns and two novel uses of double resonance techniques and difference NMR, viz. the separation of through-space and through-bond coupling between protons, and the sequencing of peptides by double resonance. Not discussed in detail here (but implicit in the spectra) is the use of prepulse gated decoupling to study solute-solvent interactions (with gramicidin S and DMSO or DMSO-d_5) and solute-solute interactions (with gramicidin S and TMS or H_2O-impurity). Previous studies in this field involved irradiation of the solvent and not the individual protons of the molecule.[5,6,8,18] The extension to the study of side-chain-side-chain interactions is also readily envisaged. A more quantitative analysis of the data is in progress, especially to separate the scalar and dipolar contributions to the NOE.

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FOLDED CONFORMATIONS OF TETRAPEPTIDES IN HYDROGEN BONDING SOLVENTS

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IF β-TURNS SERVE AS NUCLEI for the tertiary folding of proteins,[1] it ought to be possible to demonstrate their intrinsic stability, in that some short, non-cyclic peptides should adopt this conformation when dissolved in hydrogen bonding solvents, including water. We have examined some tetrapeptide derivatives for evidence of such folding.

The most readily identified feature of a β-turn in solution is shielding, by the backbone of the turn, of the peptide (NH) proton of the fourth residue in the turn sequence.[2]

Such a sequestered proton is identified by a reduction in the sensitivity of its nuclear magnetic resonance to environmental perturbations. The commonly used perturbations are changes in temperature or solvent, and many cyclic oligopeptides have thus been studied. However, for small peptides the total of the non-bonded intramolecular interactions is not large, and when there is no cyclic constraint, solvent or temperature variations are likely to modify the distribution of conformations, reducing their value as a probe of conformation. We have therefore tried to identify a sequestered proton using only a minor change in the environment.

Addition of low concentrations of a stable nitroxyl radical (here 3-oxyl-2,2,4,4-tetramethyloxazolidine) to peptide solutions produces nmr line broadening that is less for sequestered protons than for those exposed to solvent.[3] The radical is present at low mole fraction in the solvent, and in methanol or water its interaction with

the peptide molecule is likely to be no stronger than solvent-peptide interactions, so that the distribution of peptide conformations over which the nmr measurement averages is not likely to be much affected.

Results obtained from the peptides I-IV are described below.

I. Ac-Gly-d_2-L-Pro-D-Val-Gly-$NHNH_2$

II. Ac-L-Val-L-Pro-Gly-Gly-$NHNH_2$

III. Ac-Gly-d_2-L-Pro-L-Asn-Gly-$NHNH_2$

IV. Ac-Gly-d_2-D-Ala-L-Val-Gly-$NHNH_2$

The sequence of I is expected to be especially favorable for a β-turn because of the constraints introduced by the pyrrolidine ring of Pro^2 and by the side chain of the residue of opposite configuration that follows.[4,5] Recently we have shown that for Z-Gly-d_2-L-Pro-D-Ala (or Val)-Gly-NHNH-Boc in methanol, not only is the peptide proton of Gly^4 shielded from the solvent, but so also is that of Gly^1 (Figure 1).[6] This is consistent with a major contribution from a favored conformation containing the β-turn plus an additional pair of extended residues, as shown in Figure 1. Figure 2 shows the current result with the less hydrophobically terminated analog I. Peptide I shows a sequestered Gly^4 NH in methanol, but the NH of Gly^1 is not sufficiently shielded to give an observable effect.

Pro^2-Gly^3, as in peptide II, is another sequence expected to adopt the β-turn structure readily.[4,5,7,8] A structure that buries one of the glycine NH's is observably (see Figure 2) more favored for II in methanol than is a comparable folded structure for I, judged by the extent of the differential line broadening at a given radical concentration. What is more, the differential effect persists in aqueous solution of II, but not of I, suggesting that the N-terminal valine stabilizes a bend in the chain. There is a related experimental result: For aqueous solutions of the peptides, the resonance of the N-terminal NH disappears when the water resonance is saturated, *except* in the case of II. The valine NH resonance of II is not saturated in this manner, indicating that NH^1 in this case exchanges more slowly with solvent water.[9] This is not a strictly local effect resulting from the presence of a side chain. Model studies show that saturation is transferred from water protons to the NH protons of Ac-Val-$NHCH_3$

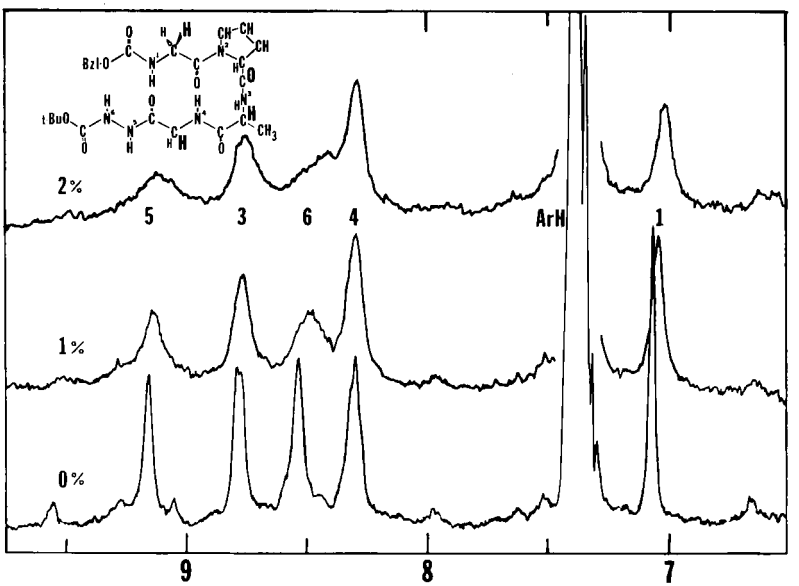

Figure 1: Effect of nitroxyl radical on N-H resonances of a methanol solution of Z-Gly-d_2-L-Pro-D-Ala-Gly-NHNH-Boc. A β-structure consistent with this result is shown.

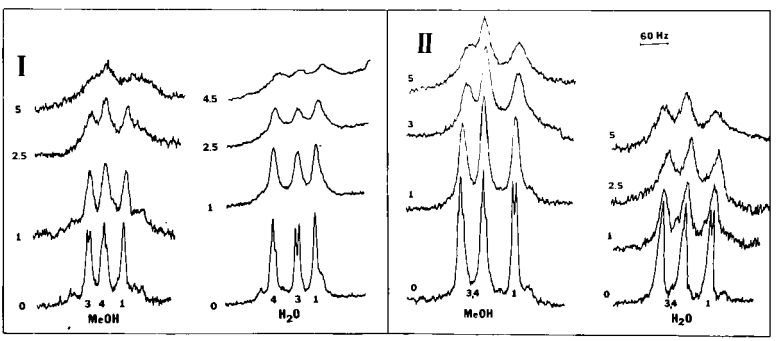

Figure 2: Effect of nitroxyl radical on N-H resonances of methanol and water (5% trifluoroacetic acid) solutions of I, Ac-Gly-d_2-L-Pro-D-Val-Gly-$NHNH_2$, and II, Ac-L-Val-L-Pro-Gly-Gly-$NHNH_2$. Volume per cent nitroxyl is indicated by the figures at left of the traces; numbering of residues is from the amino end.

as well as to those of Ac-Gly-$NHCH_3$. The slower exchange of NH^1 in II indicates some additional hindrance produced by the rest of the tetrapeptide molecule, and this argues for the contribution of a folded structure to the conformational average.

Figure 3 shows the effect of nitroxyl on the NH resonances of peptide III, which contains the Pro^2-Asn^3 sequence, one that is also likely to result in β-turns in

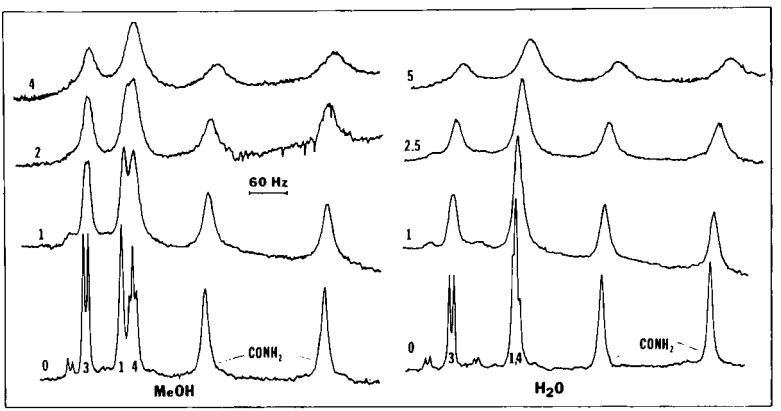

Figure 3: Effect of nitroxyl radical on N-H resonances of methanol and water (5% trifluoroacetic acid) solutions of IV, Ac-Gly-d_2-L-Pro-L-Asn-Gly-$NHNH_2$. Numbering as in Figure 2.

protein chains.[7,8] In water the backbone NH resonances of III broaden with radical as readily as do the carboxamide NH resonances; since at least one of the latter ought to be fully exposed to solvent, probably all of the NH's are. However, examination of the linewidths in the spectrum of a methanol solution shows that the NH of Gly^4, and perhaps that of Gly^1, are less susceptible to the effects of radical than is the NH of Asn^3, and that all three of the backbone protons are less exposed than the carboxamide protons. This is consistent with a type I β-turn contribution to the conformation in methanol.

The importance of the proline pyrrolidine ring in stabilizing a folded structure is readily established. When the constraint introduced by Pro^2 is removed but

the D^2-L^3 sequence is retained, as in peptide IV, there is no evidence for differential shielding of a peptide proton in methanol or water.

In summary, folding that sequesters a peptide proton, consistent with although not proving a β-turn backbone conformation, can be demonstrated for proline-containing tetrapeptide derivatives in methanol. In aqueous solution the folded conformations are less stable, and evidence for one has so far only been found for Ac-L-Val-L-Pro-Gly-Gly-$NHNH_2$.

Acknowledgment

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THE RELATIVE STABILITIES OF THE β-STRUCTURES OF MONODISPERSE SYNTHETIC LINEAR HOMO-OLIGOPEPTIDES WITH ALIPHATIC SIDE CHAINS

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SINCE THE PIONEERING STUDIES of Goodman and colleagues on the secondary structures of homo-oligoglutamates and aspartates by UV[1,2] and IR[3] absorption, ORD,[2] CD,[4] and NMR,[4] substantial work has been carried out in our laboratory on the synthesis and conformational properties both in the solid state and in solution of complete series of monodisperse, linear homo-oligopeptides derived from aliphatic amino acid residues. The aim of the present progress report is to summarize briefly the main results obtained and to indicate the unsolved problems in this particular field.

Seven series of protected homo-oligomeric peptides with aliphatic side chains, having the general formula Boc-(-L-X-)$_n$-OMe, where n=2-7 and X=Ala, Nva, Val, Leu, Ile, Cys(Me) and Met, were prepared by classical methods. We employed an urethane group (Boc) for the protection of the α-amino function and an alkyl ester (OMe) for the protection of the α-carboxyl function. The DCCI coupling method,[5,6] employing the N-protected amino acids, was used for preparing dimers and trimers; the Rudinger's acid azide method,[7] starting with N-protected dipeptide hydrazides, was used for preparing the higher oligomers. The Boc group was removed using TFA or HCl in anhydrous organic solvents. These methods are known to give monodisperse peptides of high chemical and optical purity in good yields.

The chemical purity of these oligopeptide series was assessed by C, H, N, and S elemental analytical data, thin layer chromatography in a number of solvent systems, and comparison of melting points where available. Furthermore, the linearity of the Goodman's plots (total molar rotation values of oligomers *vs* n)[8] in structure-disrupting solvents strongly suggested that little if any racemization occurred in the various synthetic steps.

We carried out a conformational analysis in the solid state on the aforementioned homo-oligopeptide series using IR absorption technique.[9] The results obtained indicate that all pentamers and higher oligomers assume essentially a β-form; trimers and tetramers present evidence of mixtures of β and unordered structures while dimers exist essentially as unordered structures. A marked difference in the propensity of the various series to attaining the β-conformation in the solid state was not unrevealed. However, the effect of molecular weight in directing peptide conformations was established; in fact, the ordered secondary structures taken by the oligomers derived from alanine, norvaline, leucine, and methionine and their respective homo-polymers are different, the former existing as β-forms while the latter as α-helical forms.[10] In this context it should be pointed out that we were not able to observe the presence of the vibrational bands characteristic of the α-helical structure in none of oligomers of the above mentioned four series. It is evident that they have a chain length below the critical one for α-helix formation in the solid state. In this study the amide I and amide V absorption bands turned out to be by far the most sensitive to conformational changes of the peptide chain.[11]

In addition, all oligopeptides exhibit a well-defined, although weak, bank near 1690 cm^{-1}. A band in this region is usually assigned to the parallel component of the splitted amide I band of polypeptides in the antiparallel β-form.[11] However, in the present case a conformational assignment on these bases would be ambiguous because we demonstrated that the urethane chromophore of the Boc N-protecting group also absorbs at about 1690 cm^{-1}.[9] Consequently, we were forced to remove this group from our oligomers (by HCl in anhydrous methanol). Preliminary IR results on the homo-heptamers in the ammonium form indicate that the 1690 cm^{-1} band is still present in the alanine, norvaline, leucine, S-methyl-cysteine, and methionine derivatives. In contrast, we did not obtain a clear-cut

proof of the occurrence of this band in the N-deblocked homo-heptamers derived from valine and isoleucine, which still do show evidence of β-structure formation.

Detailed clues to which secondary structure our linear aliphatic homo-oligopeptide series might assume in solution were obtained using CD. For these spectral investigations we employed essentially TFE since it is suitable for use in the far-UV and an effective structure-supporting solvent.[4] Among all protected oligopeptides examined only Ala_7, Val_7, Ile_7 and $Cys(Me)_7$ show evidence of CD curves typical of an ordered structure (at 1.2×10^{-3} M or higher concentration in TFE). The intense negative maximum in the 217-234 nm region and the more intense positive maximum in the 197-207 nm region strongly indicate the occurrence of β-type conformations.[12] The marked red shift of the overall CD spectrum of $Cys(Me)_7$ if compared with those of heptamers derived from amino acid residues with hydrocarbon side chains should be related to the contribution of the thioether optically active transitions located in the region of peptide absorption. The relative stabilities of the β-structures of the various heptamers as a function of concentration in TFE are illustrated in Figure 1. Clearly, the scale appears to be $Ile_7 > Val_7 > Cys(Me)_7 > Ala_7$.

Our CD study also showed that the ordered forms taken by the four heptapeptides in TFE can be destroyed by increasing the temperature (Figure 1) or adding HFIP, a solvent which is known to disrupt the secondary structures of oligopeptides. Also in these two cases the stabilities of the β-associated conformations decrease in the order $Ile_7 > Val_7 > Cys(Me)_7 > Ala_7$.

In addition, as water is added to solutions of Nva_7, Leu_7 and Met_7 in TFE, these heptamers also do show dichroic spectra indicative of β-structure formation. The stabilities are as follows: $Nva_7 > Leu_7 > Met_7$ (Figure 2). Again, dilution or heating disrupts these β-type conformations.

In summary, we demonstrated that all seven heptapeptides examined by us can exist as β-aggregated forms in solution, provided that solvent-solute interactions do not successfully compete. By means of optical titrations, in mixed solvents, we established that β-branching, presence of a heteroatom in γ-position, and small linear side chain all have a relevant effect in stabilizing β-aggregated structures of oligopeptides. It is important to note that these factors are also effective in inducing β-structure formation in high molecular weight peptide molecules.[10,13,14]

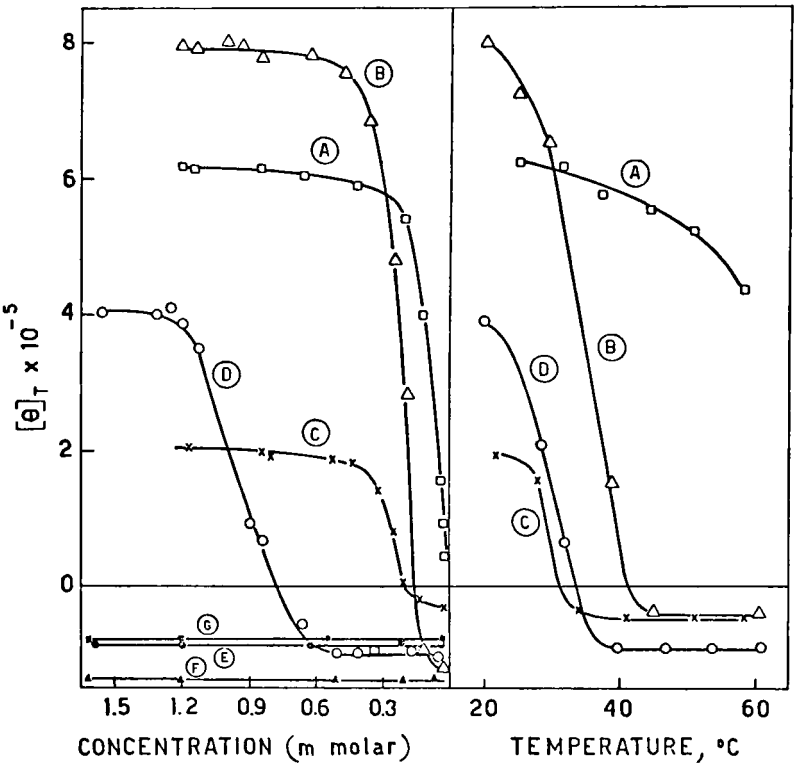

Figure 1: Left, total molar ellipticity values of protected homo-heptapeptides *versus* concentration in TFE at 21°C; *right*, total molar ellipticity values *versus* temperature at 1.2 x 10^{-3} M in TFE. A, Ile_7 (197.5 nm); B, Val_7 (202.5 nm); C, $Cys(Me)_7$ (207.5 nm); D, Ala_7 (197.5 nm); E, Nva_7 (200 nm); F, Leu_7 (202 nm); G, Met_7 (202 nm).

At this stage of our research one important point remains to be clarified. The wavelength maxima of the CD Cotton effects of protected Val_7 in TFE appear at 222 nm (negative) and 202 nm (positive). Thus, this spectrum can be classified as B-type according to Woody[15] or β-II-type according to Fasman and Potter.[16] However, Ala_7 exhibits the two CD maxima at 217 nm and 197 nm, respectively. These positions are typical of the A-type[15] or β-I-type[16] spectra. Interestingly enough, a single

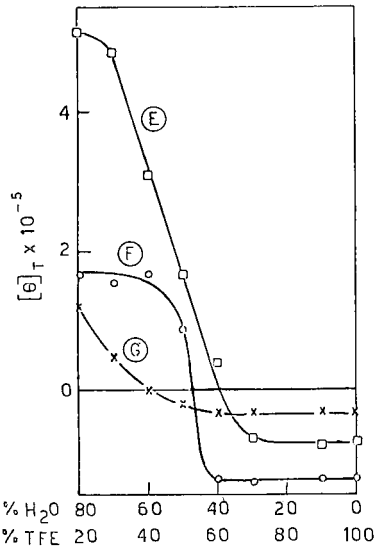

Figure 2: Total molar ellipticity values of protected homo-heptapeptides *versus* solvent composition (TFE-water) (conc. 0.3 x 10^{-3}M) at 21°C. E, Nva_7 (197.5 nm); F, Leu_7 (200 nm); G, Met_7 (197.5 nm).

preparation of Ile_7 showed both types of spectra. We believe that more detailed experimental investigations coupled with theoretical studies are needed to distinguish among the various possible explanations of this different behavior. In fact, the two types of CD spectra could arise because the oligopeptides have parallel or anti-parallel structures,[17-19] and/or different widths of their associated structures,[17,19,20] and/or different fractions of their residues in β-turn regions,[15] and/or different side-chain polarizabilities.[21] In particular, the solution of this problem is important for a correct prediction of protein conformation using CD data.[20]

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THE SOLUTION CONFORMATION OF BRADYKININ

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THE RECENT YEARS ARE WITNESS to an intensification of interest in the conformational states of peptide systems. Hitherto the most considerable progress in such studies has been for the cyclic members of this class, the considerably higher flexibility of the linear peptides making the acquisition of data concerning their structural organization a formidable task. The present paper deals with the spatial structure of a biologically active linear nonapeptide bradykinin:

1 2 3 4 5 6 7 8 9
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

The complexity of the problem caused us to make full use of our composite approach involving a variety of spectral methods combined with theoretical conformational analysis for its solution.

The CD curves of bradykinin (Figure 1) display chiroptical effects at 200 nm (amide $\pi \longrightarrow \pi^*$ transition) and a series of aromatic $A_{1g} \rightarrow B_{2u}$ bands at 250-270 nm (not shown in the Figure). The 230 nm band is due to the amide $n \longrightarrow \pi^*$ transition, and at 222 nm at least partially to the $A_{1g} \rightarrow B_{1u}$ transition of the phenyl groups. The latter assignment is confirmed by disappearance of the band in the cyclohexyl-bradykinin analog we have synthesized (Figure 1a). As can be seen from Figure 1, the position

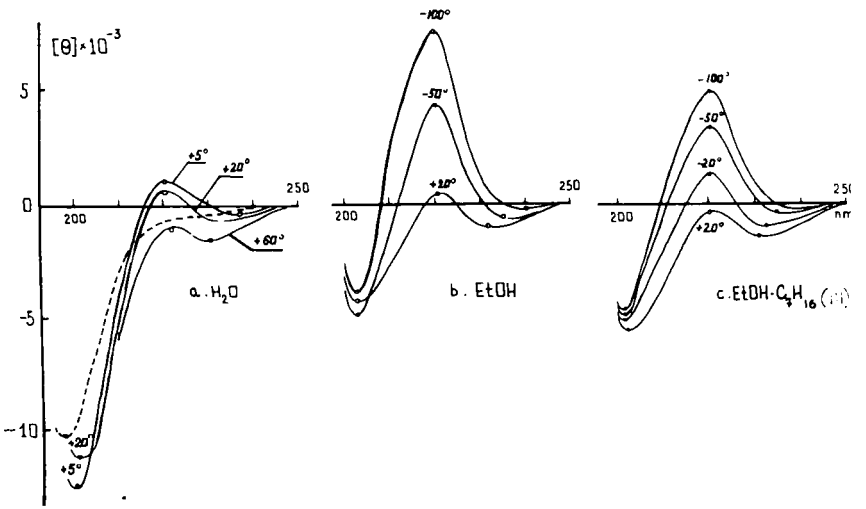


Figure 1: CD-curves of bradykinin (——) and 5,8-cyclohexylalanyl-bradykinin (---).

and sign of the Cotton effects are only weakly solvent dependent; the curve for the aqueous solution undergoing practically no change over the pH range 1.5-9.0 and for concentrations from 10^{-2}-10^{-4} *M*. It can thus be inferred that the hormone has a highly stable spatial organization and a low tendency to form aggregates. In all the solvents investigated, heating of the solution weakens the positive 222 nm band while cooling causes its intensification. This effect is most probably due to the phenyls assuming a definite spatial orientation with respect to the peptide backbone on cooling and its "disordering" on heating.

It has been shown on a large number of examples that the chemical shifts of the proline $^{13}C^{\beta}$ and $^{13}C^{\gamma}$ atoms in peptides with *trans* X-Pro bonds equal 30,5 ± 0,6 ppm and 25,1 ± 0,5 ppm (from TMS), whereas for *cis* amide bonds - 32,2 ± 0,4 ppm and 23,4 ± 0,3 ppm corresponding[1]. In the ^{13}C NMR spectra of bradykinin we have taken the C^{β} shifts are 29,0 - 30,6 ppm and the C^{γ} shifts are 25,3 - 25,9 ppm (figure 2) over the entire pD interval investigated which unequivocaly indicates on all-*trans* configuration of the X-Pro bonds of bradykinin in aqueous solutions.

An important conclusion followed from the study of the pH dependence of the bradykinin ^{13}C shifts. As could been expected, a clear cut titration picture of the NH_2

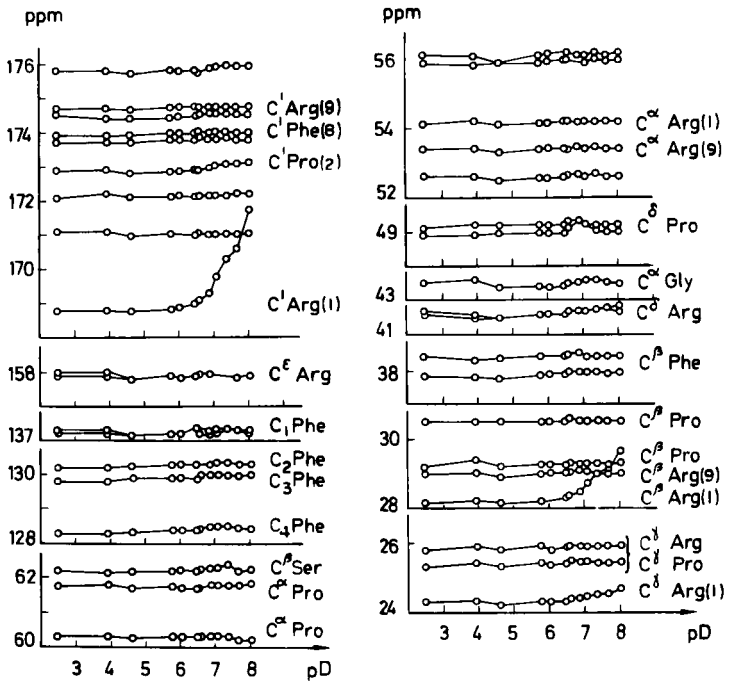
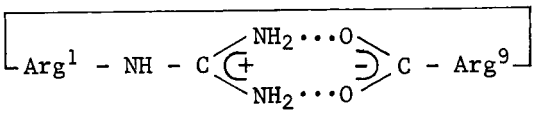


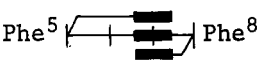
Figure 2: pD dependence of the ^{13}C chemical shifts of bradykinin (in ppm relative to TMS).

terminus appears at pD 6.5 - 8.6, manifested in diminishing order in the regions of the C', C^{β}, C^{γ} and C^{δ} atoms of the Arg^1 residue and also in the C' atom of the Pro^2 residue. It thus follows that the C^{α}-NH_2 group has the normal pK value of 8.1. However, none of the $^{13}C'$ signals was shifted in the 2.5 - 5 region at which the carboxyl group is ordinarly deprotonated;no signs of titration of the Arg^9 residue are detectable also at the other pD values up to 9.0, after which rapid hydrolysis of bradykinin sets in. Apparently the carboxyl group retains its states over the entire range of pD values investigated, a circumstance which can be explained by assuming the formation of a strong bond with the guanidine grouping. The carboxyl group titrated normally in the model peptides Phe-Arg and Pro-Phe-Arg, hence it follows that the salt bond is formed with the N-terminal rather than the C-terminal guanidine grouping. In other words the hormone apparently has a *cyclic* conformation of the following type.



Next, the dansyl derivatives of bradykinin were synthesized and from their fluorescence spectra the distances of radiationless energy transfer (3) in water at 25°C between the donor (hydroxylphenyl) and acceptor (dansyl) were determined. The results also preclude from consideration the extended structure of the hormone being in accord with the above represented cyclic type of structure (Table I).

Analysis of bradykinin molecule, bearing in mind the *trans* configuration of X-Pro bond, was carried out by the procedure described in the monograph[2]. Four energetically preferable conformations were found (see Table II and Figure 3). All contain the salt bridge predicted from NMR studies and possess approximately the same Φ,Ψ parameters of the Arg^1, Pro^2, Phe^5, Ser^6, Pro^7, Phe^8 and Arg^9 residues, differing only in the Pro^3-Gly^4 region. All four structures are capable of hydrophobic stacking via two phenyl and pyrrolidone rings. Just such



orientation of the side groups is represented in Figure 3.

Further study involved the synthesis of spin-labelled bradykinin derivatives containing two stable iminoxyl radicals (SL) and the determination of the interspin distance from measurements of the ESR spectra.[4] The results obtained are in accord with the calculated values for only one of the four conformers shown in Figure 3, namely I (see also Table I). The most essential grounds for such a conclusion was the fact that the N^{α} - Tyr^5 distance is much larger than the N^{α} - Ser^6 distance.

Table I

Comparison of the ESR and Fluorescence Determined Inter Label Distances with those from the Theoretical Conformational Analysis

Conformation	DNSArg[1]-Tyr[5]		DNSArg[1]-Tyr[8]		SLArg[1]-SLSer[6]		SLArg[1]-SLTyr[5]		SLArg[1]-SLTyr[8]		SLTyr[5]-SLTyr[8]	
	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.	Calc.	Exptl.
I	13.9		12.0		10.8		17.2		14.8		12.4	
II	11.6		10.2		11.9		15.1		15.4		10.5	
		15.5		11.1		13.3		18.9		14.8		12.1
III	11.4		10.8		13.8		14.8		14.6		10.2	
IV	10.7		16.1		13.9		13.5		17.6		13.2	

Table II

The Φ, Ψ -Parameters of the Four Energetically Preferable Bradykinin Conformations

Conformation	Arg[1]		Pro[2]	Pro[3]	Gly[4]		Phe[5]		Ser[6]		Pro[7]	Phe[8]		Arg[9]		Relative Energy kcal/mol
	Φ	Ψ	Ψ*	Ψ*	Φ	Ψ	Φ	Ψ	Φ	Ψ	Ψ*	Φ	Ψ	Φ	Ψ	
I	-169	126	122	138	89	-71	-141	147	-126	127	-43	-167	-52	-141	-23	0
II	-168	104	214	-30	-74	-63	-116	131	-91	125	-49	-147	-55	-137	-40	1.5
III	-171	109	134	127	63	34	-154	146	-131	105	-44	-146	-51	-134	-32	2.0
IV	-165	123	133	-36	102	51	-155	143	-126	119	-40	-160	-51	-136	-34	4.7

* Φ angles are fixed at -60°.

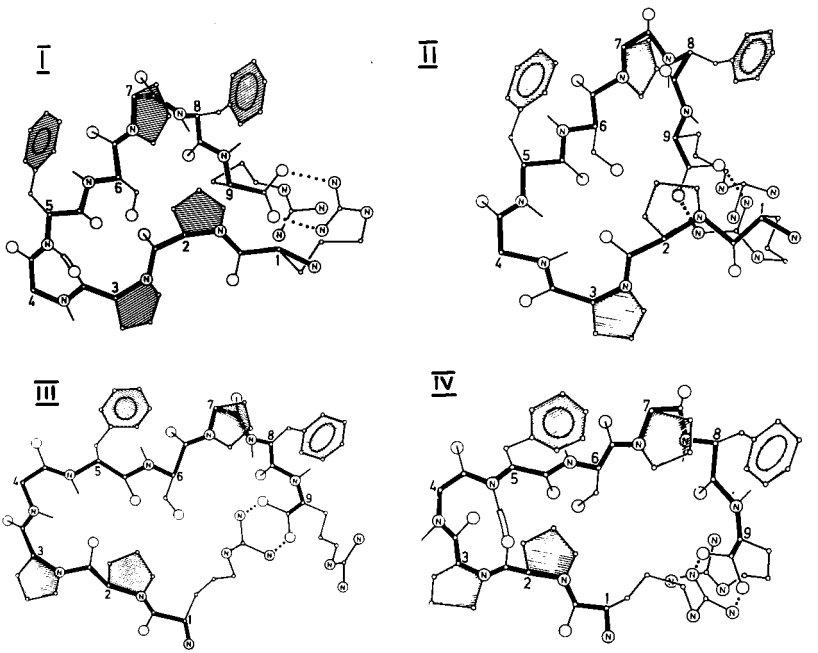
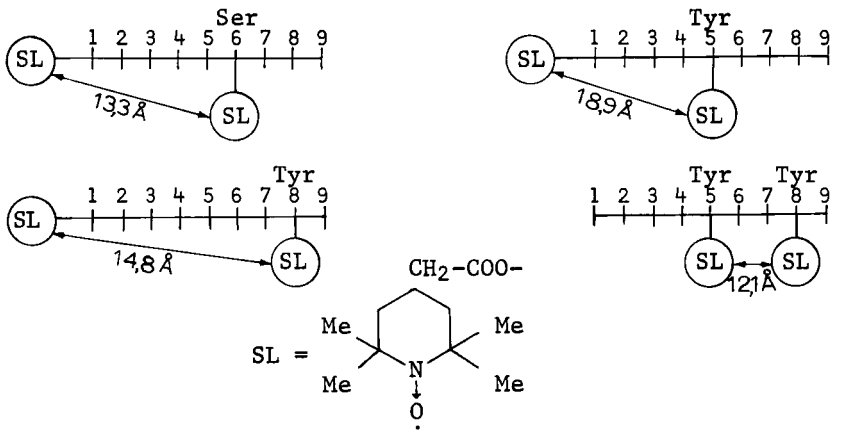


Figure 3: The four energetically preferable conformations of bradykinin.



Thus, for the first time a detailed conformational analysis of a peptide hormone in solution has been carried out. The predominant spatial structure of bradykinin which has been determined resembles the γ-model of angiotensin II with its characteristic intramolecular 3⟶1

hydrogen bond closing a seven-membered ring. Moreover a new principle of spatial arrangement of linear peptides has been discovered, in which the rings are formed by way of ionic interaction of the ionogenic groups situated at opposite ends of the molecule.

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SOLVATION OF ANGIOTENSIN II AND GRAMICIDIN S DETERMINED BY NMR SOLVENT SATURATION METHOD

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A KNOWLEDGE OF THE EXPOSURE TO SOLVENT of side chains and functional groups of an oligopeptide is fundamental to elucidating its solution conformation by nmr. The nmr solvent saturation method detects interaction between the solvent and specific solute nuclei exposed to the solvent. Saturation of the solvent resonance results in intensity perturbation of solute resonances: transfer of saturation[1-4] occurs between rapidly exchanging nuclei of the solute and solvent; intermolecular nuclear Overhauser effects[5-8] are observed for non-exchangeable solute nuclei whose dipoles are interacting with saturated solvent nuclear dipoles. To illustrate the information obtained from this type of experiment, we present studies of (Asn[1], Val[5]) Angiotensin II (AII')[9] in H_2O and of gramicidin-S in methanol, dimethylsulfoxide, and trifluoroethanol (TFE).

Figure 1 depicts a typical solvent saturation experiment for AII' dissolved in H_2O. When the H_2O resonance is irradiated, the Arg peptide N*H*, Asn trans amide N*H* and Arg guanidino N*H* resonances experience transfer of saturation and decrease in intensity by 50 $\pm$ 5%, 9 $\pm$ 3% and 7 $\pm$ 2% respectively. Overhauser enhancements are

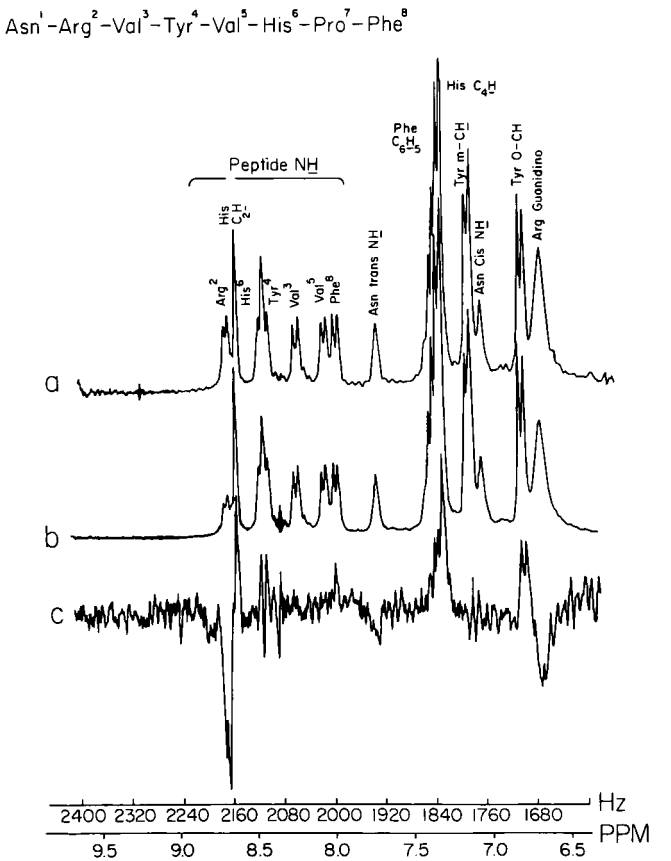

Figure 1: Solvent saturation study of Angiotensin II (AII') (6.9% w/v) in H_2O, pH 3.0 ± 1 at 30 ± 1°C. The spectrum was obtained by correlation spectroscopy[18,19] (20 scans): (a) with off-resonance irradiation 1200 Hz to high field of the H_2O resonance, (b) with saturation of the H_2O peak, and (c) the difference spectrum (spectrum b minus spectrum a amplified 13 fold). Chemical shifts are referred to the methyl resonance of 2,2 dimethyl-2-silapentane-5-sulfonate.

observed for the His C_2H, His C_4H, Tyr ortho CH and perhaps also the Phe C_6H_5 resonances. Enhancements for the His C_2H and Tyr ortho CH resonances are 17 ± 5% and 12 ± 3% respectively,but quantitative NOE measurements for the other two resonances are complicated by peak overlap. Since the NOE's result from direct dipole-dipole interaction with solvent nuclei,[10] these results suggest that the

imidazole group and the ortho protons of Tyr are well solvated. This observation is consistent with the normal pK_as of these groups.[11-14] Failure to observe an NOE for the Tyr meta C*H* indicates shielding of this portion of the phenolic ring from the solvent.

In non-symmetric solvents preferential interaction between specific solvent and solute functional groups is detected. Figure 2 illustrates such an experiment for

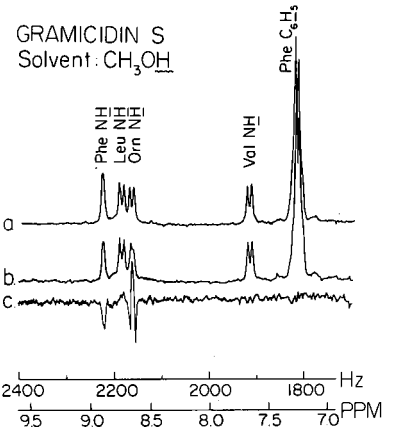

Figure 2: Solvent saturation study of gramicidin S (5% w/v) in methanol at 30 ± 1°C showing the effect of saturation of the solvent O*H* resonance (250 scans/spectrum; 1.6 sec/scan): (a) with off-resonance irradiation 400 Hz to low field of the CH_3OH peak, (b) with saturation of the CH_3OH peak, and (c) the difference spectrum (spectrum b minus spectrum a amplified 3 times). Chemical shifts are relative to internal TMS.

gramicidin S dissolved in methanol. When the CH_3OH peak is saturated, the resonance intensity of the solvent exposed Phe N*H* proton decreases by 24 ± 2% due to saturation transfer; no change in the intensities of other resonances is observed. The signal with dispersion character in Figure 2-c at the Orn N*H* resonance position results from partial decoupling of this N*H* from its $C^{\alpha}H$, whose chemical shift coincides with that of CH_3OH. Because of this effect, no attempt was made to measure intensity changes in the Orn N*H* resonance.

Deuterium exchange experiments[15] indicate that the

exchange rate of this proton is significantly slower than that of the Phe N*H* although several orders of magnitude faster than the intramolecularly hydrogen bonded Leu and Val NH protons.

When the C*H*$_3$OH resonance is saturated, the intensity of the Phe C$_6$*H*$_5$ resonance increases by 5 ± 1%, while the intensity of the Phe N*H* peak diminishes in intensity by 8 ± 2% (Figure 3). The NOE observed for the ring protons results from dipole-dipole interaction with solvent CH_3

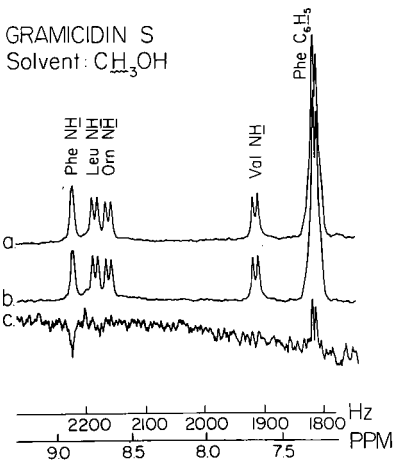

Figure 3: Solvent saturation study of gramicidin S in methanol showing the effect of saturation of the solvent C*H*$_3$ peak. Conditions are the same as in Figure 2 except that the C*H*$_3$ resonance rather than the O*H* resonance of methanol is saturated. The amplification factor in (c) is 5.

protons. The inverse sixth power dependence of dipolar coupling on internuclear distance[16] indicates intimate contact between the hydrophobic Phe ring and solvent methyl groups. The decreased intensity of the Phe N*H* resonance results from exchange-transfer of magnetization from the CH$_3$O*H* proton. The methanol O*H* resonance decreases in intensity by 22% upon saturation of the C*H*$_3$ peak, because the hydroxyl proton is relaxed by exchange modulation of its scalar coupling[16] with the methyl hydrogens.

Saturation of the methyl resonance of dimethylsulfoxide results in a nuclear Overhauser enhancement of 10 ± 1 % for the Phe ring protons, suggesting intimate contact

between methyl protons and ring CH protons. Since dimethylsulfoxide has no exchangeable hydrogens, no perturbation is observed in the intensities of other resonances.

When the ^{19}F resonance of TFE is saturated, an enhancement of 5 ± 1% is observed for the Phe C_6H_5 peaks. Irradiation of the CH_2 or OH protons produces no intensity changes in the gramicidin S spectrum. Exchange of the OH proton is too slow[17] to produce measurable saturation transfer. These observations indicate that solvation in TFE is such that the trifluoromethyl group is closer to the Phe ring than either the methylene or hydroxyl group.

It is evident that the solvent saturation method is potentially a very powerful tool in conformational studies. In addition this technique may yield important information about intermolecular interactions as well as clues about the balance between intermolecular and intramolecular forces which determine the solution conformation of a biomolecule.

Acknowledgments

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CONFORMATIONAL STUDIES ON THE LUTEINIZING HORMONE RELEASING FACTOR (LRF) AND RELATED COMPOUNDS

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STUDIES OF THE STRUCTURE-ACTIVITY relationships in LRF have emphasized the importance of several positions in the decapeptide chain. Among them, position 6 (symbolized by X in *Figure 1*) certainly seems to be critical. Many variations of substituent X have been reported. To mention only two of these, when glycine is substituted by D-alanine, the

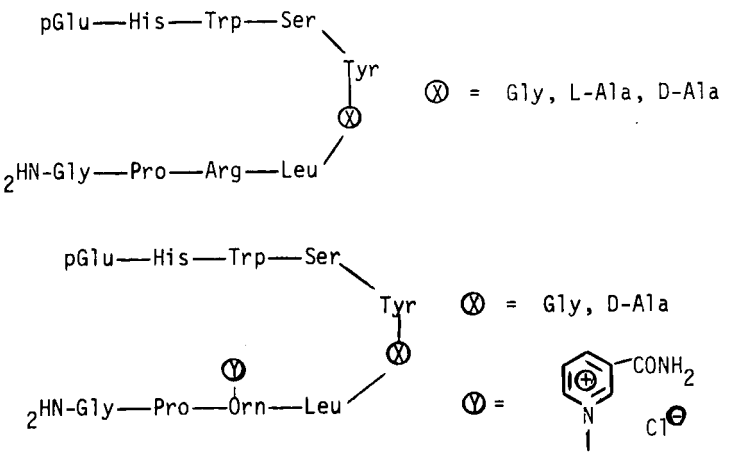

Figure 1: A schematic representation of the LRF molecule and analogs in a β-turn.

potency is increased 4-fold, whereas substitution of glycine by L-alanine drastically decreases the LRF activity.[1] Are these differences related to changes in the stability of the preferred conformation of the hormone? Monahan *et al.*[2] have proposed that such a correlation might be explained by a β-bend occurring at the sequence Ser-Tyr-Gly-Leu. It is well known that a β-turn of type II, as defined by Venkatachalam,[3] is stabilized by the introduction of a D-amino acid residue at the third position of the turn, and destabilized by the introduction of an L-amino acid residue in the same position. Using semi-empirical energy calculations we have been able to confirm these results for the specific sequence Ser-Tyr-X-Leu. In order to investigate this possibility experimentally, we have prepared a variety of analogs which were designed to yield specific information about side-chain side-chain interactions and consequently concerning the allowed conformations of the backbone. For instance, we prepared the two analogs [$Nic^{+}Orn^{8}$]LRF·Cl^{-} and [D-Ala^{6},$Nic^{+}Orn^{8}$]LRF·Cl^{-} in which the arginine residue in position 8 was replaced by ornithine, and the δ-amino group of ornithine then quaternized to a nicotinamidium residue. This residue is known to give a specific charge transfer interaction with the tryptophan side chain. The electronic absorption characteristics of this complex were originally investigated by Deranleau and Schwyzer,[4] Bosshard,[5] and Donzel,[6] using model compounds. The stabilization energy of the complex is small and is not sufficient to induce new allowed conformations, at least for the oligopeptides studied. In the case of our LRF analogs, it was expected that the intensity of the intramolecular CT effect, if it occurred, would be dependent upon the stability and location of the β-turn.

Spectroscopic Studies on LRF, [D-Ala^{6}]LRF and [L-Ala^{6}]LRF

Circular Dichroism Studies. The far ultraviolet circular dichroic spectra of LRF, [L-Ala^{6}]LRF and [D-Ala^{6}]LRF in water at pH 8.6 and 3.6 are shown in *Figure 2*, together with the spectra of the analogs [DesHis, Ala^{2}]LRF and [DesTrp,Ala^{3}]LRF, recorded under similar conditions. A positive shoulder at 245 nm, a strong positive band at 230 nm, a negative shoulder at 214 nm and a strong negative band below 200 nm characterize the CD spectrum of LRF at pH 8.6 (*Figure 2a*). Acidification produces a blue shift and an increase in molar ellipticity of the 230 nm band and causes the shoulder at 214 nm to disappear.

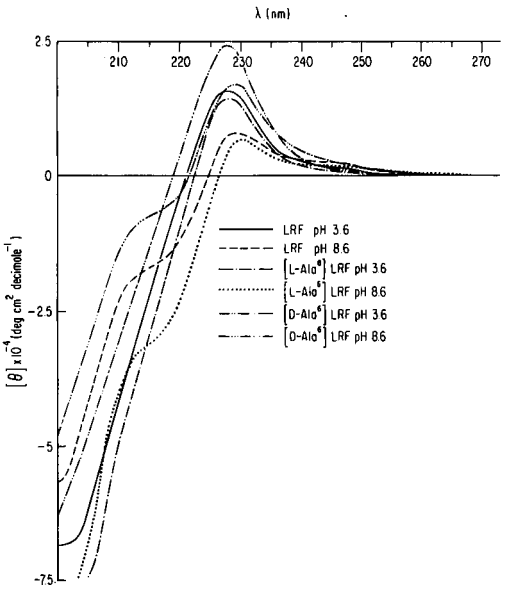

Figure 2a: Circular dichroism in the far UV of LRF, [D-Ala^6]LRF and [L-Ala^6]LRF in water at pH 3.6 and 8.6.

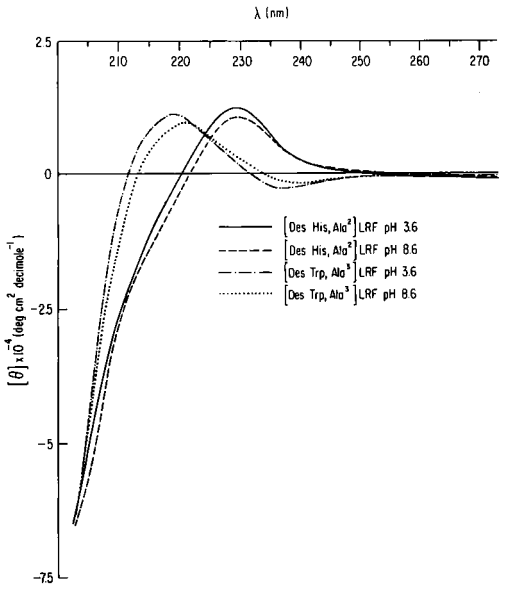

Figure 2b: Circular dichroism in the far UV of [DesHis, Ala^2]LRF and [DesTrp, Ala^3]LRF in water at pH 3.6 and 8.6.

The CD spectra of LRF and [DesTyr, Ala^5]LRF (not shown) are very similar at both basic and acidic pH's. Since the tyrosine side chain chromophore is known to contribute strongly to CD spectra between 200-240 nm if held in an asymmetric environment[7] its small contribution to the CD of LRF in this pH range suggests that it does not interact with any other side chain or peptide chromophore. (This result is in agreement with the nmr data, see below).

As can be seen from *Figure 2b*, there is very little change in the CD spectrum of [DesHis,Ala^2]LRF between pH 3.6 and 8.6, indicating that the histidine side chain is the major cause of changes with pH in the CD spectrum of LRF.

The CD spectrum of [DesTrp,Ala^3]LRF (*Figure 2b*) differs considerably from that of LRF showing a weak negative band at 240 nm, a positive band at 219 nm, and a strong negative band below 200 nm. It is affected only slightly by changes in pH, the small intensification of the molar ellipticity at 213 nm at basic pH probably being due to deprotonation of the imidazole chromophore.[8] The pronounced difference between the spectra of LRF and [DesTrp,Ala^3]LRF suggests that the 230 nm and the 214 nm bands are dominated by the tryptophan aromatic transitions and that the rotational freedom of the tryptophan side chain in LRF could be restricted. Nmr studies (see below) show that there is a reciprocal interaction between unprotonated histidine in position 2 and tryptophan in position 3. Since the strong negative shoulder at 214 nm is observed only when LRF contains unprotonated histidine together with tryptophan, it is possible that the 214 nm band is primarily due to a His-Trp interaction.

It is interesting to note that the CD spectrum of [D-Ala^6]LRF shows an increased molar ellipticity of the 230 nm band at both pH's whereas in [L-Ala^6]LRF the opposite effect is observed.

NMR Studies. The resonance positions of the tryptophan (left part) and tyrosine (right part) aromatic protons in LRF at three different pH's at a concentration of $3 \times 10^{-3} M$ are shown schematically in *Figure 3*. The same resonances are shown as references for the model compounds Ac-Trp-NH_2 and the tetrapeptide Ac-Ser-Tyr-Gly-Leu-$NHCH_3$. At acidic pH the indole resonances in LRF are slightly perturbed, particularly in the region of the C^2-, C^5-, and C^6-protons. Upon deprotonation of the histidyl side chain, however, drastic changes occur, especially in the resonance position of the indolyl C^7-proton, which coalesces, at pH 8.5 with

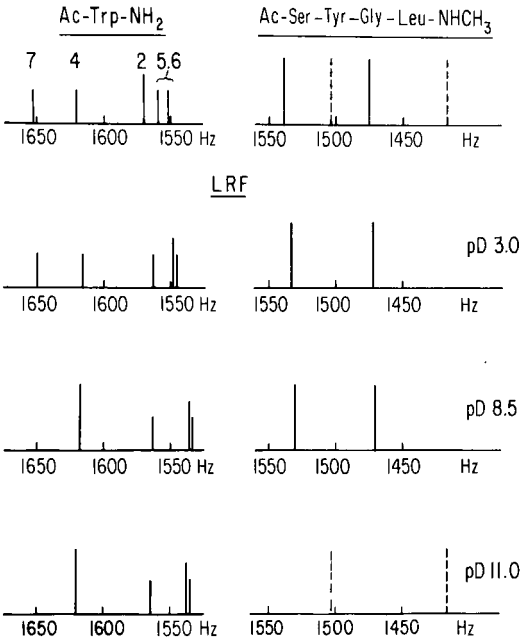

Figure 3: Chemical shifts (Hz) of Trp and Tyr aromatic protons in LRF at 220 MHz. (D_2O, internal ref. HMDS, T = 22°). The dashed lines indicate the resonance positions of the ionized tyrosine side chain.

the C^4-H resonance. This shift approximately follows the titration curve of the histidyl side chain. No significant changes are observed upon ionization of the tyrosine side chain (pH 11). In contrast, the resonance positions of the tyrosine side chain in LRF are close to their positions in the model compound at all three pH's (the dashed lines indicate the resonance positions of the tyrosinate ring), which suggests that the tyrosine side-chain in LRF is not involved in a strong intramolecular interaction with the other aromatic side chains. The tryptophan side chain is certainly influenced by the adjacent histidyl residue. From these results, however, we are not able to exclude the possibility of an effect of the arginine side chain in position 8. In [L-Ala^6]LRF and [D-Ala^6]LRF one observes similar shifts of the indole resonances to those found for LRF.

Spectroscopic Studies on [Nic⁺Orn⁸]LRF.Cl⁻ and [D-Ala⁶,Nic⁺Orn⁸]LRF·Cl⁻

Chemical Shifts of the Nicotinamidium Protons as a Function of Temperature. The temperature dependence of the chemical shifts of the nicotinamide resonances in the Gly⁶-analog (circles) and in the D-Ala⁶-analog (crosses) is illustrated in *Figure 4* and compared with the temperature effects observed in N-methylnicotinamide chloride (triangles). The experiments were conducted at a 3 x 10^{-3} molar concentration. One can see that in both decapeptides the slopes of the lines are positive for all resonances, in contrast

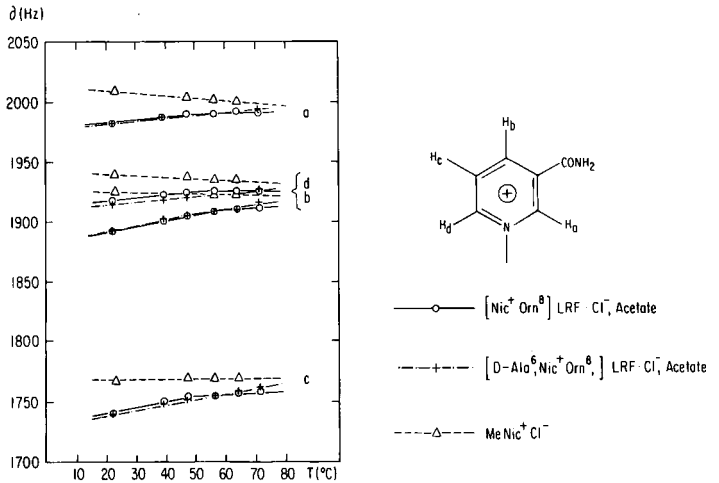

Figure 4: Chemical shifts (Hz) of Nic⁺-protons in [Nic⁺ Orn⁸]LRF.Cl⁻ and [D-Ala⁶,Nic⁺Orn⁸]LRF.Cl⁻ as a function of temperature (220 MHz, D_2O, internal ref. HMDS). N-methylnicotinamide chloride resonances (triangles) are shown as references.

to the reference compound for which they are negative or equal to zero. Between approximately 50 and 60°C, the lines tend to flatten. This effect seems to be less pronounced in the case of the D-Ala⁶-analog. Furthermore, it was observed that the slopes were concentration-dependent and therefore may reflect, at high concentration at least, the cumulative effects of *intra-* and *inter*molecular interactions.

Charge Transfer Experiments. *Figure 5* represents the Beer's law test of the two nicotinamide analogs at both 360 and 400 nm, which are outside the absorption region of the decapeptides. For comparison the OD-values were taken from the normalized absorption spectra. In both LRF-analogs, the increase in optical density with concentration is linear up to 0.5 x 10^{-2} molar. At higher concentrations, the lines become slightly non-linear. Most interesting is the fact that the absorbance at a given wavelength is slightly higher for the D-Ala^6-analog than for the Gly^6-analog. It is evident that we have to accumulate more data before drawing definite conclusions. However, this small difference was estimated to be outside the confidence limits of the measurements. For comparison, the *inter*molecular CT-absorbance at 360 nm between Ac-Trp-NH_2 and N-methylnicotinamide chloride[4] was calculated for the same concentrations (lower curve). The *inter*molecular CT-effect is much less pronounced and the deviation from the straight line is detectable at much lower concentrations. *Figure 6* shows the electronic absorption spectra of [Nic^+Orn^8]LRF.Cl^- and [D-Ala^6,Nic^+Orn^8]LRF which are compared with the addition

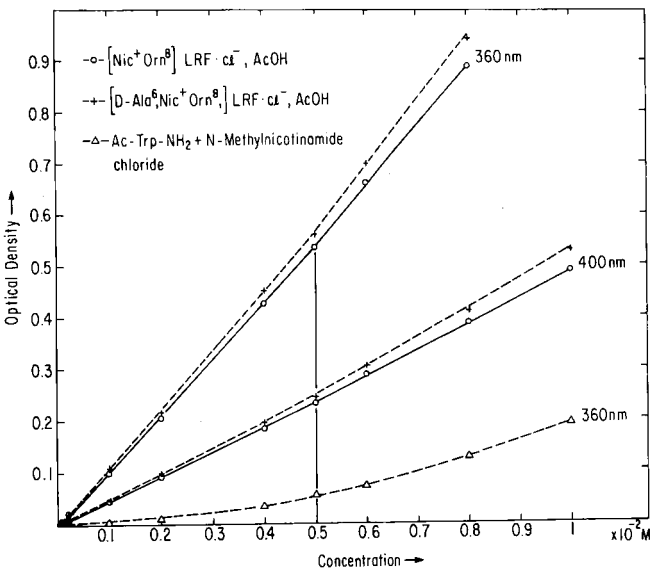

Figure 5: CT-absorption of [Nic^+Orn^8]LRF.Cl^- and [D-Ala^6, Nic^+Orn^8]LRF.Cl^- at 360 and 400 nm as a function of concentration (H_2O, T = 25°). The intermolecular CT-absorbance at 360 nm of Ac-Trp-NH_2 and N-methylnicotinamide chloride is presented with triangles.

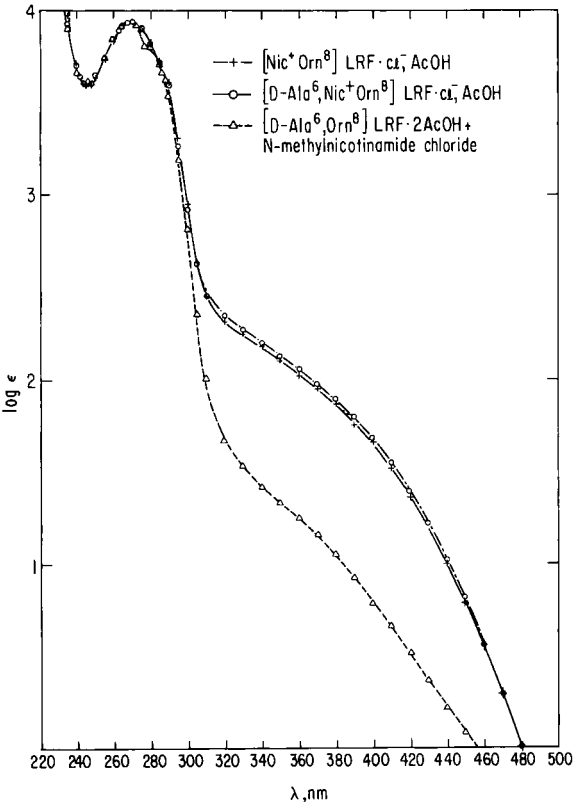

Figure 6: UV/VIS-spectra of [$Nic^{+}Orn^{8}$]LRF.Cl^{-} and [$D\text{-}Ala^{6}$, $Nic^{+}Orn^{8}$]LRF.Cl^{-} in H_2O at 25°. The addition spectrum of [$D\text{-}Ala^{6}$,Orn^{8}]LRF and N-methylnicotinamide chloride is presented with triangles.

spectrum of the unlabelled [$D\text{-}Ala^{6}$,Orn^{8}]LRF and N-methylnicotinamide chloride (triangles). This illustrated the CT-shoulders that we believe to be essentially *intra*molecular in nature.

From these studies we conclude that a folding of the peptide backbone in these two analogs seems to be energetically allowed in water. Our present goal is to determine the extent of this folding.

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PHYSIOCHEMICAL CHARACTERISTICS AND A PROPOSED CONFORMATION OF SOMATOSTATIN

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SOMATOSTATIN IS A HYPOTHALAMIC TETRADECAPEPTIDE of known sequence[1] which inhibits the secretion of growth hormone, insulin, and glucagon.[1,2] It and a number of analogs have been synthesized and assayed.[3] No studies, however, have appeared on the physicochemical aspects of somatostatin. In this communication some spectral and hydrodynamic characteristics of somatostatin are reported and a possible conformation is suggested.

The CD spectra of (cyclic) somatostatin and S-carboxymethylated (CM) somatostatin are shown in Figure 1A. Above 250 nm, the negative ellipticity observed in somatostatin probably arises mainly from the disulfide chromophore while the positive ellipticity exhibited by CM-somatostatin is due to the aromatic residues. Both compounds exhibit a negative extremum between 236-240 nm and a positive extremum at 225 nm, although the magnitude of the former extremum is greater for the S-carboxymethylated derivative. These CD bands are assigned to the aromatic chromophores, with the possibility of some peptide contribution to the 225 nm band. The negative CD band at 238 nm may arise from phenylalanyl residues which are partially shielded from solvent.[4]

The magnitude of the CD extrema is sensitive to guanidine hydrochloride (GuHCl) concentration as also shown in Figure 1A. A plot of [θ] at 242 nm for somatostatin vs. GuHCl concentration has the appearance of a cooperative-like transition with a mid-point at about 3 *M* GuHCl

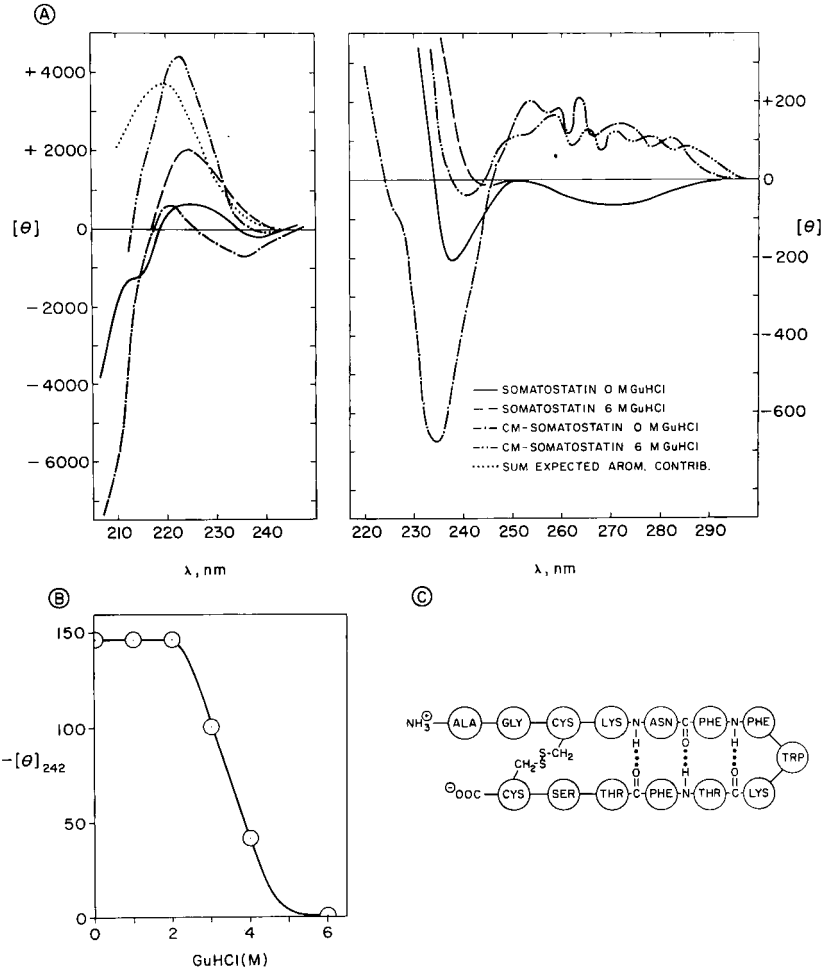

Figure 1: (A) CD spectra (reduced mean residue) at 25.0° of somatostatin (Pierce cyclic somatostatin, lot 5164-8) in 0.1 *M* KCl, 10 m*M* tris-HCl, pH 7.0 (——) and in 6 *M* GuHCl (---); CM-somatostatin in the same KCl-tris buffer (—·—) and in 6 *M* GuHCl (—··—). The calculated CD spectrum of reduced somatostatin in an aperiodic conformation using the CD spectra of tryptophanyl and phenylalanyl model compounds in 6 *M* GuHCl[5] is also shown (. . .). (B) [θ] at 242 nm vs. GuHCl concentration at 25.0°. (C) Schematic of the proposed somatostatin conformation; the principal structural features involve a hairpin loop and a section of anti-parallel β-pleated sheet.

(Figure 1B). Thus, the denaturant appears to induce a conformational change from one or more stable conformers to a less ordered conformation. This, in turn, means that somatostatin possesses a stable structure(s) in aqueous solution.

From model compound studies, it is possible to estimate the contribution of aromatic residues to the ellipticity in the vicinity of 225 nm.[5] Using this information and the somatostatin aromatic content *i.e.*, 1 tryptophan and 3 phenylalanines,[1] the expected CD spectrum of reduced somatostatin in an aperiodic conformation has been computed between 210-240 nm. The expected disulfide contribution to the far uv CD is more difficult to assess since the band positions and magnitudes are critically dependent on the dihedral angle of the -S-S- chromophore.[6]

The generated curve near the 225 nm extremum does not agree with the spectrum of either somatostatin or CM-somatostatin in aqueous buffer; the calculated spectrum is much more positive at 225 nm than that of the cyclic or linear tetradecapeptide. However, as shown in Figure 1A, the CD spectrum of the CD-derivative in 6 *M* GuHCl approaches that expected based an the aromatic chromophores.

This provides additional evidence that a stable conformation exists in aqueous solution. A more detailed analysis of the CD spectra of both somatostatin and the CM-derivative indicates that the presence of limited β-structure, but not α-helix, could account for the observed CD spectral differences in tris-KCl solutions and 6 *M* GuHCl.

To eliminate the possibility that quaternary structure is responsible for a stable ordered or semi-ordered conformation, sedimentation equilibrium[7] data were obtained (12 hours, 30,000 rpm, 20°) for somatostatin (0.11 mg/ml) in 0.1 *M* KCl, 10 m*M* tris-HCl, pH 7. From a ln(O.D.) vs r^2 plot and a calculated partial specific volume of 0.723, a M_w of 1610 + 36 was obtained. This is in excellent agreement with the molecular weight of 1636 calculated from the amino-acid sequence[1], and demonstrates that under these conditions somatostatin exists primarily in the monomeric form. Consequently, any stable conformation must arise from intramolecular interactions. A diffusion constant, $D^{o}_{20,w}$[8] of 1.66 + 0.3 x 10^{-6} cm^2/sec was estimated for somatostatin. These data are consistent with an ellipsoid of axial ratio 5 (1 g bound water/g peptide). This latter value must, however, be considered tentative in view of the assumptions used in estimating axial ratios for such a small molecule.

The results of the present study provide evidence that somatostatin contains a stable conformation(s) in aqueous solution. Interestingly, the disulfide bond does not appear essential for formation of a stable structure, although it may, of course, be important in biological activity. Also, the conformations of somatostatin and CM-somatostatin probably differ somewhat. Indeed, it appears that CM-somatostatin may contain more residues in β-structure than does somatostatin.

A model consistent with these data and with semi-empirical rules for formation of secondary structure[9] has been constructed and is shown schematically in Figure 1C. The structure is somewhat elongated, forms a hairpin loop, and has 4 residues in an anti-parallel β-pleated sheet yielding 3 peptide-peptide hydrogen bonds. A hydrophobic domain arising from trp 8 and the phenylalanines occurs on one end and a hydrophilic domain containing the disulfide is on the other end. The phenylalanyl residues tend to provide a hydrophobic shield for the peptide hydrogen bonds. The proposed conformation should provide a useful guide for analog synthesis.

Acknowledgment

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NMR CONFORMATIONAL STUDIES OF ACTINOMYCINS CONTAINING SARCOSINE IN PLACE OF PROLINE

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SEVERAL PROTON NMR STUDIES of actinomycin D in organic solvents,[1-4] aqueous solution,[3,5] and in complexation with nucleotides,[3,6] have been reported, and conformations for the cyclopeptide moieties were proposed[1,2,7] with all the peptide bonds *trans*. X-Ray crystallography[8] of the deoxyguanosine-actinomycin D complex revealed a different conformation having *cis* Val-Pro and Pro-Sar peptide bonds. The influence of $Eu(FOD)_3$ upon N-methyl and chromophoric methyl chemical shifts is shown in Table I. The $N-CH_3$

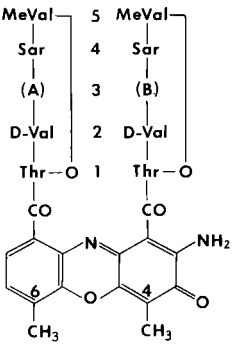
Table I

Lanthanide Shifts* of Actinomycin D Methyl Singlets

	$\delta 0$	$\delta 0.5$	$\Delta 0.5$	$\delta 1.0$	$\Delta 1.0$
Sar $N-CH_3$	2.81	2.88	0.07	3.04	0.23
Sar $N-CH_3$	2.81	2.88	0.07	3.01	0.20
MeVal $N-CH_3$	2.66	2.69	0.03	2.75	0.09
MeVal $N-CH_3$	2.61	2.65	0.04	2.71	0.10
$6-CH_3$	2.37	2.30	0.01	2.41	0.04
$4-CH_3$	2.05	2.10	0.05	2.25	0.20

* Subscripts to δ and Δ refer to relative molar concentrations of $Eu(FOD)_3$/actinomycin D in $CDCl_3/C_6D_6$ (3:1).

downfield shift of Sar is twice that of MeVal, and equal to that of the sensitive chromophoric 4-CH_3 group. When correlated with shifts in related model peptides,[9] these data are compatible with *cis* Pro-Sar and *trans* Sar-MeVal peptide bonds. The same conclusion results from the benzene shift data[1,3] (MeVal>>Sar N-CH_3), since the lanthanide effect invalidates the explanation[1] based on steric inaccessibility of the Sar N-CH_3 group. In addition, ^{13}C NMR studies[10,11] favor a *cis* Val-Pro peptide bond, and it is concluded from all the above data that the conformation of actinomycin D in solution resembles that established for the crystalline deoxyguanosine complex. NMR studies indicate a relatively inflexible peptide backbone conformation, as expected from space-filling models.



ACTINOMYCINS:

II: A=B=Sar

III: A=Pro, B=Sar or vice-versa *

IV(=D): A=B=Pro

V: A=Pro, B=4-Ketopro

* During separation of the actinomycin complex produced by *Streptomyces antibioticus*, III was further separated into 2 isomers yet to be structurally assigned. The major isomer was used in this study.

In actinomycins II and III, in which sarcosine replaces both or one proline respectively,[12] the possibility of different conformations exists due to the absence of the restrictive effect of the prolyl ring on the dihedral angle ϕ. Proton NMR spectra of actinomycins II-V were obtained at 220 MHz in $CDCl_3$ and various $C_6D_6/CDCl_3$ mixtures. Typical spectra of II, III and IV (=D) are shown in Figure 1. Assignments of NH and certain CαH-protons were made by track-field decoupling. The following remarkably parallel conformationally-dependent parameters were observed throughout the series: (a) NH-CαH coupling constants (Table II). (b) N-CH_3 chemical shifts and their

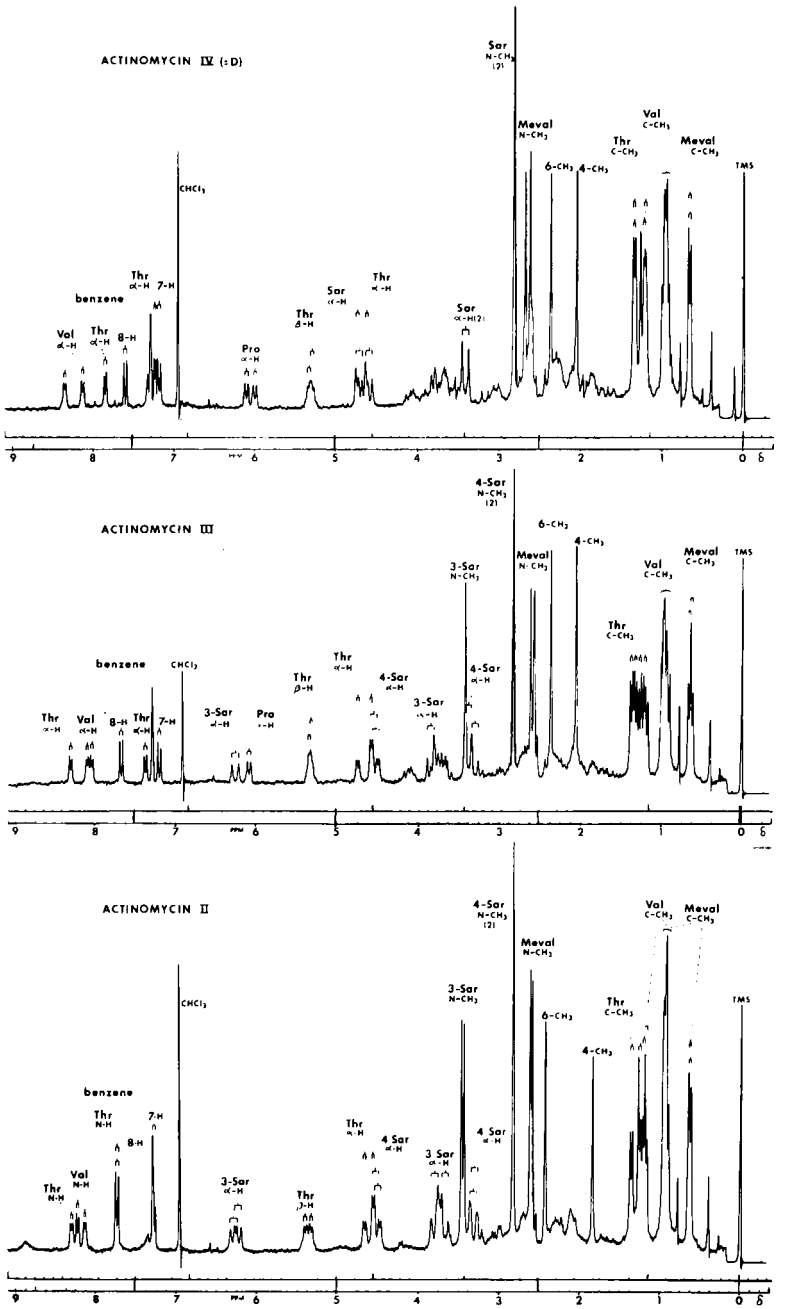


Figure 1: Proton NMR spectra of 0.05M solutions of actinomycins in $CDCl_3/C_6D_6$ (3:1) at 18°.

Table II

NH-CαH Coupling Constants (Hz)

Actinomycin	*D-valine*		*threonine*	
II	5.7	6.1	6.4	6.7
III	5.8	6.0	6.2	6.8
V	5.8	5.6	6.0	6.8
IV[1]	5.7	5.7	5.7	6.3
IV[3]	5.7	6.0	6.2	6.8

sensitivity to benzene (Table III). (c) 3 and 4-site α-proton chemical shifts (Table IV). The latter include unusually large shift separations for geminal Sar

Table III

N-Methyl Proton Chemical Shifts (δ)

Solvent	*Actinomycin*	*3-Sarcosine*		*4-Sarcosine*		*N-Methylvaline*	
$CDCl_3$	IV	-	-	2.87	2.87	2.90	2.95
	V	-	-	2.89	2.89	2.93	2.94
	II	3.35	3.39	2.88	2.88	2.79	2.82
	III	3.33	-	2.88	2.87	2.80	2.93
$C_6D_6/CDCl_3$ (1:1)	IV	-	-	2.74	2.75	2.35	2.47
	V	-	-	2.70	2.73	2.34	2.37
	II	3.50	3.52	2.75	2.77	2.40	2.42
	III	3.44	-	2.75	2.75	2.33	2.46

α-protons. These results indicate that when proline in actinomycins is replaced by sarcosine, the geometry and flexibility of the peptide backbone conformation is maintained. On the other hand, the chemical shift of the chromophoric 4-methyl protons in actinomycin II was anomalously high-field and concentration-dependent, approaching normal values on dilution. This phenomenon is typical of the stacking dimerization previously observed[5] for actinomycin D in aqueous solution, but is unexpected in organic

Table IV

Chemical Shifts (δ) of 3 and 4-Site α-Protons (in $CDCl_3$)

Actinomycin	*3 - Sites**				*4 - Sarcosines*			
IV	5.97(p)	(β-H)	6.03(p)	(β-H)	3.64	4.73	3.65	4.80
V	5.97(p)	(β-H)	6.57(k)	(β-H)	3.65	4.61	3.65	4.73
II	6.25(s)	3.63(s)	6.20(s)	3.79(s)	3.52	4.59	3.55	4.59
III	5.98(p)	(β-H)	6.20(s)	3.83(s)	3.56	4.66	3.64	4.59

* Abbreviations: p = proline, s = sarcosine, k = 4-ketoproline.

solvent. Examination of space-filling models suggests that replacement of proline by sarcosine increases the accessibility of the chromophore to complex formation.

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ANALYSES OF CYCLIC PEPTIDE CONFORMATION BY LANTHANIDE-ASSISTED NMR

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CYCLIC DIPEPTIDES containing an aromatic amino acid may preferentially populate the rotamer that places the aromatic side chain in a folded position with respect to the diketopiperazine backbone.[1]

In order to investigate whether aliphatic side chains might also prefer specific orientations, we have examined a series of cyclic dipeptides by proton and ^{13}C nmr using ytterbium shift reagents. The dipeptides chosen all contained L-proline and either L- or D-phenylalanine, valine, leucine, isoleucine, and *allo*-isoleucine -- all hydrocarbon side chains. Proline is used to increase peptide solubility, to decrease the flexibility of the diketopiperazine backbone, and to provide additional invariant atom positions for the lanthanide analysis.

We have described our general method in an earlier communication.[2] The shifts for each proton and carbon atom in the molecule (excluding the non-prolyl side chains) are plotted versus the molar ratio of $Yb(fod)_3$ to peptide. The least square slopes of these lines are then used in equation [1]

$$\frac{\Delta\nu}{\nu} = K\left(\frac{3\cos^2\theta - 1}{r^3}\right) \qquad [1]$$

to locate the best positions for the lanthanide ion at each binding site by computer iteration. Using these positions, the shifts of side chain atoms are predicted at 10° increments for rotation about the $C_\alpha - C_\beta$ (χ_1) and $C_\beta - C_\gamma$ (χ_2) bonds. Comparison between predicted and observed shifts using R factor[3] analysis allows us to determine a best match or preferred rotamer within a certain confidence level.

To test further the hypothesis that a preferred rotamer exists for each dipeptide, a second computer program to determine the mole fraction of each rotamer that gives the best averaged match was utilized. The rotamers considered for each compound include the preferred rotamer from the R factor analysis and up to four of the rotamers whose intramolecular potential energies, determined from conformational energy calculations, were within 3 kcal/mole of the global minimum. For all the dipeptides the bicyclic backbone, consisting of diketopiperazine and pyrrolidine rings, was assumed to have identical coordinates -- those obtained from conformational energy calculations on *cyclo*(L-Pro-Gly).

The relative populations estimated from the ytterbium analysis for each of the L,L and L,D dipeptides are presented in Table I for various rotamers about the $C_\alpha - C_\beta$ bond. The italicized rotamer is that computed to have the lowest intramolecular potential energy including only van der Waals and torsional energy terms.

If we use the criterion that a conformation is "preferred" whenever it accounts for more than 50% of the population, then all three L,L cyclic dipeptides listed in Table I have a preferred rotamer, and in each case this rotamer has the side chain extended to nitrogen (Figure 1). For the L,D dipeptides four of the five compounds also adopt a preferred conformation. The preferred rotamers are: extended to nitrogen for *cyclo*(L-Pro-D-Leu)

Table I

Fractional Population of Rotamers

Lanthanide Analysis of *Cyclo*(L-Pro-X)

X	*Rotamer*[a,b]	*Fractional Population*	*R Factor*[c,d,e]
L-Phe	Extended to N	0.67	0.063
	Folded	0.33	
L-Val	*Extended to N*	0.90	0.054
	Folded	0.10	
L-Leu	*Extended to N*	0.87	0.072
	Folded	0.13	
D-Phe	*Folded*	0.85	0.051
	Extended to N	0.15	
D-Val	*Unfolded*	1.00	0.081
	Extended to O		
D-Leu	*Extended to N*	1.00	0.088
D-*allo*-Ile	*Extended to N*	0.58	0.051
	Unfolded	0.25	
	Extended to O	0.17	
D-Ile	Extended to N	0.40	0.042
	Unfolded	0.34	
	Extended to O	0.26	

[a] See Figure 1 for explanation of nomenclature.

[b] Italicized rotamer is lowest energy rotamer (see text).

[c] See Reference 3.

[d] R factor of best average match.

[e] ca 0.3 *M* in $CDCl_3$.

and *cyclo*(L-Pro-D-*allo*-Ile), unfolded for *cyclo*(L-Pro-D-Val), and folded for *cyclo*(L-Pro-D-Phe). For *cyclo*(L-Pro-D-Ile) there seems to be a nearly equimolar mixture of three conformers.

For *cyclo*(L-Pro-L-Leu), the one compound whose crystal structure has been determined, ytterbium analysis yielded a solution conformation ($\chi_1 = -65°$, $\chi_2 = 175°$) in good agreement with the crystal structure[4] ($\chi_1 = -72.3°$, $\chi_2 = 177.7°$).

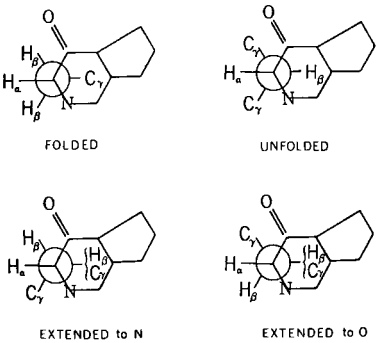

Figure 1: Diagrams of the stereochemistry of amino acid side chain rotamers in *cyclo*(Pro-D-X) dipeptides. For clarity, only the diketopiperazine functional groups adjacent to the side chain of the non-prolyl residue are shown. The rotamers are classified "folded", "extended to nitrogen", and "extended to oxygen" according to the location of the C_γ atom of the non-prolyl side chain. For side chains with two C_γ atoms, the rotamers are named according to the orientation of the extended C_γ; if both C_γ atoms are extended, the rotamer is designated "unfolded".

For six of the eight cyclic dipeptides, the preferred rotamer from the lanthanide analysis is the one which was calculated to have the lowest conformational energy for that compound. In fact, the experimental preponderance of one conformer is often greater than might be expected from the calculated energy differences. Nevertheless, the correlation between experimental and theoretical results suggests that steric interactions are the dominant conformational determinant -- especially for the alkyl side chains.

Cyclo(L-Pro-L-Phe) is the only one of the eight compounds which has a preferred conformation in chloroform solution which differs from one computed to have the lowest energy. The rotamer predicted by the ytterbium analysis for *cyclo*(L-Pro-L-Phe) is extended toward nitrogen, as concluded by Blaha and co-workers.[5] This rotamer may be stabilized by interaction of the electropositive amide proton with the π electrons of the aromatic phenylalanine ring. Consistent with the attenuation of electronic interactions between molecular groups in more polar solvents, proton coupling constants and CD spectra indicate that *cyclo*(L-Pro-L-Phe) adopts the folded conformation (which has the lowest steric conformational energy) in water and methanol solutions.[6]

Our success with cyclic dipeptides led us to try the lanthanide technique with two cyclic hexapeptides, *cyclo*(D-Ala-L-Pro-Gly)$_2$ and *cyclo*(D-Ala-L-Pro-D-Ala)$_2$. Larger peptides are more complicated to analyze due to the increase in possible binding sites and the larger number of possible conformations in solution. However, the shift reagent might bind selectively to one of these conformations, thereby providing us with information on this species even though it may not be the most highly populated one. Provided that the lanthanide reagent binds to a single conformer, analysis of nmr spectra should yield conformational information on the bound peptide species and provide a means for assigning peptide nmr signals.

Several different β-turn conformations may be considered for cyclic hexapeptides containing proline. We have considered one conformation in which both peptide bonds preceding proline are *cis* and three in which all bonds are *trans*. These latter three include one in which the proline residue occupies the *i + 2* corner position of a β-turn[7] (the so-called II' conformation). The remaining two β-turn conformations have proline in the *i + 1* corner position and differ only in whether the proline carbonyl oxygen is *trans* (type II) or *cis* (type I) to the proline α proton. Clearly, many more conformations are possible, and the analysis is limited, at the very least, by the number of conformations considered.

The lanthanide data for *cyclo*(D-Ala-L-Pro-D-Ala)$_2$ and *cyclo*(D-Ala-L-Pro-Gly)$_2$ are summarized in Table II. The best match for both peptides occurs for the type I β conformation. For *cyclo*(D-Ala-L-Pro-D-Ala)$_2$ the R factor for this match, 0.103, is at the upper limit of a satisfactory correlation and suggests that the bound peptide is not well-matched by this single conformation. For *cyclo*(D-Ala-L-Pro-Gly)$_2$ the R factor for the type I match, 0.046, is much better, and the type I conformation is likely to be a better approximation to that of the bound peptide in this case. In neither situation can the type II structure be ruled out on the basis of R factors. The two-*cis* conformation examined only for *cyclo*(D-Ala-L-Pro-D-Ala)$_2$ and the type II' structures, examined for both, can be ruled out with 99% certainty on the basis of their high R factors.

In order to help us determine whether the addition of lanthanide changes the conformation of the peptides and, thus, whether the conformation we are examining is that of the unperturbed peptide or that of a different peptide-Yb complex, we have added Yb(fod)$_3$ to an acetonitrile solution of *cyclo*(D-Ala-L-Pro-Gly)$_2$ and examined the CD

Table II

R Factors for Selected Cyclic Hexapeptide Conformations

Peptide	*Conformation*	*R Factor*[a]
cyclo(D-Ala-L-Pro-D-Ala)$_2$ (0.11 *M* in $CDCl_3$-CD_3CN (1:1,v/v))	Type I	0.1034
	Type II	0.1639
	Type II'	0.2184
	Two *Cis*	0.2566
cyclo(D-Ala-L-Pro-Gly)$_2$ (0.15 *M* in $CDCl_3$)	Type I	0.0456
	Type II	0.0945
	Type II'	0.2255

[a] See Reference 3.

spectrum. Up to the highest molar ratio obtainable for Yb/peptide (*ca.* 6/1), there was no detectable change in the CD spectrum. Other work in this laboratory indicates that this peptide does bind strongly to calcium, magnesium, and even europium perchlorate in acetonitrile. La(fod)$_3$, however, causes changes in the CD spectrum similar to those caused by calcium. It also affects the ^{13}C nmr spectrum in a manner similar to calcium and quite different from Yb(fod)$_3$, although much higher molar ratios are necessary for the nmr effect of La than Yb. Work is presently in progress to ascertain whether these changes reflect a lower binding constant for ytterbium, or whether the two lanthanides bind the peptide in different ways.

We believe that lanthanide analyses can yield conformational data for small peptides with a limited number of binding sites. Using this type of analysis, we have shown that steric interactions dominate in selecting preferred rotamers for aliphatic side chains in cyclic dipeptides. Electronic interactions are more important for aromatic side chains. Nmr signal assignment and conformational analysis of cyclic hexapeptides is also possible by the lanthanide method, but selection of a single preferred solution conformation may require data from additional methods of analysis.

Acknowledgments

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SANDWICH COMPLEXING OF METAL BINDING CYCLOPEPTIDES AND ITS BIOLOGICAL IMPLICATIONS

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MUCH CURRENT INTEREST is being shown in cyclic peptides capable of complexing metal ions. Among them are the antibiotic ionophores valinomycin and the enniatins, the antitoxin antamanide (see[1] and references therein) and numerous synthetic analogs.[2,3] They have been widely known as 1:1 complexones, but recently the enniatins have been shown to form also sandwich complexes and even to undergo more complicated "stacking" processes, whereby the macrocycle to cation ratio becomes 2:1 and 3:2. It is just such "stacks" that perhaps are responsible for the K^+ and Cs^+ transport across black lipid films. In the present paper the results will be discussed of our further investigations into the complexing stoichiometry in solution as observed on valinomycin, antamanide and their analogs.

CD-controlled titration of 10^{-3} *M* ethanolic valinomycin with KCl solution gave a curve markedly above the theoretical titration curve for an equimolar complex with an infinitely high stability constant (Figure 1a, 1b). The empirical curve approached the theoretical one for a 1:1 complex at low antibiotic concentration, whereas as the concentration was augmented it approached more and more that for a 2:1 complex (Figure 2a). Similar results were obtained in UV-controlled titrations (Figure 2b). The probable structure for a valinomycin sandwich is shown in Figure 3. As in equimolecular structure, the ion interacts

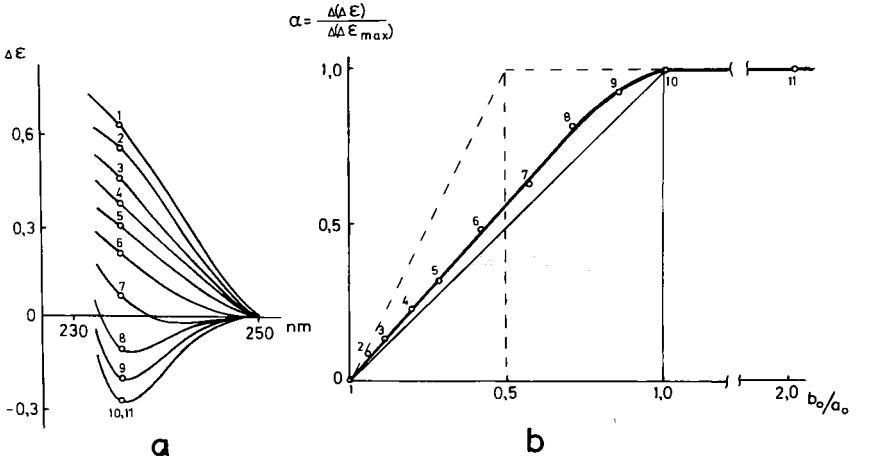

Figure 1: Titration of valinomycin with KCl in 96% aqueous ethanol at 25°. a - experimental CD curves; b - titration curve.

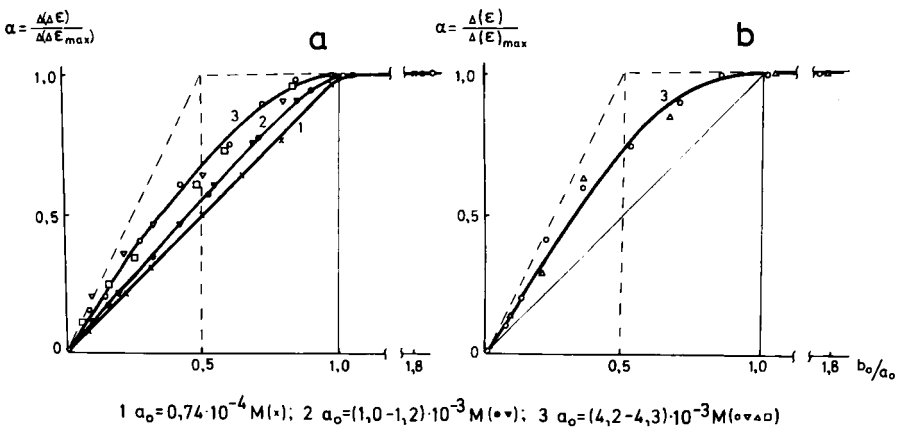

Figure 2: CD controlled (235 nm) (a) and UV controlled (240 nm) (b) titration curves of valinomycin with KCl in 96% aqueous ethanol at different antibiotic concentrations. The different designation refer to different runs.

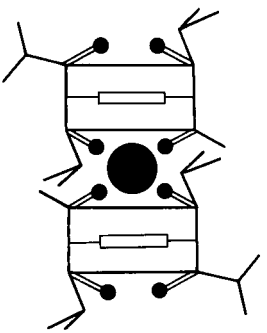

Figure 3: Proposed structure of the valinomycin sandwich.

with six ester ligands (three from each valinomycin molecule). Molecular models show that the sandwich structure is stabilized by Van der Waals interactions of the isopropyl side chains, the more advantageous site of contact being the lactyl-containing ends of the cylinders.

The results obtained seem to indicate possible involvement of the sandwich complexes in the transmembrane potassium transport process. For example, under certain conditions the concentrational dependence of the valinomycin-induced K^+-permeability of lipid bilayers is superlinear; earlier this was ascribed to the existence in the transport process of a stage whereby the potassium ion passes from one valinomycin molecule to another (p. 290 in [1]). Now these results could be explained in terms of sandwich formation. Very likely on penetrating the lipid zone of the membrane valinomycin does not just move about randomly within it (Figure 4a) rather becoming incorporated as a constituent part without affecting to any considerable extent its hydrocarbon organization. One of the possible ways of such incorporation is of valinomycin in the form of clusters as shown in Figure 4b. Such clustering would

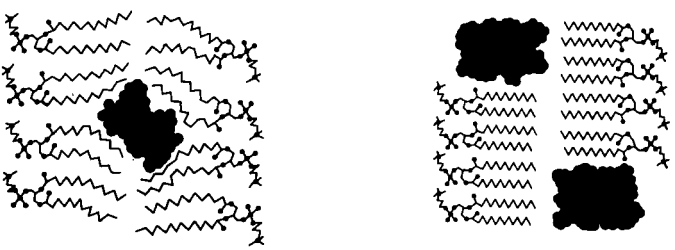

Figure 4: "Floating" valinomycin (a) and valinomycin in the form of stacks (b) in a lecithin bilayer.

actually provide the framework for sandwich formation and thus in the limiting case could impart to the sandwiches a leading role in the potassium transport process.

In the second part of this report new findings concerning antamanide, *cyclo*(-Val-Pro-Pro-Ala-Phe-Phe-Pro-Pro-Phe-Phe-) will be presented. This antitoxin and some of its synthetic analogs are known to form complexes with alkali and alkaline earth metal ions, the ability to bind Na^+ or Cs^+ being necessary though not sufficient condition for the manifestation of antitoxic activity against the poisonous principles of the mushroom *Amanita phalloides*. (p. 35-41 in [1]). The relationship between the antitoxic activity of antamanide and its complexing properties had for some time been obscure. Conformationally the equimolar complexes of antamanide with Li^+, K^+, Na^+ or Ca^{2+} were similar in both solution and the crystalline state[7], having a saddle-like form with the cation bonded by four amide carbonyl oxygens situated approximately at the four corners of a square[7]. A distinguishing feature of the equimolar complexes of antamanide from those of enniatin B or valinomycin is the partial substitution of the ion solvent sheath by ligand groups of the macrocycle. Thus Li^+ complexes with antamanide retains an acetonitrile solvate molecule[8] (Figure 5a), and Na^+ complexed with

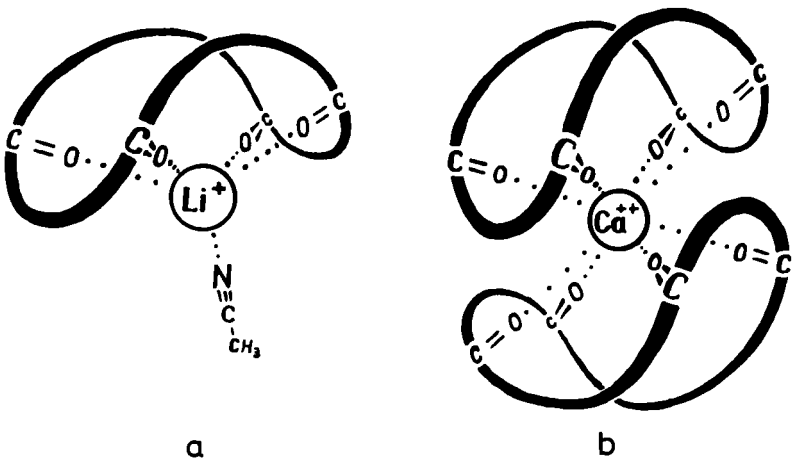

Figure 5: Schematic structure of the Li^+.antamanide complex (a) and possible structure of the complex $(antamanide)_2 \cdot Ca^{2+}$ (b).

[Phe^4,Val^6] antamanide is still capable of additionally binding ethanol.[9] Such partial unsaturation of the cation coordination sphere is manifested also in the sandwich complexing of antamanide as revealed in the case of valinomycin by CD controlled titration. Convincing evidence of sandwiching has been obtained in the NMR-monitored titration of [Val^5,Ala^9] antamanide with $Ca(ClO_4)_2$ in acetonitrile. Figure 6 shows that in the early stages of the salt

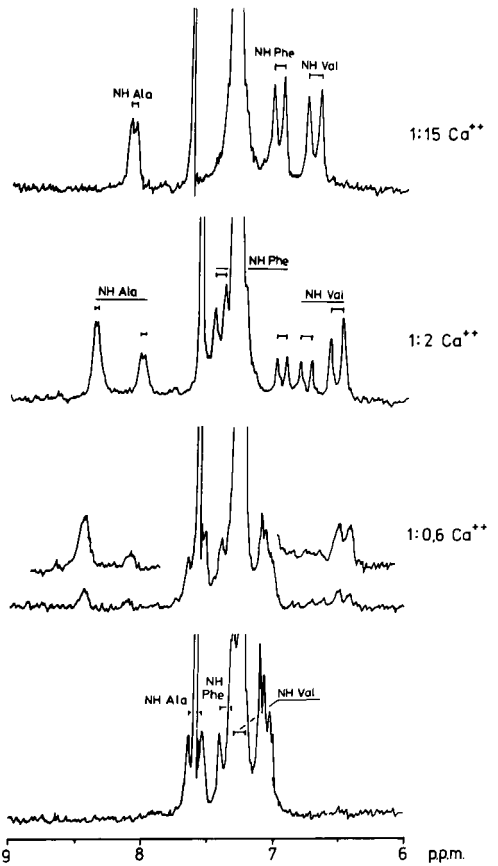

Figure 6: The low field (6-9 ppm) region of the NMR spectra of [Val^6,Ala^9] antamanide and its Ca^{2+} complexes in acetonitrile. The lowest spectrum refers to the antibiotic in salt-free solution, each following spectrum refers to increasing concentration of $Ca(ClO_4)_2$.

addition every NH-proton gives three signals, one produced by the free complexone and two others due to two complexed forms. As could have been expected, further addition of salt decreases the signal intensity of the free form until complete disappearence at large salt excesses. From the intensity changes of the two other signals one could follow the course of two opposing processes, the initially more prominent signal gradually disappearing and the less prominent one gaining in intensity until it becomes the sole signal at large salt excess. From this it may be inferred that apparently the complex first had a macrocycle to cation ratio of 2:1, which eventually gave way to the usual 1:1 complex. A possible structure of the 2:1 complex is presented in Figure 5b. From what has been said one might envisage the following molecular mechanism for antamanides antitoxic activity (see Figure 7). Becoming attached to

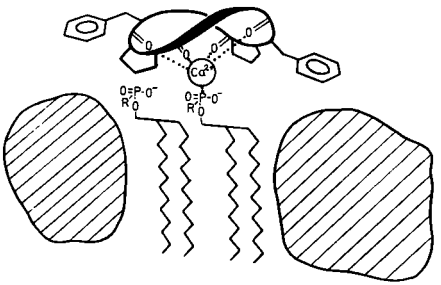

Figure 7: Proposed principle of antamanide interaction with a biomembrane (hatched areas refer to protein globules).

the membrane by the surface Na^+ and Ca^{2+} ions, antamanide "blocks" a considerable portion of its area, which should modify some of its properties including permeability to the toxins of *Amanita phalloides*. Since the antitoxic activity of antamanide is stereospecific (enantio-antamanide is less active than the naturally occuring antitoxin by an order of magnitude[10]) apparently some of the protein components of the membrane may also be involved in the antamanide binding.

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^{13}C NMR STUDIES OF COMPLEXES BETWEEN AMINO ACIDS AND CYCLIC PEPTIDES

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WHEN AN AMINO ACID (ester salt) and a *cyclo*(Pro-Gly)$_n$ peptide (n = 3, 4, 5) are mixed in chloroform solution at comparable molar concentrations, significant chemical shift changes in most resonances of both components are observed in ^{13}C spectra.[1] In addition, certain resonances of D- and L-enantiomers of the amino acids are split in the presence of cyclic peptide. These results suggest not only that the components interact, but that they do so with sufficient specificity that one could readily postulate the formation of a discrete cyclic peptide-amino acid "complex" of the type shown schematically in Figure 1. The interactions stabilizing such a structure -- principally the electrostatic attraction of the protonated amino group for the oriented array of amide carbonyl groups in the peptide cavity -- are similar to those which may be expected to stabilize enzyme-substrate or hormone-receptor complexes.

However, in view of the fact that ^{13}C chemical shifts are known to be affected by a combination of conformational and electronic factors, chemical shift changes alone may not always provide sufficient proof for the presence

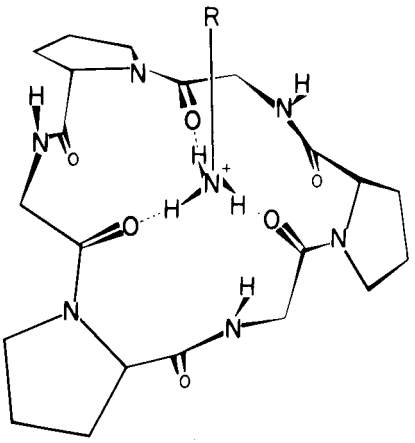

Figure 1: Proposed binding scheme of an amino acid (ester salt) to *cyclo*(Pro-Gly)$_3$. In the present work, RNH_3^+ represents Val-OMe. For Pro-OBz, one ammonium proton is replaced by the methylene group of the proline pyrrolidine ring. The N-R bond is elongated for clarity. All peptide bonds of the cyclic peptide are *trans*. Anion is Cl^-.

(or absence) of a discrete complex in solution. Consider an instance where the molecular conformation of the peptide did not change upon complexing with the amino acid or with a given metal cation; would the "complex" have the same ^{13}C spectrum as the free components? Would only carbonyl carbons or atoms in proximity to binding sites be affected? Since both components in the present systems contain carbon atoms, it was realized that ^{13}C spin-lattice relaxation measurements could be employed, as described below, to verify the existence of an amino acid-cyclic peptide complex in solution.

Since the molecular weight of any complex is larger than the individual molecular weights of its components, the rotational correlation time of the complex should be longer (*i.e.*, slower motion) than the corresponding values for its individual components. In the extreme narrowing limit an increase observed in the rotational correlation time, as manifested by a decrease in experimentally-determined spin-lattice relaxation times (NT_1's)[2], should provide good evidence for the formation of a molecular complex. With specific reference to the present systems, one might expect that, in the absence of internal motion,

NT_1 values of both amino acid and cyclic peptide should converge toward a common value if these components comprise a species which is tumbling as an entity in solution.

The results of ^{13}C relaxation experiments with *cyclo*-(Pro-Gly)$_3$ in the presence of proline benzyl ester hydrochloride (Pro-OBz) are given in Table I. Indeed, at a

Table I

NT_1 Values (Sec.) of HCl·Pro-OBz and *Cyclo*(Pro-Gly)$_3$

Mole Ratio Salt/Peptide	*Salt*	*Peptide*	
	Pro_α	Pro_α	Gly_α
Free	0.59	0.44	0.47
4:1	0.50		
2:1	0.40	0.32	0.30
1:1	0.34	0.31	0.32

1:1 molar ratio of salt/peptide, the NT_1 values found for Pro-OBz C_α and *cyclo*(Pro-Gly)$_3$ Pro C_α and Gly C_α are equal within experimental error. Other carbon atoms in this system also displayed decreased NT_1 values upon complex formation.

Similarly, the α-carbon NT_1 value of valine methyl ester hydrochloride (Val-OMe) decreases with increasing cyclic peptide concentration (Table II). However, in the Val-OMe case there are two important differences:value of

Table II

NT_1 Values (Sec.) of HCl·Val-OMe and *Cyclo*(Pro-Gly)$_3$

Mole Ratio Salt/Peptide	*Salt*	*Peptide*	
	Val_α	Pro_α	Gly_α
Free	0.62	0.44	0.47
4:1	0.41	0.27	0.28
2:1	0.34	0.28	0.28
1:1	0.48	0.29	0.32

Val-OMe C_α NT_1 at 1:1 mole ratio salt/peptide is 0.48 sec., while the NT_1 values for cyclic peptide α-carbons are *ca.* 0.3 sec. This result suggests that significant internal rotation occurs about Val N-C_α single bonds. This mode of rotation is not possible for Pro N-C_α because this bond is locked into the pyrrolidine ring. Second, the NT_1 values for Val C_α do not decrease monotonically but, rather, go through a minimum at a 2:1 salt/peptide mole ratio. This progression of NT_1 values for Val C_α provides an indication that a complex of other than 1:1 stoichiometry might be present.

Further evidence for this notion was obtained from plots of chemical shift changes versus salt/peptide ratios[3]. In the Pro-OBz case no further chemical shift variations were noted between a 1/1 and 4/1 salt/peptide ratio. However, in the Val-OMe case both Pro and Gly cyclic peptide carbonyl chemical shifts continued to change beyond a 1/1 mole ratio of salt/peptide (Pro C=O downfield 0.81 ppm. Gly C=O upfield 0.49 ppm). Since *cyclo*(Pro-Gly)$_3$ has two potential binding sites -- the triad of Gly carbonyls on one face and the triad of Pro carbonyls on the other face of the disc-shaped molecule -- the results with Val-OMe suggest the formation of an amino acid-cyclic peptide "sandwich" of 2/1 stoichiometry. The higher molecular weight of the valine sandwich complex versus the 1:1 *cyclo*(Pro-Gly)$_3$/Val-OMe complex (to which the amino acid sandwich complex reverts with increasing peptide concentration) would account for the rapid drop of Val C_α NT_1 values at the 2/1 salt/peptide ratio. The lack of evidence for the formation of a 2/1 Pro-OBz/*cyclo*(Pro-Gly)$_3$ complex is consistent with the expectation that there will be less interaction of an immonium salt with *cyclo*(Pro-Gly)$_3$ relative to an ammonium salt. Analogous complexes of *cyclo*-(Pro-Gly)$_3$ with magnesium ion (*i.e.*, 2/1 Mg^{2+}/*cyclo*(Pro-Gly)$_3$) have been described.[4] Accumulated evidence from studies on *cyclo*(Pro-Gly)$_3$ suggests that the binding of the first Val-OMe causes the major conformational changes in the peptide as well as the major chemical shift changes, particularly in resonances remote from binding sites. It is believed, however, that binding of the second Val-OMe takes place with only a minor conformational change in the peptide.

Preliminary experiments with Sar-OMe·HCl and acetylcholine in the presence of *cyclo*(Pro-Gly)$_3$ also indicate the formation of discrete complexes.

Acknowledgments

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MOLECULAR DESIGN OF A CYCLIC OCTAPEPTIDE MIMICKING THE ZINC(II) BINDING SITE OF CARBOXYPEPTIDASE A. THEORETICAL STUDIES ON THE CONFORMATION OF THE DESIGNED CYCLIC PEPTIDE-ZINC(II) COMPLEX

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THE PRESENT STUDY is a part of our continuing investigation of molecular design of the functional sites of biological macromolecules.[1-6] We have focused on the problem of mimicking a portion of the active site region of carboxypeptidase A: a zinc containing proteolytic enzyme whose primary structure was worked out by Bradshaw, *et al.*,[7] and its tertiary structure was determined by Lipscomb, *et al.*,[8]. We have attempted to mimic the zinc binding site comprised of His-69, Glu-72 and His-196. Initially, using model building as a first step, an octapeptide molecule was designed, incorporating the two histidines and a glutamic acid in a reasonable geometry as it occurs in the crystal structure. The spaces in between the ligand amino acid residues were filled with glycyl residues. Glycyl residues were chosen mainly because of their being structurally the simplest of residues. The sequence of the designed cyclic octapeptide is shown,

Gly-L-Glu-Gly-Gly-L-His-Gly-L-His-Gly

The first phase of our study consisted of generating an acceptable set of atomic coordinates for the linear octapeptide. This was accomplished by using a cartesian coordinate program from standard bond lengths, bond angles and dihedral angles.[9] The peptide units were assumed to

be in a planar transconformation. Standard tetrahedral geometry was assumed for sp^3 carbon atoms. A contact scan was made, using the procedure of Nemethy and Scheraga[10] to select "sterically allowed" conformations, whose end to end distance, *i.e.*, between the two loose ends of the octapeptide, were within 1.5 Å to 4.0 Å. Ring closure was attempted by systematically incrementing the backbone angles, ϕ and ψ, until the two loose ends were within reasonable bonding distance to each other, taking care not to violate the steric criteria. Selected conformations satisfying the ring closure constraint were minimized, using a total optimization of molecular geometry program, based on a modified Newton-Raphson method, which was applied earlier successfully for a number of amino acids, peptides and their metal complexes[11,12] in our laboratory.

Using the complete neglect of differential overlap method (CNDO/2)[13], the monopole charge distribution was obtained for the cyclic octapeptide molecule, by appropriately segmenting it. The molecular electrostatic potential for the cyclic octapeptide was generated, using the CNDO/2 charge distribution, following the procedure suggested by Bonaccorsi, *et al.*,[14]. The iso potential curves generated for the cyclic octapeptide will be reported in detail elsewhere.[15] From the detailed contours of the molecular electrostatic potential, the regions most vulnerable for attack by zinc may be readily discerned. This method has been used by Kollman *et al.*,[16] for mapping electrostatic potential around carboxypeptidase A and its active site.

However, it was realized that, in order to obtain a reasonable geometry for the metal complex with standard metal-ligand bond distances and angles,[17] a slight distortion of the conformation of the free cyclic octapeptide was necessary. Fixing zinc at the origin of a right handed coordinate system, a systematic attempt to increment the backbone and side chain torsional angles, (*i.e.*, ϕ_i, ψ_i and χ_i^n) in order to achieve standard zinc-ligand bond lengths and bond angles, resulted in several conformations of the metal complex. By repeating the minimization process on the satisfactory conformations, a minimum energy conformation for metal complex was obtained.

One of the minimum energy conformations obtained is schematically shown in Figure 1. The H_2O molecule is not shown. The conformation is stabilized by intramolecular hydrogen bonding (not shown in the figure). The zinc has a distorted tetrahedral coordination geometry. The predicted bond lengths and bond angles involving zinc for the conformation shown in Figure 1, is presented in Table I.

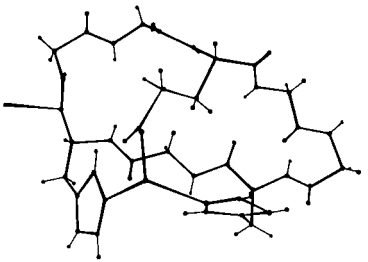

Figure 1: Conformation of the Zinc-cyclic octapeptide complex.

Table I

Some of the Predicted Bond Lengths and Bond Angles Involving Zinc and Ligand Atoms

Bond Lengths		*Bond Angles*	
$Zn - O_{72}$	2.05 Å	$N_{69}- Zn - N_{196}$	102°
$Zn - N_{69}$	2.01 Å	$N_{69}- Zn - O_{72}$	110°
$Zn - N_{196}$	2.07 Å	$N_{196}-Zn - O_{72}$	150°

Most important aspect of our study concerns the relative role of different intramolecular forces contributing to the stability of the complex. The primary role of electrostatic forces in directing specificity, stressed in a number of similar situations such as enzyme-substrate interactions in molecular biology[18], is once again brought to light in the metal-ligand interactions. Non-bonded interactions also play an important role in stabilizing the complex. A comparison of the torsional angles, *i.e.*, ϕ_i, ψ_i and χ_i^n of the free cyclic octapeptide and its zinc complex reveals the conformational changes induced by the metal atom. We would like to mention here that we have treated the metal atom (or ion) as a point charge and as a result we have not considered the detailed electronic arrangement of the metal atom as such. A complete MO calculation in the *ab initio* or semi empirical framework with geometry optimization for the metal complex is not possible at the present time, although we are currently undertaking an attack on this problem by treating segments of the zinc complex. An investigation of the solution conformation of this metal complex, using a theoretical method developed recently,[19] is presently underway in our laboratory.

Acknowledgment

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SYNTHESIS OF A CYCLIC OCTAPEPTIDE DESIGNED TO MIMIC THE ZINC-BINDING SITE OF CARBOXYPEPTIDASE A

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IN OUR CONTINUING INVESTIGATION on the molecular design of peptide molecules to mimic the functional sites of biological macromolecules,[1-4] we have now designed a molecule[5,6] to mimic a segment of the active site region of carboxypeptidase A that involves the zinc-binding ligands (His-69, Glu-72 and His-196).[7,8] Theoretical analysis on the designed molecule, a cyclic octapeptide, *cyclo*- (Gly-L-Glu-Gly-Gly-L-His-Gly-L-His-Gly)(I) further suggested a conformation in which zinc binds the three native ligands in an approximate tetrahedral geometry similar to what is found in the native enzyme.[9]

The designed cyclic octapeptide (I) was obtained from the corresponding linear octapeptide, Boc-Gly-γ-OBu^t-L-Glu-Gly-Gly-L-His-Gly-L-His-Gly-OBZlNO$_2$ (II) which was synthesized according to the scheme shown in Figure 1. Mixed anhydride procedure (4-methylmorpholine and isobutylchloroformate) was exclusively used to build the fragment Boc-Gly-γ-OBu^t-L-Glu-Gly-Gly-ONp (XII). Condensation of glycylglycine with the mixed anhydride of N-benzyloxycarbonyl-γ-*t*-butyl-L-glutamic acid followed by catalytic hydrogenation of the product afforded the tripeptide X. Repetition of the coupling using X and the mixed anhydride of N-*t*-butyloxycarbonylglycine furnished the tetrapeptide XI which was converted to the active ester XII by treatment with p-nitrophenol and DCC. Both the histidine residues in the fragment L-His-Gly-L-His-Gly-OBZlNO$_2$(VIII) were introduced through the intermediacy of N-benzyloxycarbonyl-L-histidyl azide and the glycine *via* its N-hydroxysuccinimide

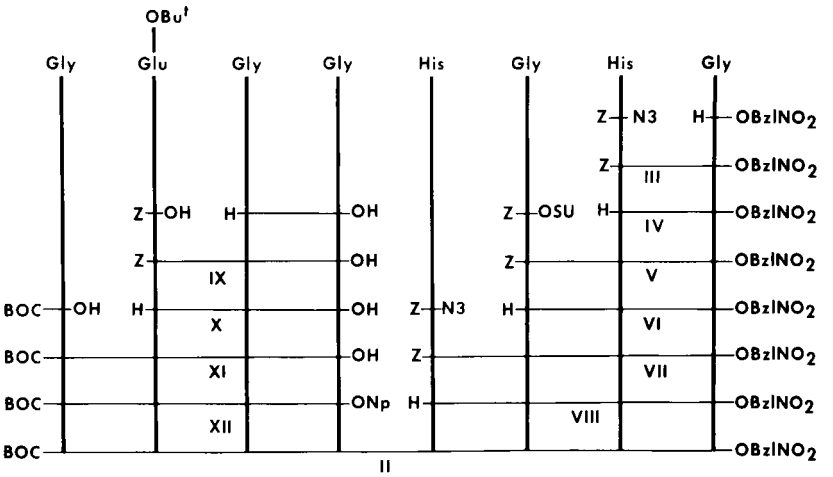

Figure 1: Scheme of synthesis of the linear octapeptide II.

ester. Hydrogen bromide in acetic acid was used to cleave the Z group used to protect the α-amino function of the various intermediates in the synthesis of this fragment. Coupling of the tetrapeptide fragments XII and VIII proceeded smoothly to produce the linear octapeptide II in good yields.

The linear octapeptide II was converted to the corresponding hydrazide which was cyclized *via* the azide following the procedure of Kopple, *et al.*[10] Figure 2 illustrates

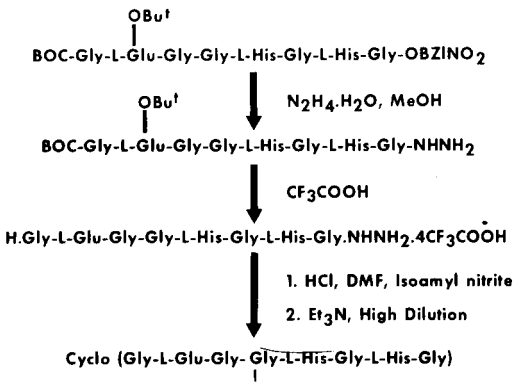

Figure 2: Synthesis of the cyclic octapeptide I.

the various steps involved in the cyclization of the linear peptide. A homogeneous material was isolated from the cyclization reaction by repeated gel filtration followed by purification by countercurrent distribution. The product obtained was found to be both ninhydrin negative and Pauly's positive. Further confirmation of the structure of this material was obtained from the 220 MHz proton magnetic resonance spectrum (Figure 3) of this material

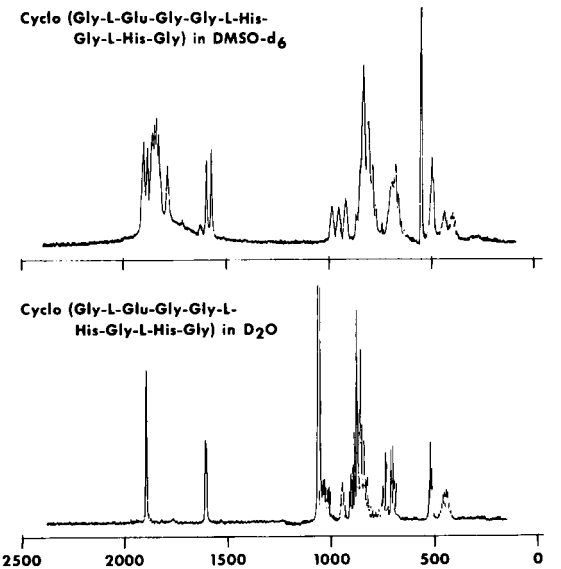

Figure 3: 220MH$_z$ PMR spectra of the cyclic octapeptide I in DMSO-d_6 and D_2O.

which, upon integration, indicated the proper ratios of the various kinds of protons to be expected of the cyclic octapeptide I.

Preliminary Zinc-binding studies showed definite binding of the imidazole side chains of both the histidine residues to the zinc. Involvement of the carboxyl side chain of the glutamic acid residue remains to be determined. Detailed structural studies are now underway.

Acknowledgment

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CONFORMATIONAL STUDIES ON L,D-ALTERNATING POLYPEPTIDES

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REGULAR POLY-L,D-PEPTIDES are the subject of recent investigations. Although they do not appear useful as models of proteins which contain only L-amino acid residues, they are strictly related to some naturally-occurring peptide and depsipeptide antibiotics involved in ion transport across biological and synthetic membranes. Some examples are given by Gramicidin A, Enniatins and Valinomycin. Assuming the condition of conformational equivalence between all the amino acid residues so that an inversion of conformation corresponds to a change in configuration, we have shown[1] that the general structure of an alternating L,D-polypeptide is a cyclic one and characterized by a reflection-rotation symmetry S_n: n represents the number of amino acid residues in the ring and can assume only integer even values and among these only when n = 6,8 and ∞ are compounds of physico-chemical interest. This selection rule, however, limits severely the possible conformations of the amino acid residues as shown in Figure 1 where the values of n are given in terms of the angle of rotation (within the sterically allowed regions) ϕ and ψ. Only an α-helix type conformation generates extended chains (n = ∞) and α-pleated sheets can be stabilized by intermolecular hydrogen bonds (see Figure 2); otherwise a β-type conformation gives rise to rings stabilized by transannular hydrogen bonds.

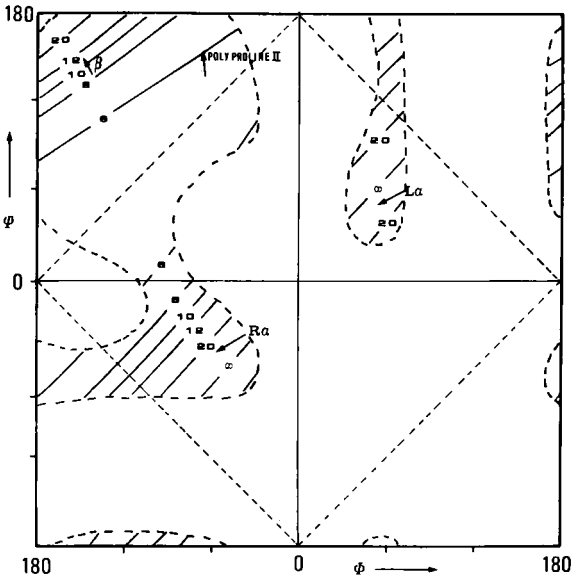

Figure 1

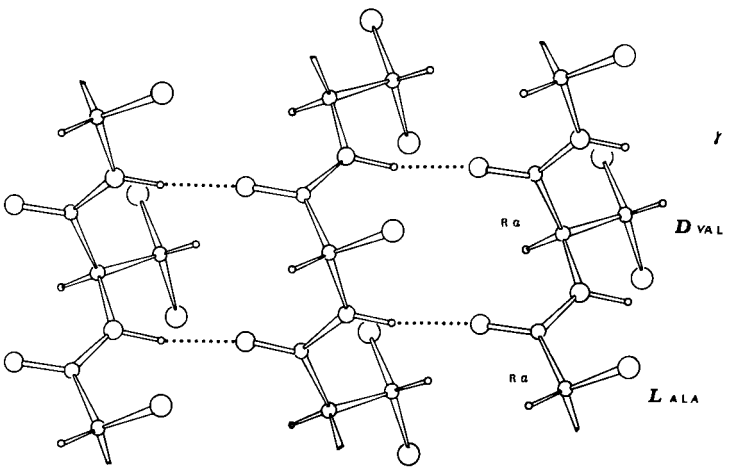

Figure 2

However, L,D β-helical structures (stabilized by intramolecular hydrogen bonds) can be formally obtained since the rigid conformational equivalence is slightly relaxed (see Figure 3). Therefore the alternation of configurations along a peptide chain results in the stabilization

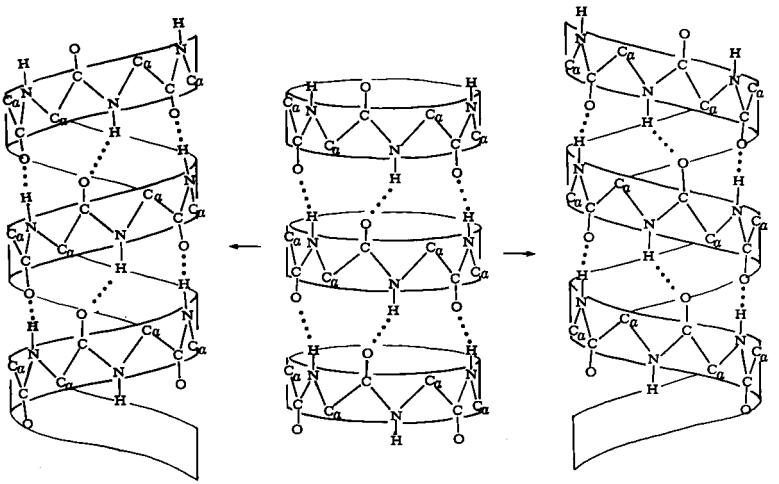

Figure 3

of two general structures, the α-pleated sheets and the β-helices, which can be considered as the secondary structures of the L,D-alternating peptides. We have recently been able to show the presence of these structures in poly-L,D-peptides corresponding to the formula Z-(L Ala-D Val)$_m$OCH$_3$ m = 2, 3, 4, n^2. The CD spectra in MeOH, trifluoroethanol and dioxane are shown in Figure 4. Two conformations can be hypothesized to be present in solution when the solvent is changed. One of these can be identified as a β-type conformation owing to the close similarity of the CD spectral patterns with those of β-structures in poly-L-peptides. The general features of NMR spectra of the oligomers in DMSO (which are characterized by equivalence of Ala and Val NH signals and by high $J_{NHC\alpha H}$'s, see Figure 5) further support the presence of β-type conformations and rule out the possibility of an α-helical structure which was previously found in poly-L,D-benzyl glutamate.[3,4] In the solid state however the X-ray

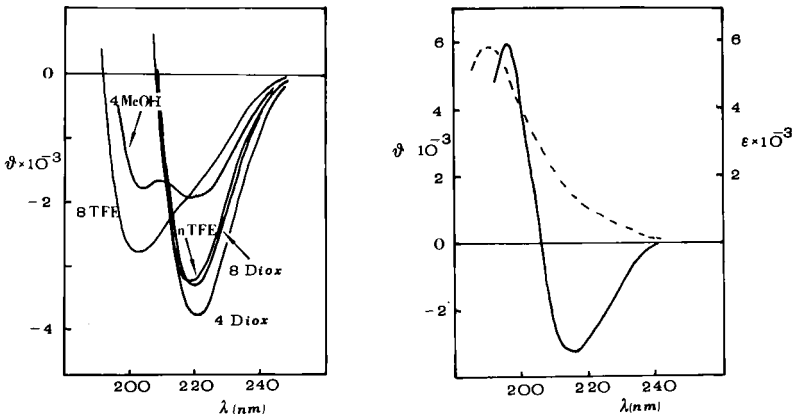

Figure 4: CD spectra of Z-(L-Ala-D-Val)$_m$OCH$_3$ m = 2,4,n in MeOH, trifluoroethanol (TFE) and dioxane (Diox). The molar ellipticities were divided by the number of amino acid residues indicated (left); absorption and CD spectra in TFE of the polymer (right).

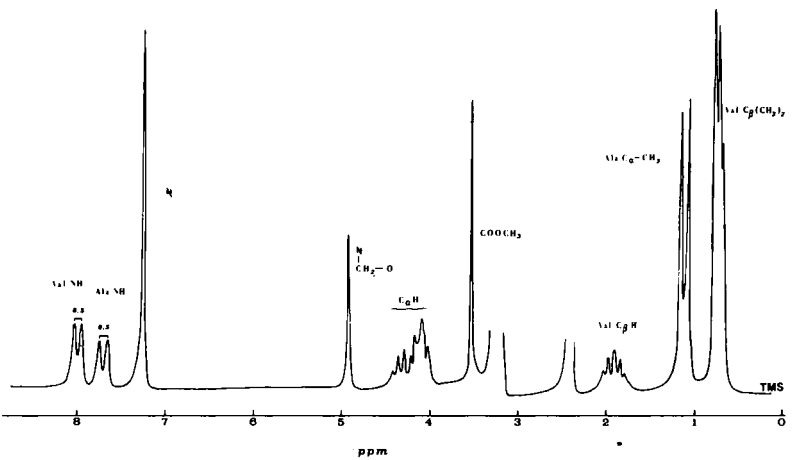

Figure 5: 100 MHz PMR spectra of Z-(L-Ala-D-Val)$_4$OCH$_3$ in DMSO.

powder diagrams show a quite similar structure of the polymers (except for the tetramer) and the cell parameters are only consistent with an α-pleated sheet. On the other hand IR spectra are also very similar and show a strong splitting of the Amide I band (1635, 1675(sh) cm^{-1}). This finding also suggests an extended conformation as opposed to the α-helix. It should be remembered that a similar structure was proposed by Lotz *et al.*[5] for poly-D,L-benzyl glutamate on the basis of electron and X-ray diffraction studies and by Aubert and Loucheux-Lefebvre for the poly-(β-para-nitrobenzyl-L-aspartate-β-benzyl-L-aspartate)[6] by CD and NMR spectra, since these amino acid residues tend to stabilize right-handed and left-handed structures, as shown several years ago by Goodman *et al.*[7]

As in related poly-L-peptide, poly-L,D-proline assumes a different type of structure due to both the intrinsic conformational rigidity of the pyrrolidine ring and the strong steric interactions with the next residue. As a result of conformational calculations, prolyl residues can assume only the poly-proline II local conformation irrespective of the configuration of the adjacent residues. This is illustrated in Figure 6 where the general

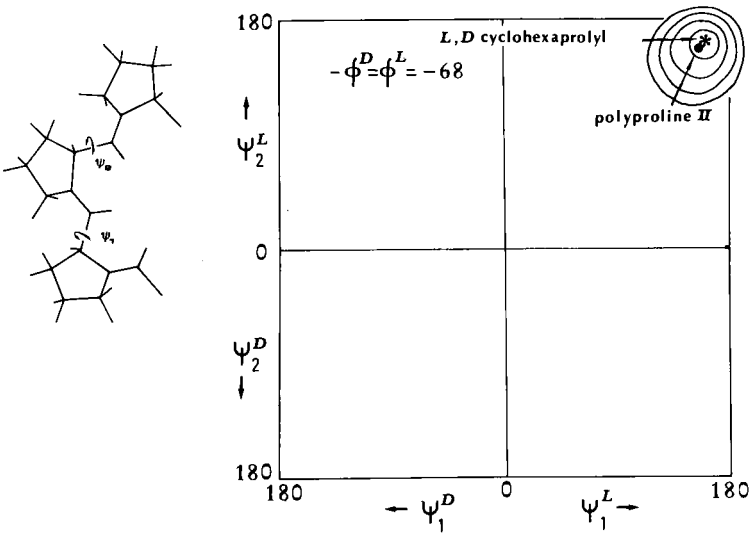

Figure 6: Van der Waals conformational energy map of the tri-prolyl fragment indicated in terms of the angles of rotation ψ_1 and ψ_2. Changes in the configurations (indicated as L and D) of the prolyl residues result in the inversions of the sense of rotation.

conformational energy map of the three-prolyl fragments for all the possible configurational sequences is shown in terms of ψ_1 and ψ_2 angles of rotation.

The diagram of Figure 1 shows that the polyproline II type conformation fits exactly a six member ring. This structure is characterized by an octahedral cavity similar to that found in Valinomycin, Enniatins and other related ion carriers and it is expected to be conformationally rigid and possibly highly selective in the ion complexation. In fact peculiar differences in the NMR spectra were observed even in the dimers Z-L-Pro-D-Pro-OCH_3 and Z-L-Pro-L-Pro-OCH_3, the former being an intermediate in the synthesis of the L,D-cyclohexaprolyl in progress in our laboratory. As it is shown in Figure 7, the -O-CH_3

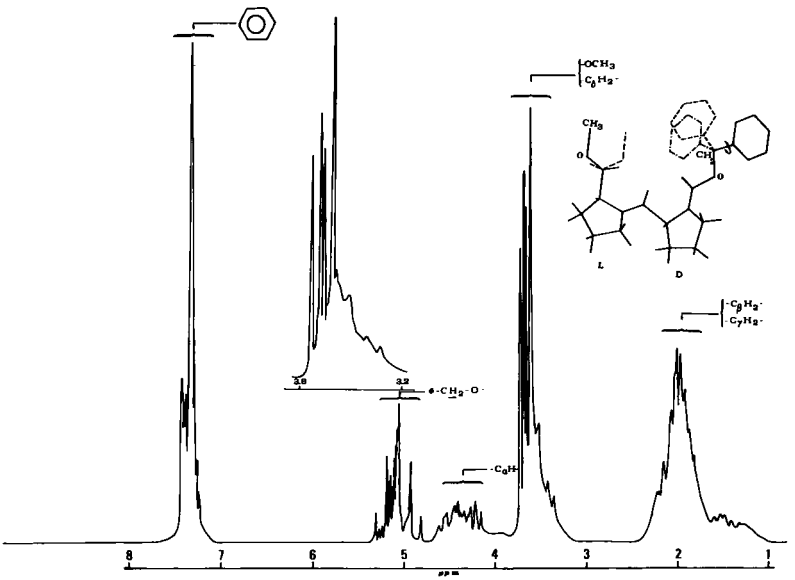

Figure 7: 100 MHz PMR spectrum of Z-D-Pro-L-Pro-OCH_3 in $CDCl_3$. The possible conformations are indicated in the insert.

signal is split in a quartet in the L,D case suggesting that this group experiences four different chemical environments, whereas this splitting is absent in the L,L compound (Figure 8). A possible model is illustrated in the inserts of Figures 7 and 8, where the favorable conformations of

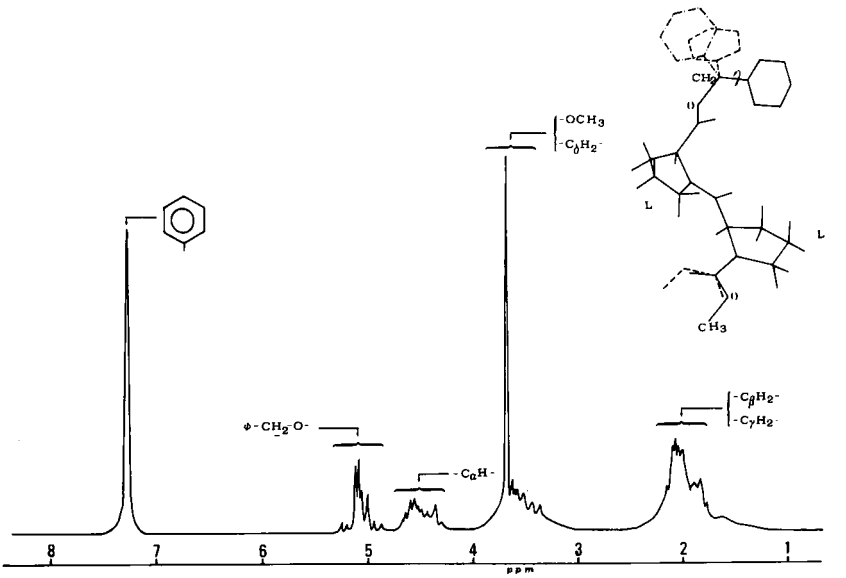

Figure 8: 100 MHz PMR spectrum of Z-L-Pro-L-Pro-OCH_3 in $DCCl_3$. The possible conformations indicated in the insert.

the di-prolyl derivatives are represented. **U** shape structure characterizes the L,D compound which allows a close contact between the terminal groups. On the contrary shielding by the phenyl group is prevented in the **S** shape conformation which characterizes the L,L derivative. Studies of conductance in bimolecular lipid membranes treated with solutions of our peptides as well as the analysis of the induced fluctuation current are in progress.

Acknowledgment

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RESONANCE AND NON-RESONANCE RAMAN SPECTROSCOPY: A PROBE OF PEPTIDE AND PROTEIN CONFORMATION

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WHEN DR. WALTER INVITED ME to address the American Peptide Symposium on the topic of laser Raman spectroscopy, he wrote "Since this is the first time we cover this subject in a Peptide Symposium, I suggest that you present a brief history focusing primarily on the state of the art, development of the method to date, and a terse outlook into the future." What follows is therefore rather pedagogical: firstly we describe the basic phenomenon of the Raman effect, secondly we interpret the effect on a microscopic level, emphasizing the sort of structural information it provides -- and contrasting Raman with other forms of spectroscopy -- and thirdly we shall illustrate the points made by brief reference to selected results from our laboratory.

Basic Phenomenon

Suppose a beam of highly monochromatic light passes through a solution. For the sake of specificity, suppose the light is blue-green laser light of wavelength 500 nm. (Figure 1a).

What is the frequency of the light that is scattered? Three regions of the observed spectrum are identified in Figure 1b. The first region is a single peak centered about the incident frequency; the scattering is called

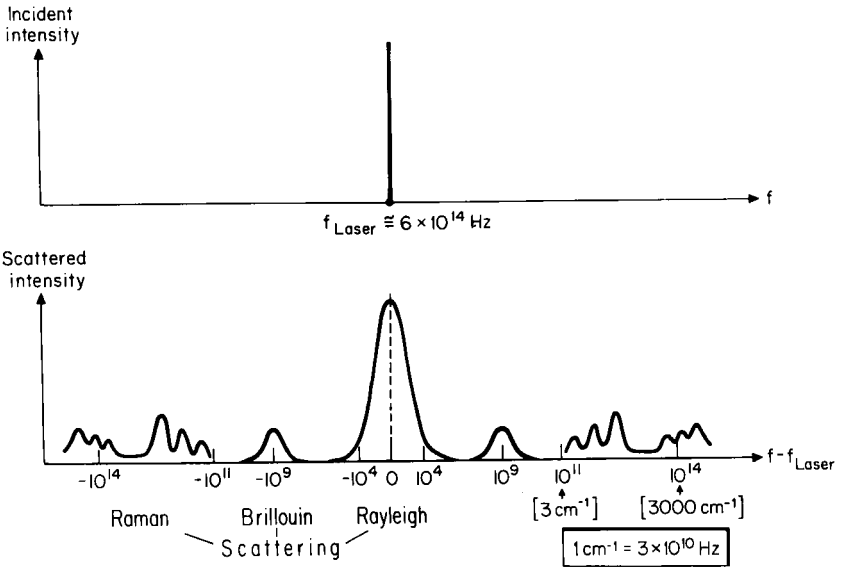


Figure 1: The light scattered off a material is divided into 3 regions, according to the change in frequency of the scattered light.

"Rayleigh" or "quasi-elastic" scattering, because the change in frequency is so small that the scattering is almost perfectly elastic. The half-width of the Rayleigh peak can give information on the size and shape of the molecules doing the scattering. The second region of the spectrum is a doublet symmetrically situated 10^{8}-10^{10} Hertz about the laser frequency; this "Brillouin" scattering gives information about collective modes of oscillation in the material. The third region and the region that concerns us here is the multipeaked Raman spectrum situated 10^{11} - 10^{14} Hertz on either side of the laser frequency. The frequency *shifts* correspond to the vibrational frequencies of the scattering molecules and hence are a source of information on molecular structure.

The Raman effect is named after C. V. Raman, who first observed it. Raman's experiment, shown schematically in Figure 2, is instructive if only because the essential technique has changed little in the 50 years since his discovery. Raman used filtered sunlight to produce monochromatic light, and demonstrated that a complementary

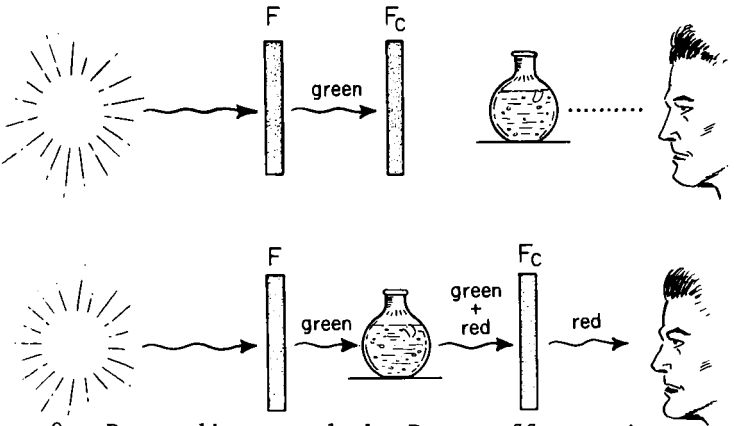


Figure 2: Raman discovered the Raman effect using filtered sunlight.

filter would blot out all the light (Figure 2a); when he placed a beaker of solution in between the two filters, he was able to detect minute amounts of red-shifted light (Figure 2b).[1]

The principles in today's Raman scattering experiment are of course no different than in Raman's first experiment, but the technique has improved greatly (Figure 3).

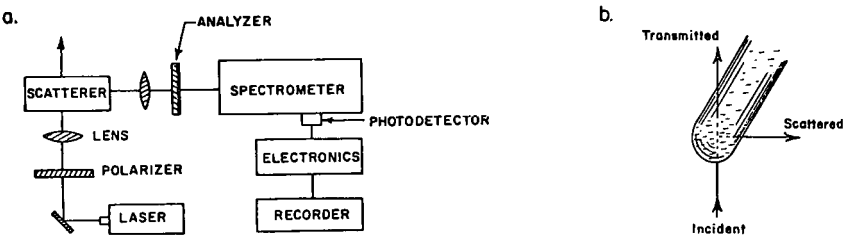


Figure 3: Today we replace filtered sunlight by a laser for greater monochromaticity; the second filter in Figure 2 is replaced by a double grating spectrometer.

We now use a laser for greater monochromaticity. The sample can be as small as a microliter, held in a glass capillary. The light scattered by the sample is collected by a large lens and frequency analyzed by a double grating monochromator.

Its Interpretation

We now give an elementary interpretation of the Raman effect. Figure 4 is a schematic representation of an isolated hydrogen atom, its nucleus shown as a solid circle. What happens if we suddenly apply an electric field to the hydrogen atom? The electron cloud responds almost instantly to the field, with the center of the cloud shifting a distance b from its original position.

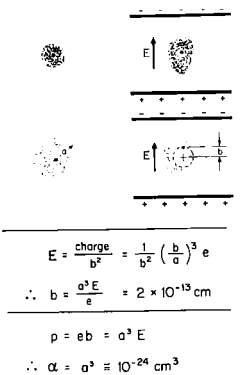


Figure 4: A hydrogen atom's electronic charge cloud in an electric field of 100 statvolts = 30,000 volts/cm. is displaced only twice the radius of the nucleus; the polarizability is the proportionality constant between the dipole moment p induced by this displacement and the electric field.[2]

How much is the hydrogen atom deformed by the field? That is, how large is b? To answer this question, let us approximate the electron cloud by a sphere of radius a, and calculate the displacement b of the center of this negatively charged sphere. The field due to the electron cloud at a point inside a sphere of charge at a distance b from the center is equal to the charge inside a sphere of radius b, divided by b^2.[2] This charge is just the fraction of an electronic charge contained within the sphere of radius b, so that

$$b = (a^3E)/e = (10^{-8})^3\ 10^2\ /\ (5 \times 10^{-10}) = 2 \times 10^{-13}\text{cm}. \quad [1]$$

This distance is only twice the radius of a bare proton, a very small distance indeed! However we shall see that this minute displacement gives rise to all light scattering, including the Raman effect. The physical reason for this fact is that the displaced electron charge cloud creates a small dipole moment p (of magnitude eb) so that if the electric field is created by an oscillating electromagnetic wave, then the dipole oscillates and therefore radiates an electromagnetic wave.

From Eq.[1] we see that p (= eb) is directly proportional to the field strength E, with proportionality constant a^3. This proportionality constant is called the atomic polarizability and is denoted α. For our extremely crude model of hydrogen, we estimate that $\alpha = a^3 = 10^{-24}$ cm^3, an estimate close to the experimental value.

Now we are ready to understand the Raman effect (Figure 5). What happens when we subject a triatomic molecule to an electromagnetic field? If the polarizability

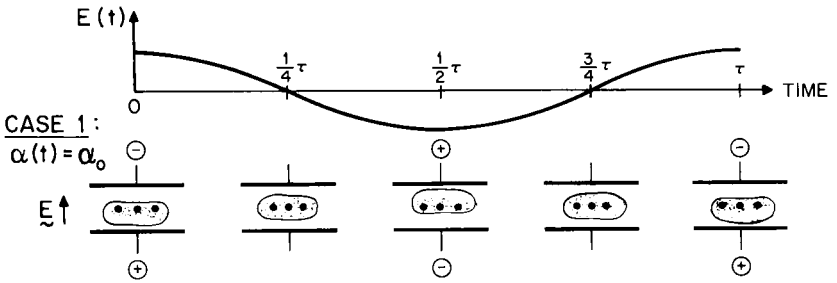
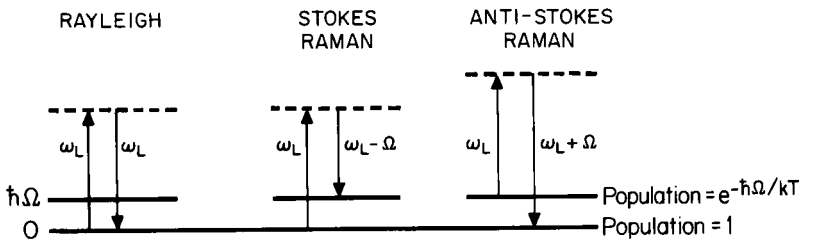


Figure 5: The Raman effect arises only because the molecular polarizability changes as the molecule vibrates; elementary trigonometry predicts that there should be both Stokes and Anti-Stokes Raman lines.

α were independent of time, then the electron cloud would simply slosh up and down with the oscillating field, creating an oscillating electric dipole moment that in turn radiates light of the same frequency. *We would have elastic Rayleigh scattering, but no Raman scattering.*

In reality, however, the polarizability is by no means constant. Rather α oscillates with frequency Ω, the symmetric vibration frequency of the molecule; this happens because when the atoms come apart, the electron cloud is less tightly held than when the atoms come together. What is the effect of this oscillating polarizability? Elementary trigonometry tells us that the resulting dipole radiation will now be at two *additional* frequencies, $\omega - \Omega$, and $\omega + \Omega$, called the Stokes and Anti-Stokes frequencies respectively.

Consideration of the energy level diagrams in Figure 6 shows that the intensities of the Stokes and Anti-Stokes Raman lines are not equal, as suggested by the preceding



SAMPLE TEMPERATURE :

$$\frac{kT}{\hbar} \cong 200\ \text{cm}^{-1} \text{ at } 300\ \text{K}$$

$$\frac{I_{AS}}{I_S} = e^{-\hbar\Omega/kT} \cong \begin{cases} \frac{1}{2} & \Omega \cong 150\ \text{cm}^{-1} \\ \frac{1}{100} & \Omega \cong 900\ \text{cm}^{-1} \end{cases}$$

Figure 6: The ratio of the Anti-Stokes to the Stokes intensities is a measure of sample temperature.

classical analysis. Rather the Stokes intensity will be stronger than the Anti-Stokes intensity, because while the Stokes Raman light arises from the excitation of a ground state vibrational energy level, the Anti-Stokes scattering

arises from the excitation of a vibrational level at energy $\hbar\Omega$ relative to the ground vibrational level, and the population of the excited vibrational level is less than the population of the ground vibrational level by a factor $\exp(-\hbar\Omega/kT)$. Here $\hbar$ is Planck's constant divided by 2π, k is the Boltzmann constant, and T is the absolute temperature. Thus by measuring the relative intensities of a pair of corresponding Stokes and Anti-Stokes Raman lines, we can obtain a direct measure of the temperature of the sample. The ability to continuously monitor the sample temperature is a feature not possessed by many other forms of spectroscopy.

Figure 7 compares Raman spectroscopy with infrared spectroscopy (IR). In IR, one illuminates the sample with a variety of individual wavelengths of infrared light, and

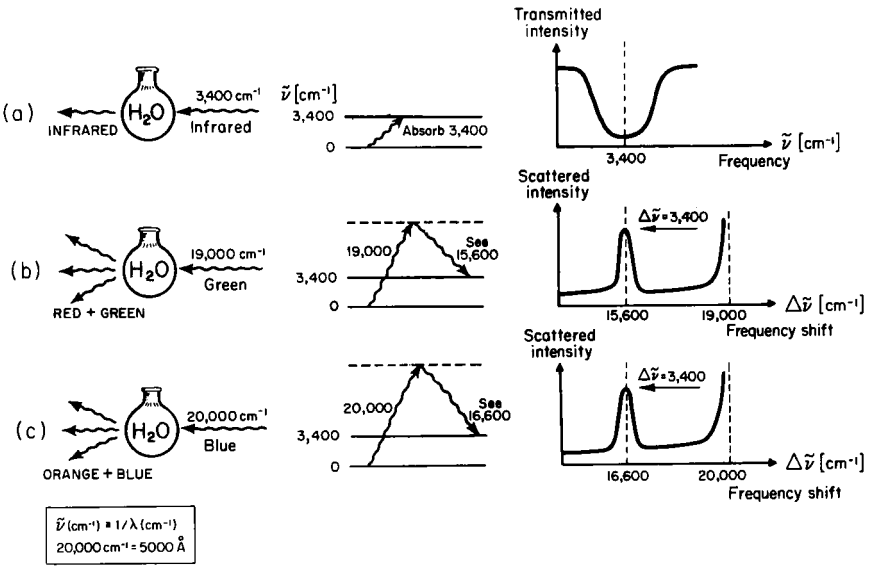


Figure 7: IR spectroscopy measures transmitted intensity as a function of incident frequency, while Raman spectroscopy measures scattered intensity as a function of frequency shift from a fixed incident light frequency.

measures the transmitted intensity at different incident frequencies. On the other hand, in Raman spectroscopy one illuminates the sample with a fixed wavelength and measures

the scattered intensity as a function of the frequency *shift* from the incident frequency. Both techniques give information about the vibrational energy levels, and hence both provide clues to molecular structure. For biological applications, however, infrared spectroscopy has the drawback that water is extremely opaque to infrared light, whereas water hardly obscures visible light at all. To be precise, water environments all but prohibit useful infrared measurements for vibrational frequencies below about 1500 cm^{-1}, whereas water interferes with Raman measurements only in 2 regions near 3400 cm^{-1} and 1650 cm^{-1}; by replacing H_2O by D_2O we can make Raman measurements even in these regions. The bottom line of Figure 7 shows that one can repeat Raman experiments using a laser line of different frequency, and the resulting spectrum is identical so long as one plots ones data as a function of the shift in frequency.

One reason for using different laser lines is to obviate problems due to fluorescence. Figure 8 contrasts the physical processes underlying fluorescence phenomena

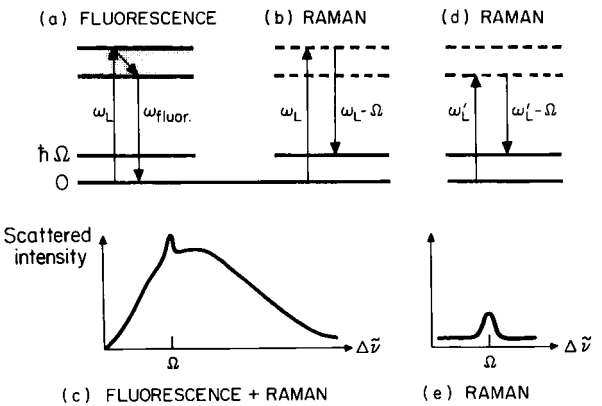


Figure 8: Fluorescence phenomena are much stronger than Raman scattering; they can be removed in many cases by choosing a different laser frequency.

with those underlying Raman scattering. In fluorescence phenomena the laser excites an electronic level in a region where there are many closely-spaced excited levels to which the molecule may decay. Then there is a final decay from some electronic level to the vibrational ground state. Thus the fluorescence signal competes with the Raman signal

(Figure 8c), and since fluorescence phenomena are generally intense (and since the noise is roughly proportional to the total light scattered), one may have difficulty seeing the Raman-scattered light superposed on the fluorescence signal. One solution to this problem is to vary the frequency of the laser light until one is below the "band" of Figure 8a; this solution is shown in Figure 8d and 8e. A second solution would be to measure the Anti-Stokes Raman spectrum, but at room temperature the Anti-Stokes spectrum becomes weaker with increasing shift, being 1% as strong as the Stokes spectrum for a shift of 900 cm^{-1} (Figure 6).

Applications of the Raman effect are often limited by the fact that in order to see a given structural feature -- *e.g.*, a hydrogen bond -- one needs a concentration in excess of about 1 m*M* of the moieties responsible for that feature (of course, one need not necessarily have molecular concentrations in excess of 1 m*M* -- and later we shall see that many structural features of the large protein myosin are evident when the concentration is only 0.1 m*M*). Now if the vibrational modes of interest are associated with a chromophore, then we can choose (by using, *e.g.*, a tunable dye laser) a frequency ω' that selectively excites the chromophore-associated modes (Figure 9). Our group

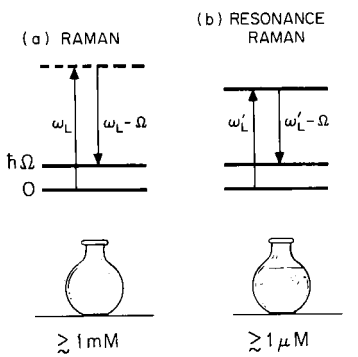


Figure 9: Resonance Raman spectroscopy can be used in much more dilute solutions of scatterers by tuning the incident laser frequency to an electronic absorption band.

has done such "resonance Raman" experiments to study *in vitro* and *in situ* hemoglobin and certain of its mutants, and to study the membrane protein rhodopsin. One can get interpretable spectra from a drop of blood in a 10 ml. beaker of distilled water, so dramatic is the sensitivity improvement of resonance Raman over nonresonance Raman spectroscopy.

Selected Applications

Conformational Information on Membrane-Active Ion-Specific Molecules

How can an ion get through a membrane? This basic question engages the attention of increasing numbers of researchers in recent years. Many workers are focusing on simple systems in an attempt to gain insights regarding the mechanisms governing membrane conductivity and membrane selectivity. A lipid bilayer (or "black lipid membrane") by itself has an ionic conductivity about 10^5 lower than observed membrane conductivities and is not selective for one ion over another. However, if the bilayer is presented with small amounts of certain membrane-active materials -- such as the prototype pore-forming antibiotic gramicidin A or the prototype carrier antibiotic valinomycin (Figure 10)

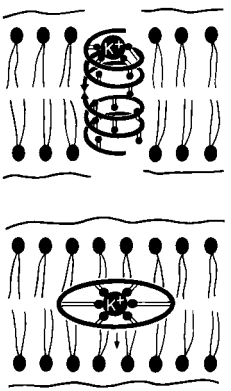


Figure 10: Gramicidin is a prototype pore-forming antibiotic, while valinomycin is a prototype carrier antibiotic.

-- then the bilayer acquires a remarkable degree of ionic conductivity and ionic selectivity. The primary structure of these "ionophores" is known and Figure 11 shows schematically the conformation of the 12 carbonyl groups of the valinomycin-cation complex in the crystalline state.[6] The 6 ester carbonyl groups coordinate the cation (Figure 11a), while the 6 amide carbonyl groups form intramolecular hydrogen bonds with the 6 NH groups (Figure 11b) resulting in the characteristic 'bracelet' conformation. What is the structure of uncomplexed valinomycin? Some

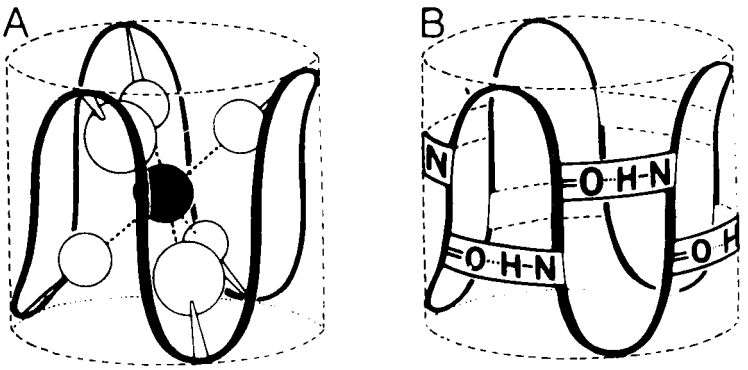


Figure 11: The x-ray structure of the valinomycin-K^+ complex reveals that the 6 ester carbonyl groups coordinate the cation, while the 6 amide carbonyl groups are intramolecularly hydrogen bonded, giving rise to the characteristic 'bracelet' conformation. After ref. 4.

have argued that -- at least in non-polar solvents -- uncomplexed valinomycin is characterized by 6 free ester carbonyl groups, with the remaining 6 amide carbonyl groups hydrogen bonded as in Figure 11b. However in 1972 Duax and collaborators proposed a x-ray structure that was rather less symmetric, being characterized by only 4 hydrogen bonded amide carbonyl groups and 2 hydrogen bonded ester carbonyl groups, leaving 2 free amide and 4 free ester carbonyl groups.[7]

Now the stretch vibration frequency of an ester carbonyl group is about 100 cm^{-1} larger than the stretch vibration frequency of an amide carbonyl group; moreover, the stretch vibration frequency of a free carbonyl group is about 25 cm^{-1} higher than that of a hydrogen bonded carbonyl group. Hence the x-ray structure for the valinomycin-K^+ complex (Figure 11) leads us to expect 2 peaks in the Raman spectrum -- one for the hydrogen bonded amide carbonyl groups and the second for the ester carbonyl groups coordinating the cation. This is indeed observed (Figure 12a). For uncomplexed valinomycin, the x-ray structure suggests that there should be 4 peaks, since both ester and amide carbonyl groups are present both in the free and hydrogen-bonded form. Four peaks are indeed observed (Figure 12b), thus confirming the x-ray findings of Duax and collaborators.

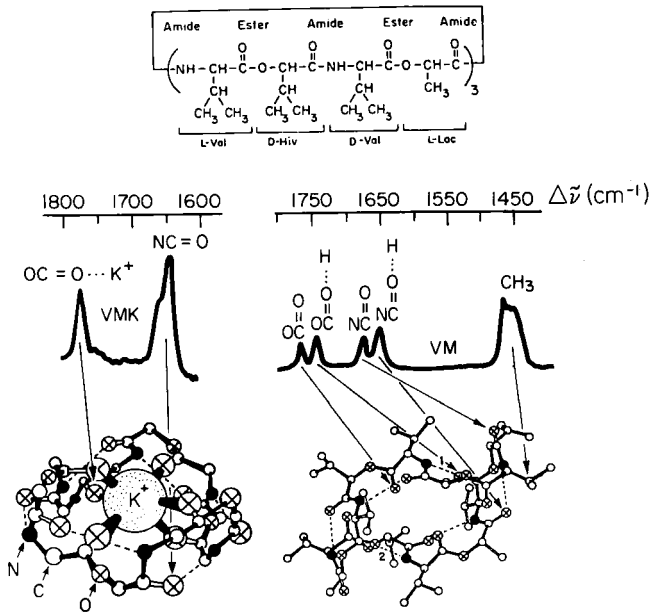


Figure 12: The Raman spectra[5] of the valinomycin-K^+ complex and of uncomplexed valinomycin confirm the x-ray crystallographic structures.[6-7]

One of the advantages of Raman spectroscopy is that it can be used to connect conformations obtained by x-ray scattering with those obtained by methods such as NMR that may be used only in solution. For example, Figure 13 summarizes what happens to the carbonyl stretch region of the valinomycin spectrum when we perturb the crystalline conformation by dissolving valinomycin in solvents of varying polarity. Although the ester carbonyl stretch vibration frequencies of the potassium complex are similar in the solid state and in non-polar solution (~1770 cm^{-1}), they are quite different in polar solvents (~1758 cm^{-1}). These results suggest that, in the complex, the ester carbonyl groups and the K^+ ion are only partially shielded from external solvent. Solvent interactions with the complexed ion may be a significant part of the ion-release mechanism in such ionophores.

For gramicidin A, no x-ray structure is known. The Raman spectra[9] are characteristic of structures with β conformation, both in the solid state and in most solvents,

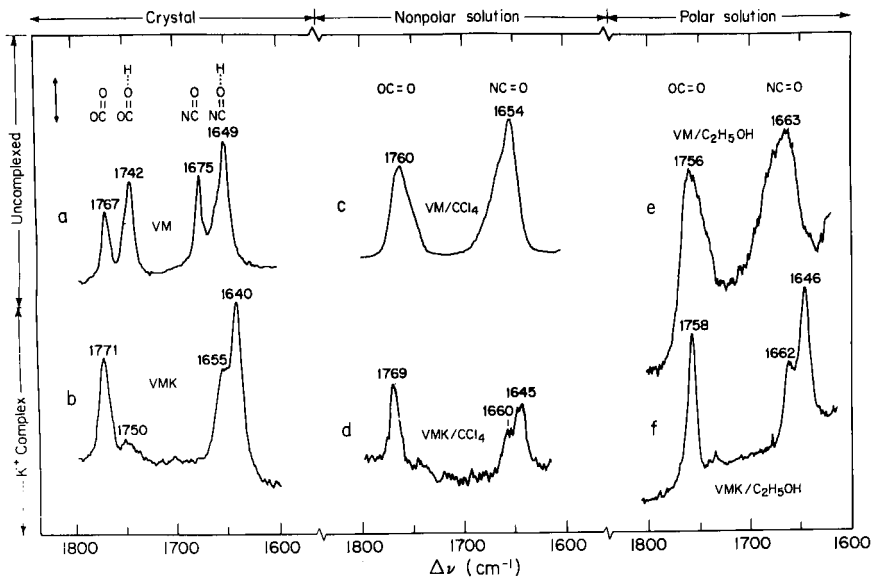


Figure 13: Raman spectroscopy can be used to connect conformations obtained in the crystalline state with those obtained in solution. Here we note that the ester carbonyl stretch vibration frequency changes as a function of solvent polarity, showing that the group that coordinates the cation is in fact susceptible to environment.[8]

thereby supporting Urry's proposed end-to-end dimer conformation (Figure 10a) characterized by parallel β hydrogen bonding.[10] Very recently Veatch and Blout[11] have proposed a different conformation that is characterized by antiparallel β hydrogen bonding, but at the present time we cannot distinguish parallel from antiparallel β hydrogen bonding; it is possible that future work with model compounds will enable the technique to make this distinction.

Thus far we have discussed the dependence of carbonyl stretch vibration frequencies upon the environment of the carbonyl groups. Now we examine the dependence of these frequencies on the properties of the ion being coordinated by the carbonyl group. To this end, we choose an ionophore that is less selective than valinomycin. Nonactin is a polyester than can coordinate a variety of different cations, because its conformation (Figure 14) is more like a 'bear trap' than a 'bracelet'.

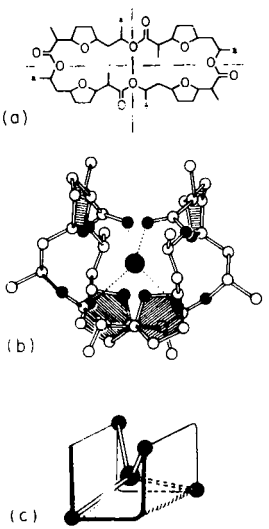


Figure 14: Nonactin is a cyclic polyester, with methyl goups at the asterisks. The conformation of its cation complexes is rather like a thesis binder clamp.[12]

Shown in Figure 15a are the carbonyl stretch vibration frequencies plotted as a function of ionic radius R for a variety of cations, and we note that there is no particular correlation with R. In Figure 15b are shown the same frequencies plotted now as a function of electrostatic interaction energy U between the ion and the carbonyl group. With 3 exceptions (Na^+, NH_4^+, and Tl^+) the data now lie on a straight line, suggesting that the interaction between the carbonyl groups and the cation is predominantly electrostatic. The three exceptions are discussed in detail elsewhere;[13] of significance here is the fact that since the stretch frequency depends on U rather than on R, electrostatic forces in nonactin-cation interactions are probably more significant than steric ('mechanical') interactions. This evidence provides experimental support for electrostatic theories of the ionic selectivity of ionophores.[14]

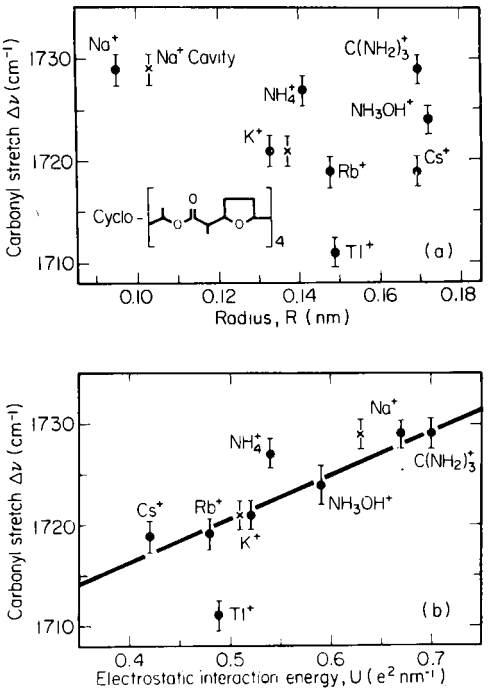


Figure 15: The carbonyl stretch vibration frequencies for the cation complexes of nonactin do not depend in any regular fashion on ionic radius R, but are -- with three exceptions -- a linear function of electrostatic interaction energy U.[12-14]

Conformational Information on Proteins

Raman spectroscopic studies of proteins may be divided into two classes, those using resonance Raman spectroscopy to study a particular region of the protein associated with a chronophore, and those using nonresonance Raman spectroscopy to study the overall properties of the entire protein. Since resonance Raman studies generally give information about one particular region of one protein, they are particularly suitable for *in situ* studies of complex tissues. For example, the resonance Raman spectrum of intact erythrocytes is almost identical to the Raman spectrum of purified hemoglobin, the Raman scattered light in both cases being due almost exclusively to molecular vibrations associated with the heme group. Resonance Raman spectroscopy can also

be used to study mutant hemoglobins, for cases in which the mutation involves residues in the vicinity of the heme group.[15] For example, hemoglobin M Milwaukee (β67 Val → Glu) involves a substitution that stabilizes the Fe atom of the abnormal β chains in the ferric form. In Hb Milwaukee this occurs because the carboxyl groups of the glutamate at E11β occupy the sixth coordination position at the Fe atom. Since the Fe atom of Hb Milwaukee is completely internally co-ordinated, a comparison of the resonance Raman spectra of Hb Milwaukee with that of normal adult Hb helps in the assignment of vibrational modes to some of the observed spectral lines.

The membrane protein rhodopsin is situated in the discs of rod outer segment cells of the retina, and is responsible for the detection of visible light. Understanding the details of the conformational changes that are consequent upon excitation by a single proton is a major unsolved problem. Rhodopsin can be studied both by resonance Raman spectroscopy -- in which case the vibrations associated with the retinal chromophore are responsible for the observed spectrum (Figure 16) -- or by nonresonance Raman spectroscopy, in which case the protein is studied as a whole.[17]

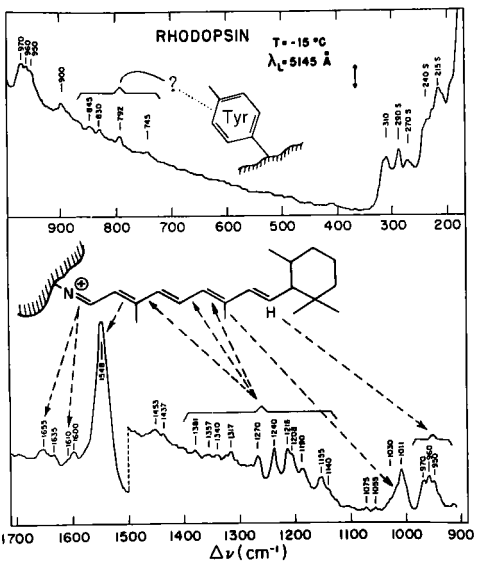
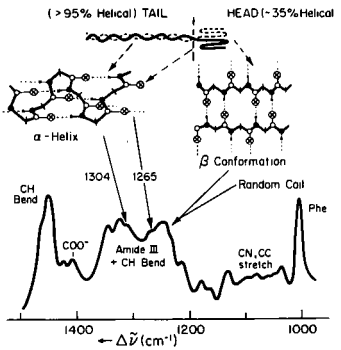


Figure 16: The resonance Raman spectrum of rhodopsin reveals details of conformation that are associated with the chromophore.[16]

Myosin is a structurally-heterogeneous protein of molecular weight 500,000 daltons, or about 10 times larger than rhodopsin. One conformationally-sensitive region of myosin is shown in Figure 17; peaks corresponding



NATIVE MYOSIN — SPECTRUM & STRUCTURE

Figure 17: The Raman spectrum of myosin can be interpreted in terms of the conformations of its subfragments, and these interpretations can be checked by separate Raman studies of these subfragments.[18-20]

to the tail and head regions of myosin are readily identified. Spectra of the isolated subfragments of myosin confirm the assignments indicated schematically in Figure 17. Identifiable spectra have also been obtained for tropomyosin and the three troponins, troponin C (the Ca^{++} binding protein), troponin I, and troponin T.[18-20]

We conclude by noting that light scattered off freshly excised rabbit psoas muscle has a Raman spectrum quite similar to that of purified myosin. The fact that Raman spectroscopy can be used for *in situ* studies makes it an appealing tool for use in probing peptide and protein conformation in the field of molecular physiology.

Acknowledgments

The authors wish to thank Professor George Eisenman and Dr. Sally J. Krasne for useful discussions of the microscopic basis of ionic selectivity. They wish to thank Dr. Vadim T. Ivanov, Academician Yuri Ovchinnikov, and their co-workers at the Shemyakin Institute for many useful discussions and helpful correspondence, and for their hospitality to one of us (IMA) during the autumn of

1974. The authors wish to thank Professor John Gergely and his co-workers at the Boston Biomedical Research Institute for their assistance on many aspects of the research on muscle proteins, and Paul Brown for help on rhodopsin. Finally, continued discussions with Prof. R. C. Lord are gratefully acknowledged. This work has been supported by grants from the National Science Foundation, the Research Corporation, and the National Institutes of Health.

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A NOVEL METHOD FOR CONFORMATIONAL ANALYSIS OF PEPTIDES IN ORIENTED POLYOXYETHYLENE BY INFRARED DICHROISM

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A RECENTLY DEVELOPED PROCEDURE[1] for determining the infrared dichroic spectra of oligo- and polypeptides is described. A peptide is dissolved in a solution of polyoxyethylene in either water or in an organic solvent such as chloroform or trifluoroethanol and a peptide:polyoxyethylene film is formed upon solvent evaporation. Rectangular strips of film are partially oriented by uniaxial stretching and infrared dichroic spectra are then recorded.

The infrared characteristics of the polyoxyethylene support allow measurement of the dichroic spectra of amide N-H stretching bands between 3500-3000 cm^{-1}, of amide I and II bands between 1700-1500 cm^{-1} and of far infrared bands below 800 cm^{-1}. Since polyoxyethylene strongly absorbs large amounts of water vapor, hydrogen to deuterium exchange reactions of incorporated molecules are readily effected by exposure to D_2O vapors. Thus, both the rate of hydrogen to deuterium exchange, which reflects accessibility to the solvent medium, and the orientation of each spectrally distinct N-H group can be determined simultaneously. The correlation of exchange kinetics and dichroism should greatly enhance the value of the separate measurements for conformational analysis.

The infrared dichroic spectrum of uniaxially oriented polyoxyethylene and of gramicidin S oriented in polyoxyethylene are compared in Figures 1A and 1B, respectively.[1] Spectra recorded with light polarized parallel to the direction of stretching are drawn with a solid line; those

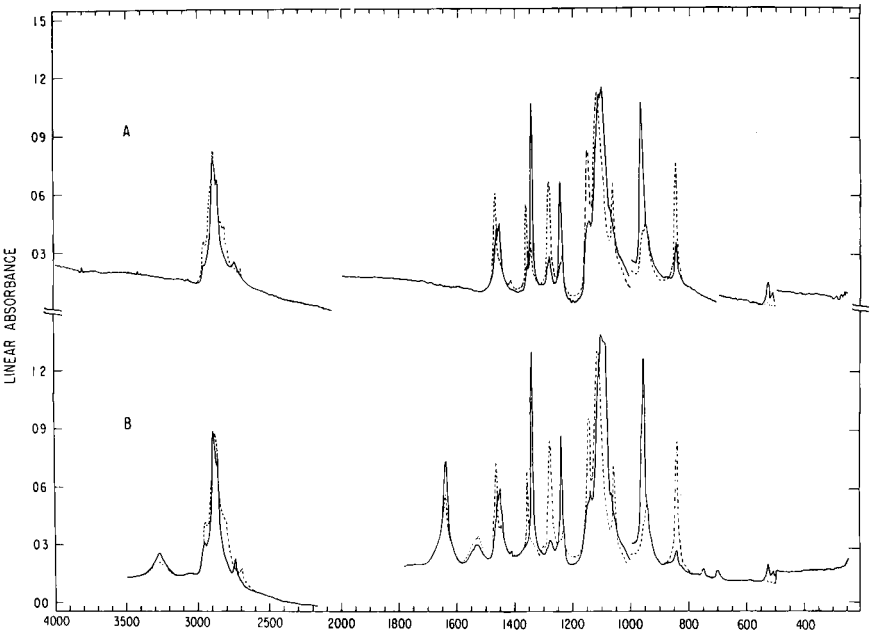

Figure 1: A. Infrared dichroic spectrum of polyoxyethylene oriented by uniaxial stretching; polarization parallel to the stretching direction, ——; polarization perpendicular to the stretching direction, ----.
B. Infrared dichroic spectrum of gramicidin S incorporated in uniaxially oriented polyoxyethylene; polarization parallel to the orientation axis, ——; polarization perpendicular to the orientation axis, ----.

recorded for perpendicular polarization are drawn with a dotted line. It is clear from Figure 1A that the amide NH stretching modes (3500-3000 cm^{-1}), the amide I and II vibrations (1700-1500 cm^{-1}) and the far infrared amide IV and V bands (<800 cm^{-1}) of suspended peptides are free from interference by polyoxyethylene absorptions.

Expanded spectra of the ν(NH), amide I and amide II bands of oriented gramicidin S are presented in Figure 2.[1] The position of the single, sharp ν(NH) band at 3265 cm^{-1} indicates that all of the NH groups of gramicidin in polyoxyethylene are hydrogen bonded. The ν(NH) band, with a dichroic ratio D_{11} = absorbance $_{11}$/absorbance$_{\perp}$ of 2.0, exhibits strong parallel dichroism. Figure 2 reveals that

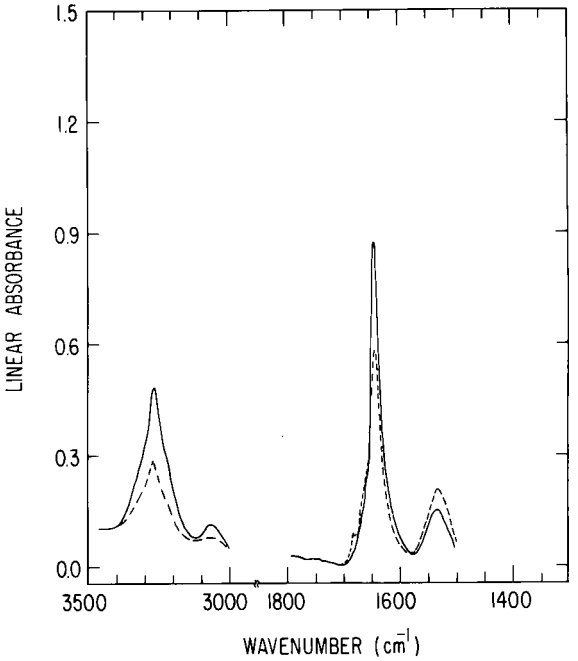

Figure 2: Infrared dichroic spectrum of the ν(NH), amide I and II regions of gramicidin S incorporated in uniaxially oriented polyoxyethylene; polarization parallel to the orientation axis, ——; polarization perpendicular to the orientation axis, ----.

the amide I band comprises a weak, perpendicularly polarized shoulder at 1680 cm^{-1} and a strong peak with parallel polarization at 1640 cm^{-1}, a pattern which is characteristic of polypeptides in the "cross-β" conformation.[2,3] The dichroic asymmetry of the 1640 cm^{-1} peak probably results from an unresolved absorption near 1660 cm^{-1} with weak or moderate perpendicular polarization due to the two tertiary Phe-Pro amide groups. The perpendicularly polarized amide II band occurs at 1530 cm^{-1}. Spectral parameters of gramicidin S in oriented polyoxyethylene are collected in Table I.

Diminution of the amide II absorption of gramicidin S upon exposure of the supporting polyoxyethylene film to vapors of D_2O saturated with DCl is expressed in Figure 3

Table I

Infrared Spectral Parameters of Gramicidin S Oriented in POE

Band	*Position, cm*$^{-1}$	*Dichroic ratio* D_{11}
ν(NH)	3265	2.0
amide I	1680 (w)	<1.0
	1640 (s)	1.4
amide II	1530	0.7

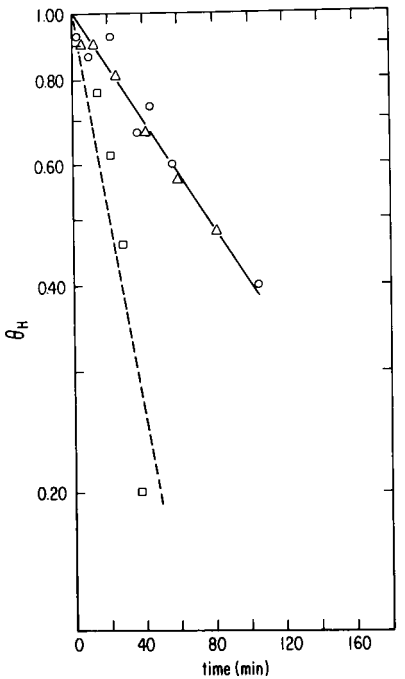

Figure 3: A log plot of θ_H, the fraction of N-H bonds that have not exchanged, against time of exposure to vapors of D_2O saturated with DCl; N-acetyl-L-leucine-N'-methylamide in uniaxially oriented polyoxyethylene, θ_H determined from the parallel polarized amide II absorption, □—□—□ gramicidin S in uniaxially oriented polyoxyethylene, θ_H determined from the parallel polarized amide II absorption, ○—○—○ , θ_H determined from the perpendicularly polarized amide II absorption, Δ—Δ—Δ.

as a logarithmic plot of $\theta_H(t)$, the fraction of the initial amide II absorbance remaining after exchange time t, against t. For purposes of comparison, results of H⟶D exchange kinetics for the model compound N-acetyl-L-Leu-N'-methylamide are also shown. The exchange kinetics for each compound are accountable by a single, first-order rate process. This contrasts with observations made in dilute solution of two or more kinetic classes of exchangeable hydrogens for gramicidin S.[4,5] In addition, the dichroic ratios of the gramicidin S ν(NH) and amide II bands were found to be independent of time during the first 60% of the exchange reaction. The small absorbancies in the latter stages of the exchange made it difficult to extend accurate measurements beyond 60% reaction. It is apparent from a comparison of the exchange half lives of 20 and 80 minutes for the model compound and for gramicidin S, respectively, that none of the amide groups of gramicidin S is readily accessible to solvation by the absorbed water.

The infrared dichroism of Table I is consistent with the conformation of gramicidin S proposed by Hodgkin and Oughton[6] and by Schwyzer[7] and supported by the subsequent investigations of Ovchinnikov *et al.*[8] The two Val-Orn-Leu chain segments of the Hodgkin-Oughton-Schwyzer (HOS) model are connected by hydrogen bonds to form an antiparallel β structure. The extended chain ends are linked by two D-Phe-Pro- bends. The dichroic ratios reported in Table I require that the secondary amide N-H and C = O bonds of gramicidin be aligned approximately parallel to the direction of stretching; the long axis of the HOS model, which connects the two D-Phe-Pro bends, must therefore be aligned transverse to the stretching direction. Polypeptides in the "cross-β" conformation[2,3] adopt a similar chain arrangement and exhibit infrared dichroism patterns[3] that are nearly identical to that observed here for gramicidin S oriented in polyoxyethylene.

It is readily apparent from examination of a molecular model that the HOS conformation of gramicidin S can aggregate to form the "cross-β" structure shown in Figure 4. Adjacent molecules in the "cross-β" aggregate are linked by antiparallel β-sheet hydrogen bonds. These aggregates are ribbon-like structures with the polar ornithine side chains on one side and the nonpolar side chains on the other side of the ribbon. There is thus a potential for formation of bilayers by association of two ribbons with opposed hydrophobic sides.

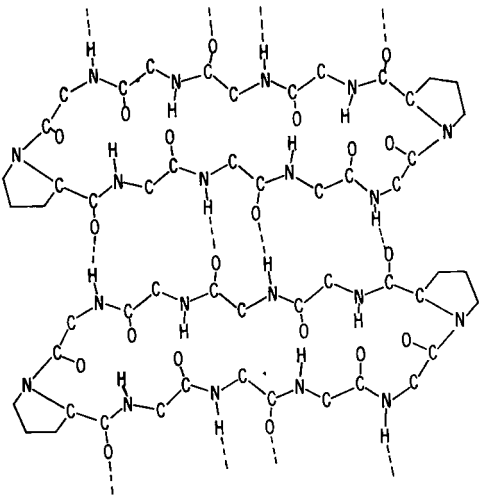

Figure 4: Illustration of the proposed "cross-β" type aggregate of gramicidin S in oriented polyoxyethylene.

Aggregates of five or more gramicidin S molecules would have their long axes parallel to the β-sheet hydrogen bonds and, in accordance with the dichroic ratios of Table I, parallel to the polyoxyethylene stretching direction.

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CONFORMATIONAL PROPERTIES OF OLIGOPEPTIDES CONTAINING TWO AROMATIC AMINO ACID RESIDUES SEPARATED BY A VARIABLE DISTANCE

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THE STUDY OF INTRAMOLECULAR INTERACTIONS in open-chain oligopeptides containing aromatic residues is presently one of the main objectives of our group. We believe that this study can contribute to the clarification of the importance of aromatic residues for stabilizing certain specific conformations in biopolymers. We are now investigating a series of oligopeptides of glycine and aromatic amino acids, having the general formula H-Gly-X-$(Gly)_n$-Trp-Gly-OH. One of the two aromatic residues is L-tryptophan, always kept in the same position and X = Trp, Phe, Tyr, His, n being 0,1,2. In this paper we will be concerned about the spectroscopic properties of the families with X = Trp or Phe, to which we will refer as I (n=0,1,2) or II (n=0,1,2) respectively. A paper describing the synthesis and the characterization of the tryptophan-containing peptides is in press elsewhere,[1] and a paper on the peptides of the phenylalanine series is in preparation.

U.V. Studies

For the compounds which contain two tryptophyl residues, the molar extinction coefficient in the region of the B_b and L-bands corresponds rather well to twice the absorptivity of tryptophan (see Table I). Analogously no hypochromic or hyperchromic effect was found for the oligopeptides of the series II, containing one tryptophyl and one phenylalanyl residue. This shows that no *strong*

Table I

Absorption Properties
of Oligopeptides of Glycine and Aromatic Aminoacids
($4 \times 10^{-3} M$ Phosphate Buffer, pH 5.9)

Peptide	λ_{max}, *nm* ($\varepsilon \times 10^{-3}$ mol^{-1} cm^2)
H-Gly-Trp-Gly-OH	287.0(4.73);278.8(5.54); 218.3(33.5);190.0(34.6)
H-Gly-Trp-Trp-Gly-OH	288.3(9.32);280.4(10.90); 219.0(64.9);190.0(63.0)
H-Gly-Trp-Gly-Trp-Gly-OH	287.3(9.19);279.1(10.75); 218.4(64.5);190.6(64.9)
H-Gly-Trp-Gly-Gly-Trp-Gly-OH	287.6(9.44);279.4(11.05); 217.5(66.4);190.0(72.1)
H-Gly-Phe-Gly-OH	256.8(0.20);187(66.1)
H-Gly-Phe-Trp-Gly-OH	287.6(4.72);279.5(5.52); 217.0(37.6);187(87.8)
H-Gly-Phe-Gly-Trp-Gly-OH	287.5(4.68);279.3(5.52); 216.8(37.7);187(92.7)
H-Gly-Phe-Gly-Gly-Trp-Gly-OH	287.0(4.72);279.1(5.52); 216.5(38.4);187(100.5)

electronic interactions are present between the two aromatic chromophores. Figure 1 reports the extinction coefficients of the absorption maxima in the 185-195 nm region as a function of the number of peptide bonds in the molecule. The experimental points fall rather well on a straight line in both cases. This indicates that the amide contribution is constant for all peptides investigated, so that the onset of regular conformations for the longer oligopeptides can be excluded. The extrapolated value at $n_A=0$ should give the sum of the contributions of the two aromatic chromophores to the molar extinction coefficient. The slope (5400 $mole^{-1}$ x cm^2 for A and 5670 for B) should give the mean absorptivity contribution of the amide bond.

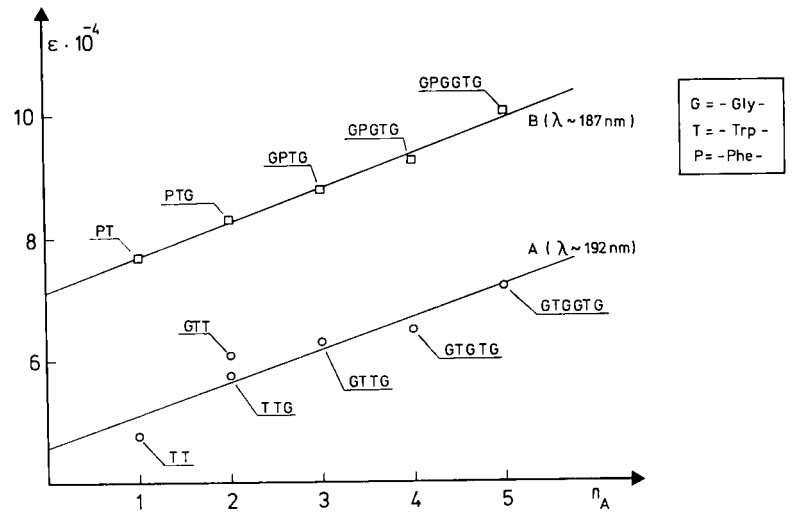

Figure 1: Extinction coefficients at the farthest U.V. maxima as a function of the number of peptide bond, n_A ($4.10^{-3}M$ phosphate buffer, pH=5.9)

CD Studies

Figure 2A shows the CD spectra in the far UV of compounds of the series I, together with H-Gly-Trp-Gly-OH as a reference. Consider the band at 225 nm, which originates mainly from the indole chromophore.[2,3] Note that I (n=2) has the same λ_{max}, and almost twice the ellipticity as H-Gly-Trp-Gly-OH, which indicates that no appreciable chromophoric interaction is present in I(n=2). On the contrary, the behavior of I(n=0) is quite striking, as the band at ~225 nm has an opposite sign. We have not found a negative band at ~225 nm for water solutions of any of the peptides which contain a single tryptophyl residue. In order to establish the origin of this band, we investigated a series of simpler oligopeptides containing two adjacent tryptophyl residues. As shown in Figure 3A, H-Trp-Trp-OH has a positive ellipticity at ~225 nm in the acid and neutral pH range (H-Trp-Trp-Gly-OH behaves analogously). The presence therefore of two adjacent tryptophyl residues is a necessary condition, but not a sufficient one, to bring about the spectroscopic behavior of I (n=0). This suggests that in I (n=0) a particular

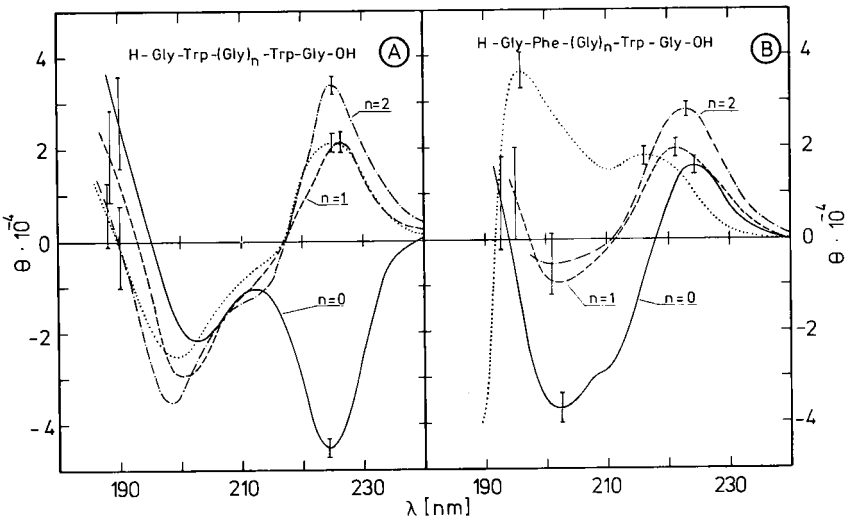

Figure 2: CD spectra of I(n=0,1,2) and II(n=0,1,2) with model compounds (dotted lines) H-Gly-Trp-Gly-OH (in A) and H-Gly-Phe-Gly-OH (in B), in $4.10^{-3}M$ phosphate buffer, pH=5.9.

conformational equilibrium is responsible for the negative band. From Figure 3A, note that for H-Trp-Trp-OH a negative ellipticity at ~225 nm is observed at the alkaline pH. Furthermore, H-Trp-Trp-Gly-OH (not reported in the figure) behaves as H-Trp-Trp-OH, whereas H-Gly-Trp-Trp-OH maintains a negative band at ~225 nm over the whole pH range. (H-Trp-OH maintains a positive ellipticity in the ~225 nm region also at an alkaline pH[4]). Therefore in order for the negative band to appear at ~225 nm in a compound containing two adjacent tryptophyl residues, it is necessary that the amino terminus of the -Trp-Trp- sequence is either involved in a peptide bond or in the form of a free - NH_2 group. The presence of a $-NH_3^+$ eliminates the negative band in H-Trp-Trp-OH and H-Trp-Trp-Gly-OH.

Figure 2B shows the CD spectra of the oligopeptides of the series II, together with that of H-Gly-Phe-Gly-OH. There is a positive band around 220 nm, containing contributions from tryptophyl and phenylalanyl chromophores. For II (n=2) the ellipticity in this region is approximately the sum of the ellipticities of H-Gly-Trp-Gly-OH and H-Gly-Phe-Gly-OH, again indicating no interaction between the

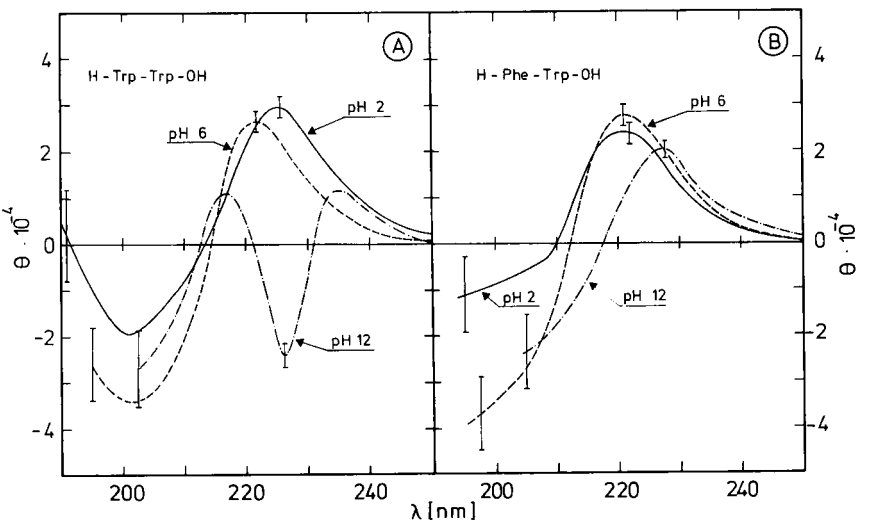

Figure 3: pH dependence of CD spectra of H-Trp-Trp-OH and H-Phe-Trp-OH.

aromatic chromophores. For II (n=0), there is a decrease of the ellipticity around 220 nm, the cross-over point is located at longer wavelength, and the ellipticity in the farther UV is much greater. The study of the pH- dependence of CD spectra of simpler peptides indicates that the difference between II (n=0) and II (n=2) in the 220 nm region has the same origin as for I (n=2) and I (n=0). In fact in this region, as shown in Figure 3B, H-Phe-Trp-OH as well as H-Phe-Trp-Gly-OH (not shown in the Figure) behaves as II (n=1 and 2) under acid conditions, whereas it behaves as II (n=0) at alkaline pH. A discussion of the circular dichroic properties in the near UV will be presented elsewhere.[3]

Preliminary Fluorescence and NMR Studies

Table II reports some fluorescence properties of I (n=0,1,2) obtained with an Aminco-1000 spectrofluorometer. The corrected wavelength of the emission maximum is at 353 ± 1 nm and the quantum yield decreases by going from I(n=2) to I(n=1) and I(n=0). We are now investigating to what extent this progressive quenching is brought about by increasing chromophoric interaction between the two aromatic chromophores. PMR analysis has shown that for

Table II

Fluorescence Properties
of Tryptophan - Containing Peptides
(pH 6, excit. 279 nm)

Compound	λ_{max} (±1 nm)	*Quantum yield* (± 4%)
H-Gly-Trp-Trp-Gly-OH	353	0,039
H-Gly-Trp-Gly-Trp-Gly-OH	354	0,046
H-Gly-Trp-Gly-Gly-Trp-Gly-OH	352	0,053

H-Trp-Trp-OH, the protons in position 2 of the indole ring, which are known to be most readily identified among the aromatic protons, absorb by 6 to 27 Hz at higher field than those in I(n=2). Furthermore, their chemical shifts are within 1 Hz identical in D_2O and acid solution, whereas in alkaline solution a modest upfield shift is observed. No pH influence is found for I(n=2). This behavior is consistent with that observed in CD.

Concluding Remarks

Our data suggest that a particular conformational equilibrium is associated with the sequence -CO-X-Trp-(X= Trp or Phe). Further investigations to elucidate the nature of this conformation are in progress. We have for example observed that by going from water to trifluoroethanol, the CD properties of I(n=2) at around 225 nm do not change appreciably, whereas for I(n=0) the negative ellipticity reaches very high values (-114'000 deg. decimole^{-1} cm^2), thus suggesting a rigid conformation. This solvent effect, together with the pH effect aforementioned, indicates that polar forces (*e.g.* hydrogen bonds) may be important for stabilizing this conformation. At the present, is not yet clear to what extent hydrophobic forces are also involved, whereas strong π-π interactions would seem to be excluded on the basis of absorption spectroscopy. The fact that a sequence -CO-X-Trp- is associated with a conformational rigidity could be relevant to some conformational problems in proteins. For instance in lysozyme there are two adjacent aromatic residues in position 62 and 63 and it would be interesting in this and

analogous cases, to see to what extent the local conformation can be interpreted on the basis of our model compounds. This research is partly supported by the Swiss National Foundation (Grant 2.2060.74).

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THE INFLUENCE OF SHORT-RANGE INTERACTIONS IN PEPTIDES AND PROTEINS

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IN AN EFFORT TO UNDERSTAND THE ROLE of local interactions in peptides and proteins, a series of 54 N-acetyl-N'-methylamide dipeptides, containing combinations of mainly Pro, Gly, Ala, Ser, Asp, Asn, Val and Phe, was examined by conformational energy calculations and compared with an analysis of conformations of dipeptide sequences in globular proteins.

The structures of such blocked dipeptides were described by the following quantities: $R_{i,i+3}$, the $C^{\alpha}_{i}...C^{\alpha}_{i+3}$ distance, where the index i is for atoms on the N-terminal blocking group (CH_3CO) and i+3 for the atoms on the C-terminal blocking group ($NHCH_3$), and where the end methyl groups are considered as models for attached alpha carbons; b, the Boltzmann factor $\exp(-\Delta E/RT)$, where ΔE is the difference between the calculated conformational energy[1] of a particular structure and the conformational energy at the global minimum; z, the statistical weight of a particular structure, taking into consideration librational entropy by the method of Go *et al.*;[2] <R>, the average value of R; and P, the bend probability of a particular dipeptide. For each dipeptide, <R> was determined in four possible ways: (1) by Boltzmann averaging, where

$$\langle R \rangle_b = \sum_{k=1}^{n} R_k b_k / \sum_{k=1}^{n} b_k$$

and the sum is taken over all n conformational energy minima with $\Delta E \leq 3$ kcal/mole; (2) by statistical averaging, where

$$\langle R \rangle_z = \sum_{k=1}^{n} R_k z_k / \sum_{k=1}^{n} z_k;$$

(3) in selected cases, by conformation-space mapping, where $\langle R \rangle_g$ is defined like $\langle R \rangle_b$ but where, instead, the sum is taken over m points at 10 or 30° intervals over all backbone dihedral angles (*e.g.*, a 10° map of Pro-Gly with 3 variables requires $m = 36^3 = 46{,}656$); and (4) by analysis of x-ray crystallographic structures of globular proteins, where

$$\langle R \rangle_e = \sum_{k=1}^{N} R_k / N,$$

R in this case is the experimentally observed $C^{\alpha}_i \ldots C^{\alpha}_{i+3}$ distance, and N is the number of occurrences of the dipeptide sequence in non-helical regions of the 19 protein structures analyzed. Similarly, four types of bend probabilities were also determined, where

$$P_x = \sum_{i=1}^{M} x_{k,R \leq 7\text{Å}} / \sum_{k=1}^{M} x_k;$$

x = b, z, g, or e and M = n, m, or N, depending on x. When x = e the quantity P_e is actually the observed fraction of occurrences of a dipeptide sequence in the i+1 and i+2 position of a bend.

The structures and energies of stable conformations of blocked dipeptides were determined by energy minimization[3] from starting conformations which included all possible combinations of blocked single-residue local minima[4] and ten bend structures.[5] From conformation-space mapping, three functions were determined: (1) V(R), the volume distribution function, defined as the total volume in conformation space with a value of R in the interval between R' and R' $\pm$ dR (where, in these computations, dR was taken as 0.25 Å). V(R) is independent of conformational energy, and depends only on geometry (bond angles and lengths). (2) P(R), the probability distribution function, defined as the probability of a dipeptide having a value

of R in the interval between R' and R' + dR. P(R) depends on the conformational energy as well as on the conformational volume within the interval. And (3) $\sigma(R)$, the mean probability density function, defined as $P(R)/V(R)$. An analysis of x-ray coordinates (excluding helical regions) of 19 globular proteins yielded the frequency of occurrence of overlapping dipeptides (independent of sequence and composition) with a value of R between R' and R' + dR, from which distribution functions P(R) and $\sigma(R)$ for dipeptides in proteins were determined.

Figure 1A is the plot of P(R) vs. R from the protein x-ray data. The curve has two regions with a clear division at R = 6.5-7.0 Å. This implies that a bend,

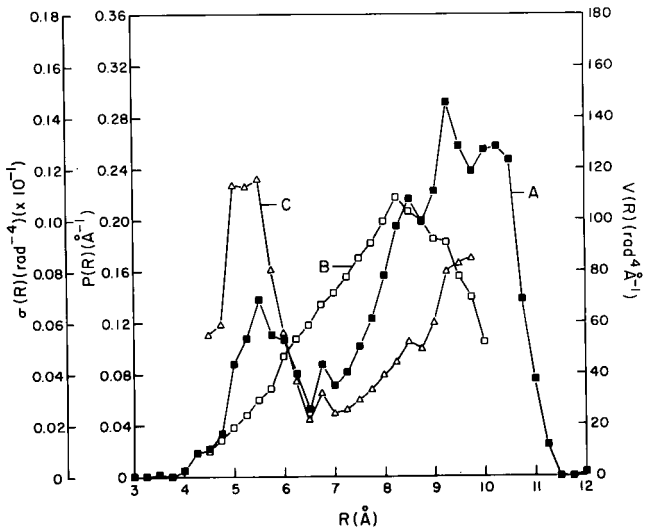

Figure 1: A. P(R) vs. R from overlapping dipeptide sequences (excluding helical regions) in 19 globular proteins. B. V(R) vs. R from a 30° conformation-space mapping of the blocked dipeptide Ala-Gly, used as a model for the backbone geometry of dipeptide sequences in proteins. This approximation and/or experimental error in the x-ray coordinates causes V(R) to approach zero faster than P(R) in the regions R<4 and R>10Å, making $\sigma(R) = P(R)/V(R)$ approach infinity. Therefore, these regions were omitted from curves B and C. C. $\sigma(R)$ vs. R obtained by dividing P(R) from the 19 globular proteins by V(R) from the Ala-Gly map in each interval between R' and R' + dR.

defined as a non-helical structure with R≤7 Å, is a physically distinguishable type of protein structure, just as helical and extended structures, and not merely an arbitrarily-defined type of "disordered" conformation. This point is even more apparent from the σ(R) vs. R curve in Figure 1C. The large peak at ~ 5 Å indicates that bend structures occur frequently in proteins not because of geometric considerations (since V(R) below R = 7 Å is very small, as shown in Figure 1B), but because of energetically favorable interactions.

These experimentally observed results can be compared with theoretical studies of blocked dipeptides. Plots of P(R), V(R), and σ(R) vs. R for Gly-Pro and Pro-Gly are shown in Figures 2 and 3. These dipeptides yield

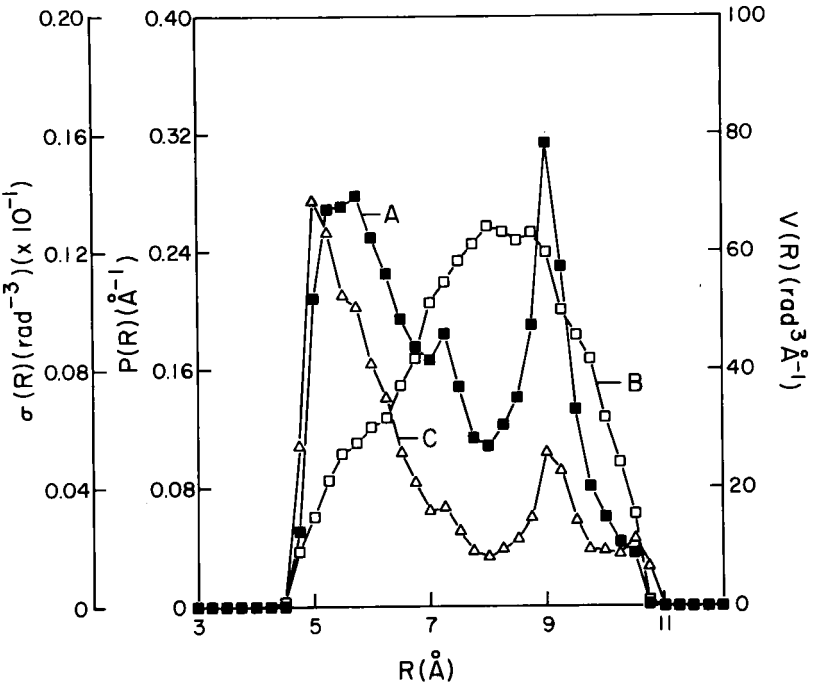

Figure 2: A. P(R) vs. R, B. V(R) vs. R, and C. σ(R) vs. R for blocked Pro-Gly, from 10° map.

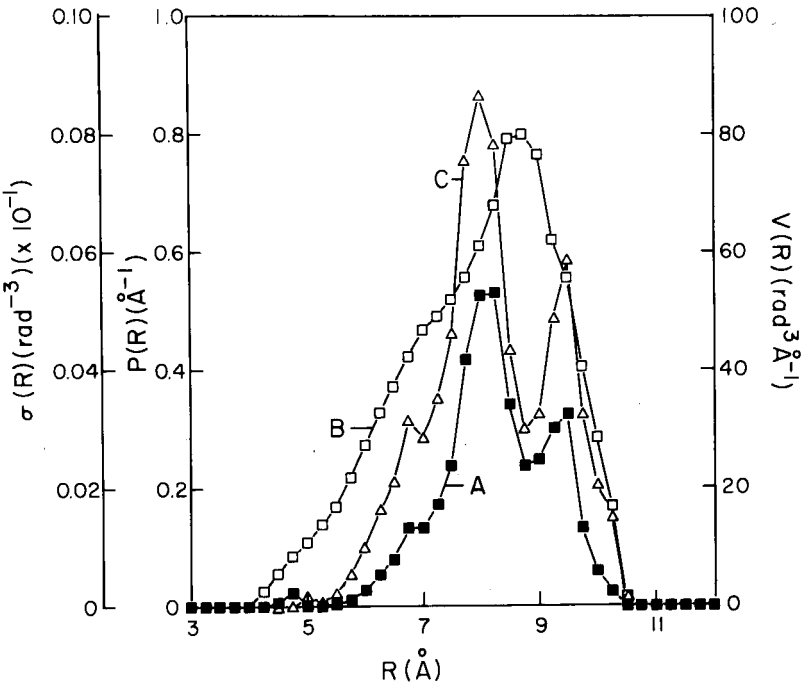

Figure 3: A. P(R) vs. R, B. V(R) vs. R, and C. σ(R) vs. R for blocked Gly-Pro, from 10° map.

distribution functions which are quite distinct but, like most other dipeptides examined, have relatively low values of P(R) in the region of R = 6.5-7.0 Å. The plot of σ(R) in the region below R = 7 Å in Pro-Gly (Figure 2C) shows that bend structures are energetically more favorable than would be expected from simple geometric considerations. These results not only support the conclusion that bends are distinguishable from other structures but also show that the forces which stabilize bends in proteins may be accounted for by interactions within residues or between adjacent residues, *i.e.*, that local interactions are sufficient to cause bends in proteins.

To show further the effects of local interactions, the calculated values of the bend probability and the average $C^{\alpha}_{i}...C^{\alpha}_{i+3}$ distances were compared with the observed values in globular proteins. Table I gives the data for Pro-Gly and Gly-Pro. First, it is observed that the values of $\langle R \rangle_z$ and $\langle R \rangle_g$ are in excellent agreement, but they are

Table I

Comparison of Calculated and Experimental Quantities*

Dipeptide	P_b	P_z	P_g	P_e	$\langle R\rangle_b$	$\langle R\rangle_z$	$\langle R\rangle_g$	$\langle R\rangle_e$	*N*	*n*
Pro-Gly	0.62	0.51	0.52	0.60	6.72	6.97	7.09	6.7	15	24
Gly-Pro	0.22	0.13	0.11	0.08	7.87	8.15	8.13	8.4	12	22

* Quantities P_x are dimensionless; $\langle R\rangle_x$ are in Å.

not in as good agreement with $\langle R\rangle_b$. This suggests that (1) the method employed here of minimization from selected starting points succeeds in locating all low-energy minima; (2) the classical librational entropy is properly accounted for by the approximation method of Go *et al.*[2] without having to resort to costly mapping of the entire conformation space; and (3) librational entropy contributes significantly in the evaluation of relative stabilities of dipeptide structures. Second, the general agreement between P_e and P_g (or P_z) and between $\langle R\rangle_e$ and $\langle R\rangle_g$ suggests that bends in proteins can be formed by local interactions and therefore may be an important driving force in protein folding.

An analysis of low-energy dipeptide structures obtained by energy minimization yields the following results: (1) Most dipeptide minima with $\Delta E < 3$ kcal/mole can be described as simple combinations of single-residue minima in which each of the single-residue minima has an energy less than 2 kcal/mole. (2) All low-energy minima obtained by minimization from bend structures were also obtained by minimization from combinations of single residues. (3) Upon minimization, the relative energies of some structures changed considerably even though the dihedral angles changed by less than 5°. (4) Minimization from combinations of single-residue minima sometimes lead to structures which are not simple combinations of single-residue minima. And (5) some combinations of single-residue minima are not stable in the dipeptide. These results not only emphasize the predominance of intra-residue interactions in determining conformation but also point out important effects of inter-residue interactions. They also show the necessity for carrying out minimization in conformational energy studies.

Further details of these results will appear elsewhere.[6,7]

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DIELECTRIC CONSTANT OR DIELECTRIC FUNCTION ?

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THE SEMI-EMPIRICAL POTENTIAL FUNCTIONS that are typically used in conformational energy calculations of peptides and proteins comprise several terms.[1] One of these, the electrostatic contribution, arises because of the presence of partial charges on certain atoms; these are determined either from quantum chemical charge density calculations,[2] or from monopole approximations to the experimental bond dipole moments when the latter are known.[3] Furthermore, the acidic and basic side-chains bear full charge. The electrostatic contribution E^{el} to the total energy for two charges q_i, q_j, separated by a distance r_{ij} is calculated as $E^{el} = q_i q_j / \varepsilon_{eff} r_{ij}$, where ε_{eff} is an effective dielectric constant. It is supposed to take into account the presence of the solvent. But does it? Since in most conformational calculations the solvent dependence is simulated only by the use of ε_{eff}, the *magnitude* of this constant can be crucial. The purpose of this work is to show that, at least in certain cases, a dielectric constant, whatever its value, may be inadequate and could usefully be replaced by a *dielectric function* $\varepsilon(r_{ij})$.

If the charges were immersed in a homogeneous continuum fluid of bulk dielectric constant ε_b, *and* if they were separated by large enough distances, then ε_b could be used in the expression for E^{el}. For a peptide or protein in solution the interacting charges are frequently too close to each other to be separated by bulk solvent. Furthermore, some charges are not exposed to solvent

molecules at all and their interactions are mediated through the bulk of the peptide or protein. Thus even if homogeneity could be assumed, ε_b of the peptide (~2.0-3.5) instead of that of the solvent should be used in the calculations. In reality the charges on a peptide or protein experience complex, inhomogeneous fields and the low ($1.0 \leq \varepsilon \leq 5.0$) effective dielectric constants generally employed can be too crude.

A dielectric *function* $\varepsilon(r_{ij})$ which depends smoothly and continuously on r_{ij} is the next simplest approximation. Qualitatively, one expects $\varepsilon(r_{ij})$ to be some ε_c at the contact distance r_c (the sum of the van der Waals radii of the charged atoms), and increase smoothly to ε_{eff}[4] (which may not have to be equal to ε_b). It has been shown[5,1] that the energy of interaction of two point charges q_1, q_2, separated by a distance d inside a spherical cabity of diameter D depends only on x, ($d/D \equiv x < 1$), d, q_1, q_2 and ε_b, where ε_b is the dielectric constant of the medium in which the sphere is immersed. It can be written in the form $E^{el} = q_1 q_2 / d\varepsilon'(x,\varepsilon_b)$, where the dielectric function ε' is generally much smaller than ε_b, tends to 1 as $x \to 0$, and to ε_b as $x \to 1$. This is in agreement with the qualitative expectation, as well as with some experimental results of Birshtein *et al.* on the relation between the first and second dissociation constants of diatomic acids and bases and effective dielectric constants.[6] In view of the approximate nature of the theory proposed by Kirkwood,[5] we decided not to adopt the computationally expensive expression he derived for $\varepsilon'(r_{ij})$. Instead, the following very simple empirical functional form was chosen (see Figure 1)

$$\varepsilon(r_{ij}) = 1 + 20\ r_{ij}^2/(r_{ij} + 7)^2.$$

This function has the correct qualitative features we discussed and it is smooth and differentiable. The lower limit, $\varepsilon_c = 1.0$, corresponds to vacuum, implying that there is no solvent between the charges. The large r_{ij} limit, here $\varepsilon_{eff} = 21.0$, should generally reflect the macroscopic dielectric properties of the solvent one simulates.

As a first test, the (ϕ,ψ) dipeptide map for alanine was calculated with $\varepsilon(r_{ij})$. It differs only slightly from the $\varepsilon = 2.0$ map, as expected for a small molecule in which the range of possible interatomic distances is small.

Next, we used $\varepsilon(r_{ij})$ to study the folding of oligopeptides. It is now well established that oligopeptides

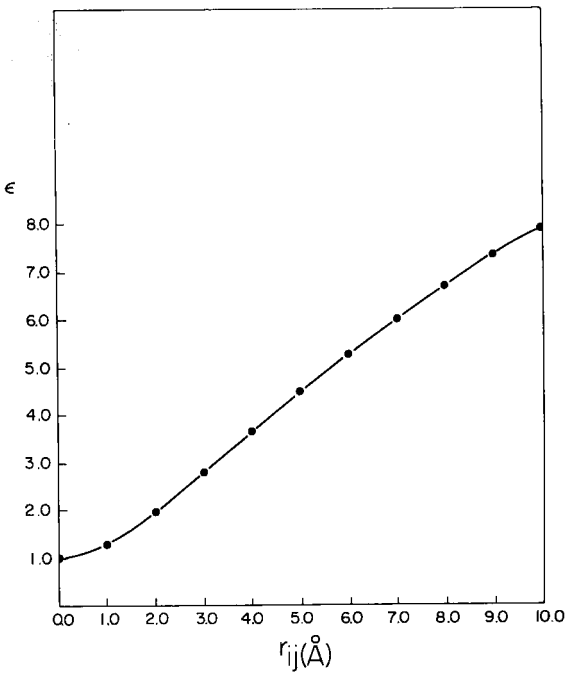

Figure 1: Dielectric function $\varepsilon(r_{ij}) = 1 + 20\ r_{ij}^2/(r_{ij} + 7)^2$.

undergo a transition from a "random coil" to a structured state at a critical chain length. This length, as well as the type of structure (α-helix, β-conformation, or other folded conformation) depends on the nature of side-chains and solvents.[7,8] For the series of peptides $(Ala)_n$, in a solvent mixture TFE/1% H_2SO_4, a transition to the α-helix has been observed at the critical length of 7 units.[7]

In previous calculations[9] the existence of a critical length for folding has been predicted; its value however is strongly dependent on the dielectric constant used in the calculations. For the series $(Ala)_n$ studied for n = 1 to 6, the critical length was found at n = 6 with ε = 3.5 and predicted by extrapolation to be at n = 9 for ε = 2.0 and at n = 12 for ε = 1.0.

What are the forces that lead to the folding of peptides? The total conformational energy of a peptide, E^{tot}, can be partitioned into two terms: E^{loc}, comprising the local energy of each unit (energy of the unperturbed molecule) and E^{int}, arising from the interactions between

different units in the peptide.[9] By definition, long-range interactions are negligible in a "random coil". Consequently, random coil states (R) are characterized by a favourable E^{loc} term and a quasi non-existent E^{int} term; folded conformations (F) are stabilized by E^{int} or by both terms. For a given peptide length, the relative importance of the average values (taken over all investigated conformations), $\overline{E^{int}}$ and $\overline{E^{loc}}$, reflects the predominance of either R or F. Therefore, we consider as the critical length for peptide folding the number n of residues at which the two plots $\overline{E^{loc}}(n)$ vs. n and $\overline{E^{int}}(n)$ vs. n cross.

Here we present calculations for the series $(Ala)_n$ (n = 1 to 8) with ε = 1.0, 2.0, 3.5 and $\varepsilon(r_{ij})$. Geometry and energy parameters can be found in 9. The plots $\overline{E^{loc}}$ (n) vs. n and $\overline{E^{int}}(n)$ vs. n are shown in Figure 2. Those

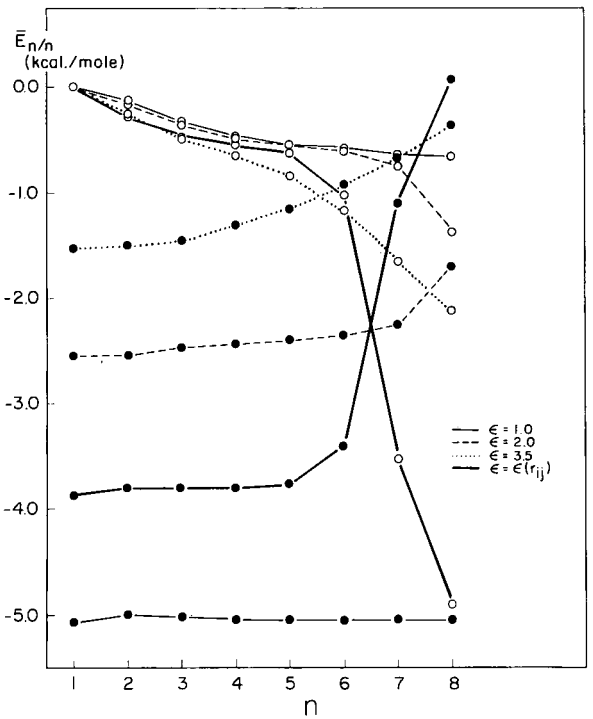

Figure 2: Plots of the average energies $\overline{E^{loc}}/n$ (•) and $\overline{E^{int}}/n$ (o) as functions of n, the number of units in the blocked oligopeptides.

obtained with $\varepsilon(r_{ij})$ differ quantitatively and qualitatively from the ones calculated with the dielectric constants. The transition is found at 7 units, in agreement with experiment. In addition, the transition is much sharper, indicating qualitatively different processes at work. To understand what is happening, let us consider the conformations that contribute most significantly to the average properties of the peptides. A large difference between $\overline{E^{loc}}$ and $\overline{E^{int}}$ (as is the case both for $\varepsilon = 1.0$ and $\varepsilon(r_{ij})$) indicates the preferential population of only one class of conformers, that which is stabilized by the energetically more favourable of the two terms. A sharp transition signals the sudden switching from one class of conformations present at equilibrium to another class. In contrast, $|\overline{E^{loc}} - \overline{E^{int}}|$ is much reduced when $\varepsilon = 2.0$ or 3.5. This means that there is no preferential population of any class of conformations. The gradual transition implies that both classes will be comparably populated for all n. The number of probable conformations existing for $\varepsilon = 2.0$ and $\varepsilon = 3.5$ increases with n, and the average plots comprise contributions from many conformations of comparable energy. The label "preferred conformation" is meaningless since no conformation has weight greater than about 15%. The transition point has no definite structural significance. Table I gives the predicted fraction of α-helix. Quantitative interpretation of CD spectra of short peptides in terms of α-helix content is difficult because of end effect

Table I

Fraction of α-helix (in %)

n \ ε	*1.0*	*2.0*	*3.5*	$\varepsilon(r_{ij})$
2	*	*	0.3	0.001
3	*	0.004	0.2	0.002
4	*	0.01	0.5	0.03
5	*	0.04	1.0	0.4
6	*	0.2	4.0	8.0
7	*	1.5	8.5	56.0
8	*	9.0	14.6	79.0

* < .001

complications. Consequently, only qualitative agreement can be claimed between the spectra (that show a predominance of α-helical conformations for peptide lengths greater than 7) and the predictions based on calculation with $\varepsilon(r_{ij})$ (which indicate a jump in % helicity from 8 (hexapeptide) to 56 (heptapeptide)). It should be emphasized that $\varepsilon(r_{ij})$ was not adjusted to fit the above experimental data! The upper bound $\varepsilon_{eff} = 21.0$ appears to simulate reasonably well the TFE/1% H_2SO_4 solvent mixture (ε_b(TFE) = 26.5).

More extensive calculations are needed to identify the critical parameters of $\varepsilon(r_{ij})$; however it seems clear that not only the limiting values (ε_c, ε_{eff}) but also $d\varepsilon(r_{ij})/dr_{ij}$, the rate of change (steepness) of $\varepsilon(r_{ij})$ between ε_c and ε_{eff} will play a crucial role. This is evident since to any conformation there corresponds a particular distribution of charge separations that is typical of that conformation. The role of distance-dependent dielectric function is to weigh differently different ranges of interaction distances; thus it can favour a certain class of conformations and not others. By changing the critical parameters of $\varepsilon(r_{ij})$, this emphasis of stabilizing a given conformation can be adjusted continuously; only crude, overall adjustments are possible with an effective dielectric constant.

We feel that the consideration of dynamical data (solvent induced transitions, etc.) is particularly important in elucidating the finer details of $\varepsilon(r_{ij})$. The disadvantage of additional parametrization should be more than compensated for by the new information we can glean about conformation changes and their dependence on the nature of the solvent, as well as of the side-chain distribution.

The interest of the results more than justifies the small increase in computer time (3% here) necessitated by the use of $\varepsilon(r_{ij})$.

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CONFORMATIONAL CALCULATIONS OF PEPTIDE CONFORMATION: SYNTHESIS OF QUANTUM MECHANICAL AND EMPIRICAL APPROACHES FOR DERIVING ENERGY FUNCTIONS

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IT IS WIDELY ASSUMED THAT the biological activity of hormonal and other peptides is related to their conformation. Thus one of the most important and active areas of research today, as seen from the proceedings of this symposium, is the determination of peptide conformation.

Since the natural environment of biologically active peptides is solution, most of the interest centers on the determination of conformation in appropriate solution phases. The main techniques being used currently to determine solution conformation are NMR, CD and IR.[1] Although these techniques give significant conformational information, unfortunately, unlike X-ray crystal analysis, the results obtained are often not sufficient to determine uniquely the intramolecular coordinates necessary to define the conformation of the peptide. The most detailed information about conformation can, in principle, be obtained from theoretical conformational energy calculations. This technique, when used in conjunction with the methods enumerated above, should provide a potentially powerful probe into the factors determining peptide conformation.

A few conformational studies involving energy calculations have been carried out. Some recent examples include the work of Blout *et al.*[2] on cyclic peptides, Goodman *et al.*[3] and Scheraga *et al.*[4] on TRH, and the work on oxytocin by Kotelchuck *et al.*[5]

Despite the fact that much useful information can be obtained from such studies, some major problems have to be resolved before the conformational energy technique can fulfill its promise and take its place on an equal footing

alongside the more developed experimental techniques. Problems are associated with the formulation of adequate derivations for potential functions and solvent effects[6] and with multiple minima (although for small peptides and when used in conjunction with experimental techniques, this latter may not be a problem).[7-9]

Up to this time, most conformational calculations have been analyzed either in terms of quantum mechanical or empirical potential methods.[8-10] These methods are often complementary (*viz.* the empirical method, by its very nature, may be easily analyzed in terms of the individual interatomic contributions, while the quantum mechanical results may provide insight in terms of electronic distribution). For example, in a recent study of the rotational potential surface about the N-C^{α} (ϕ) and C^{α}-C^{1} (ψ) bonds in substituted amides,[11] such combined analyses indicated that the barrier at $\alpha = 120°$ (C-H *syn*-planar to the C=O) was mainly due to a repulsive methyl H...carbonyl O interaction. Furthermore, it showed that minimal basis set calculations were unable to reproduce this barrier because the electrons in these basis sets were confined too close to the nucleus, *i.e.*, the atoms are "too small," and the H and O don't "overlap."[11,12]

Quantum mechanical calculations of the spatial electron distribution[13] may also be used for the formulation and derivation of empirical potential functions. Although various sources have been used for deriving potential functions,[8,9] it has recently been recognized that one of the best methods is to utilize the information on intermolecular forces implicit in the structure and energy of crystals of model compounds.[14-16] The information available from electron density distributions can be used in conjunction with the crystal data in several ways. The population analysis of several amides as carried out with CNDO/2, *ab-initio* minimal, and *ab-initio* extended basis sets are given in *Figure 1*.[13,17-19] It is clear from a comparison of the CNDO/2, minimal and extended basis sets, that the partial charges obtained from population analysis are basis set dependent. Thus the common practice of transferring these charges directly to empirical potential functions to represent the electrostatic contribution[8-10] (through Coulombs law), is a highly questionable procedure.[20] However, the comparison of charges on a given atom *within* a given basis set from molecule to molecule in different families[13] can give objective information about the transferability of potential functions for this atom from the model compounds to the biological molecules of interest. Transferability

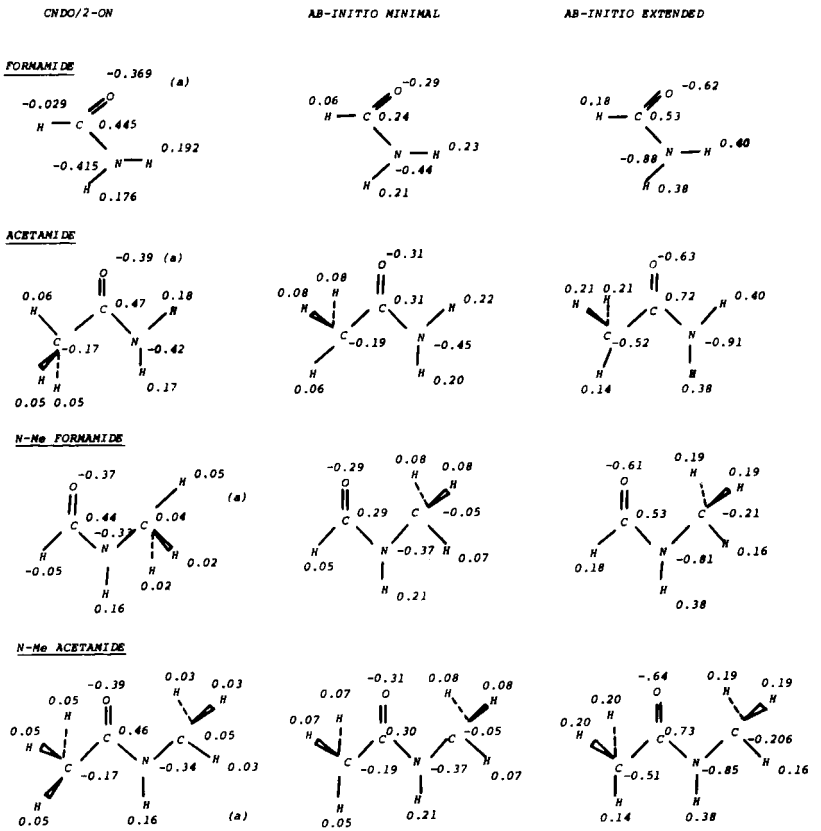

Figure 1: Net atomic charges in some primary and secondary amides as obtained from population analysis of CNDO/2, minimal and extended *ab-initio* wave functions. (Charges in electrons)

of potential functions is one of the basic assumptions on which the validity of conformational calculations rests.[8,21]

Population analysis condenses a continuous electron distribution into a discrete set of numbers. The continuous electron distribution also provides information relevant to empirical conformational calculations. In *Figure 2a*, the total electron density of N-acetyl N'-methylamine (ϕ,ψ = 180, 180) in the plane of the molecule as calculated with a minimal basis set is presented. This is compared with the electron density which results from the superposition of the electron densities of the isolated spherical atoms placed at the appropriate positions in the molecule *(Figure 2b)*.

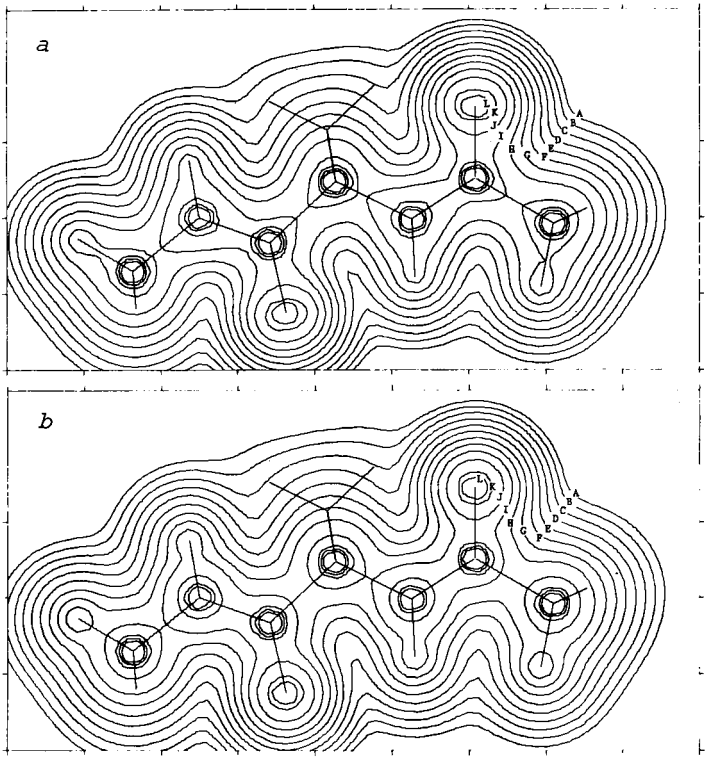

Figure 2: Contours of constant electron density in N-acetyl-N'-methylalanine. a) Total molecular density as calculated from STO-3G basis set, b) Electron density obtained from the superposition of electron densities of "spherical atoms" as calculated in the same basis set.

Comparison of the two distributions shows that for the conformationally important excluded volume effect, which is due to the nonbonded repulsion between all electrons, the spherical atom approximation is a good one. This approximation is implicit in empirical calculations. Although the spherical atom approximation is very good for nonbonded effects (which are due to all electrons), it may not be suitable for the electrostatic interactions which are due to the small perturbation of the spherical densities.

From the analysis of the deviations in calculated crystal structures, Hagler and Lifson[23] suggested that the lone pair orbitals on the carbonyl oxygen might be important in determining the crystal structure and conformation of larger molecules. Difference maps of the total electron density

minus the spherical atom density[13] bring out the electron redistribution on molecule formation (see *Figure 3* for a difference map of N-methylacetamide). Such features as the bonding densities and lone pair orbitals appear in these maps. Information on the position and number of electrons (by integration in space) which are useful in the development and improvement of empirical functions may be obtained from such maps.

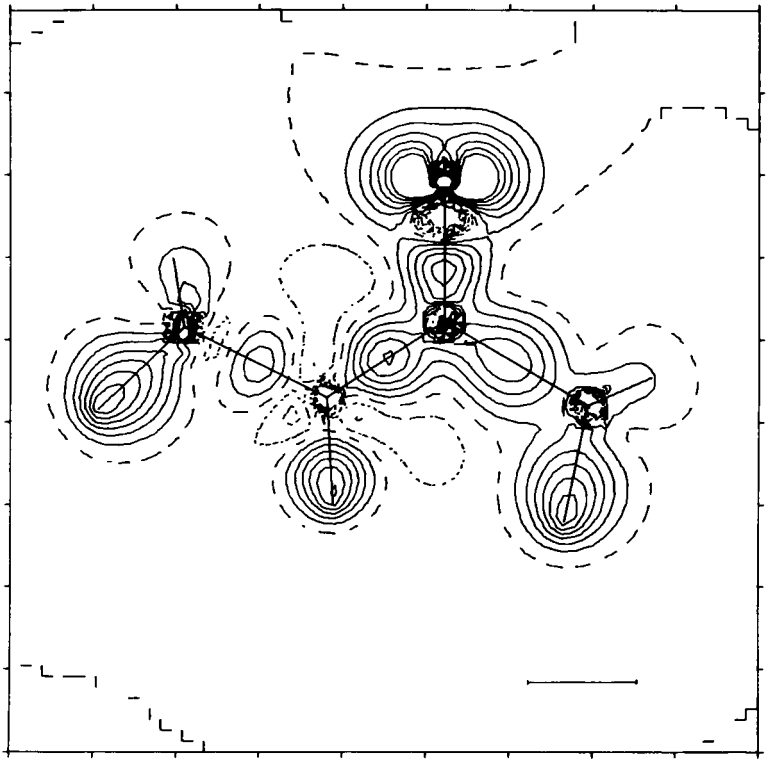

Figure 3: Difference map of the total molecular density as calculated with the extended 6-31G basis set minus the spherical atom densities. The positive difference contours are solid, while the negative are dotted and the zero contour, dashed.

In summary, from this brief survey it may be seen that from the combination of quantum mechanical and purely empirical calculations, one may gain insight into conformational behavior which is not available from either technique alone.

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RELATIONSHIP BETWEEN SECONDARY STRUCTURE OF POLY-α-AMINO ACIDS AND PLANARITY OF THE PEPTIDE GROUP

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MONOSUBSTITUTED AMIDES ARE SIMPLE MODELS for the peptide group and have been especially used as such for spectroscopic studies. The amide group is generally assumed to be planar. The purpose of this paper is to show that new infrared and Raman data lead to the conclusion that the amide group is actually not planar, and to point the consequences out for the peptide group in polyglycine.

Structure of N-Methylacetamide (NMA) Isolated as a Monomer in "Inert" Gas Matrices

Several isotopic species of NMA have been trapped in Argon and Nitrogen matrices at low temperature.[1,2] The infrared spectra indicate that both *cis* and *trans* forms are present under these conditions. The *trans* form is not planar: (1) The bonds coming out of the nitrogen atom are not planar. A pyramide is thus obtained, which can invert through tunnel effect as for ammonia.[3] The angle between the NH bond and the plane through the other two bonds has been calculated equal to 30°.[4] (2) The monomer NMA is partially or totally in the skew configuration. The CC and NC bonds are not coplanar($\omega \neq 180°$). Moreover in certain cases the amide may go from one form ($+\omega$) to the symmetrical one ($-\omega$) through tunnel effect.[3]

The existence of non planar forms of NMA has thus been demonstrated for the first time and has led us into investigating again the spectrum of crystalline NMA (where the molecules are not monomers but autoassociated).

Structure of Crystalline N-Methylacetamide

In infrared and Raman spectra of crystalline NMA between 25° and - 180° various bands display phenomena characteristic of tunnel effects. (Amide I and I', νND, Amide II, Amide VII and VII').

Figure 1 shows the Amide I' and νND bands of the $CH_3CONDCD_3$ compound, chosen as an especially clear example. In the Amide I' region when the temperature is lowered, the 1612 cm^{-1} band decreases and two weak bands appear at 1600 and 1673 cm^{-1}. Similarly in the νND region the 2455-2418 cm^{-1} band decreases while a band grows at 2375 cm^{-1}.

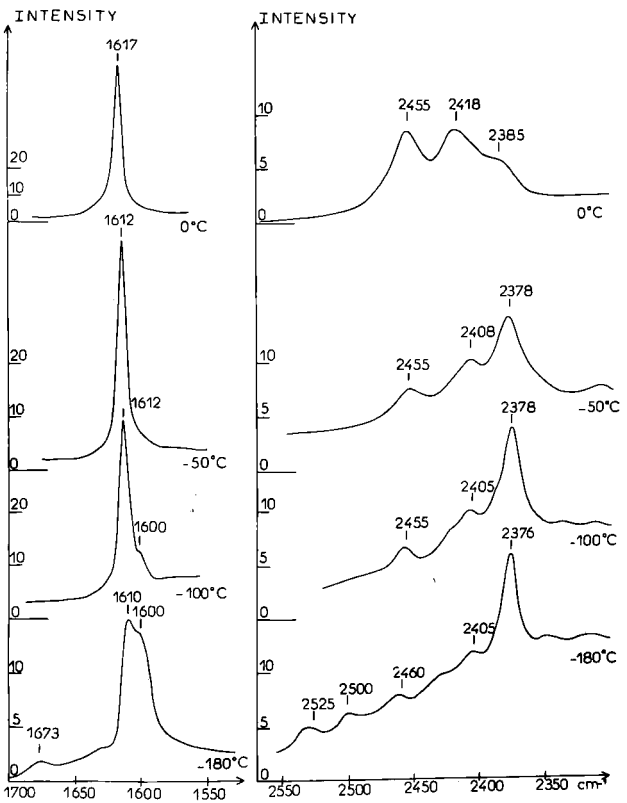

Figure 1: Raman spectrum of $CH_3CONDCD_3$ - Amide I' and νND Vibrations - Temperature effect.

These phenomena are not due to a change of crystalline phase: the intensity reversal does not occur in the same temperature range for the Amide I' and νND bands; moreover in the totally hydrogenated compound the νNH and Amide II bands are practically not perturbed between 25° and - 180°. The assumption of a band splitting due to a site effect of the crystalline field may also be ruled out since the different components of a same band do not show equal isotopic effects.

The data can again be interpreted in terms of a non-planar amide group. In this case we do not have evidence for a pyramidal nitrogen atom, but the amide group is skew and can occupy two equilibrium positions through the tunnel effect. The motions corresponding to the vibration bands may be represented by potential functions with two quasi symmetrical minima.[5] For instance the Amide I' band can be assigned as follows: $\nu_{0\to2}$ 1600 cm^{-1}; $\nu_{1\to3}$ 1612 cm^{-1}; $\nu_{0\to3}$ 1673 cm^{-1}. Similar assignments may be given for νND, Amide II and Amide VII modes.

Structure of Polyglycine

Polyglycine is known to exist in two crystalline forms I and II. Form I was thought to be the antiparallel pleated sheet but recent electron diffraction data lead to the hypothesis of an antiparallel rippled sheet.[6] The corresponding infrared spectra has been interpreted using the first order perturbation theory:[7]

$$\nu(\delta,\delta')=\nu_0 + D_{10}\cos\delta + D_{01}\cos\delta' + D_{11}\cos\delta'$$

According to Krimm *et al.*[7a] ν_0 = 1679,5 cm^{-1}; $\nu_{(0,\pi)//}$ = 1685 cm^{-1}; $\nu_{(\pi,0)\perp}$ = 1636 cm^{-1}; D_{10} = 0; D_{01} = -24,5 cm^{-1}; D_{11} = 19 cm^{-1}.

Although the infrared spectrum of polyglycine I is not perturbed between room temperature and - 180°,[8] at - 220° a very weak band appears at 1610 cm^{-1} (Figure 2) which is not predicted by the previous theory. If the same assumption of quasi symmetrical potential function is made as for NMA, a new assignment of the bands can be proposed: $\nu_{0\to2}$ 1610 cm^{-1}; $\nu_{1\to3}$ 1630 cm^{-1}; $\nu_{0\to3}$ 1684 cm^{-1}. This interpretation is consistent with the polarisation properties of the bands (transitions between wells or within a well).[5]

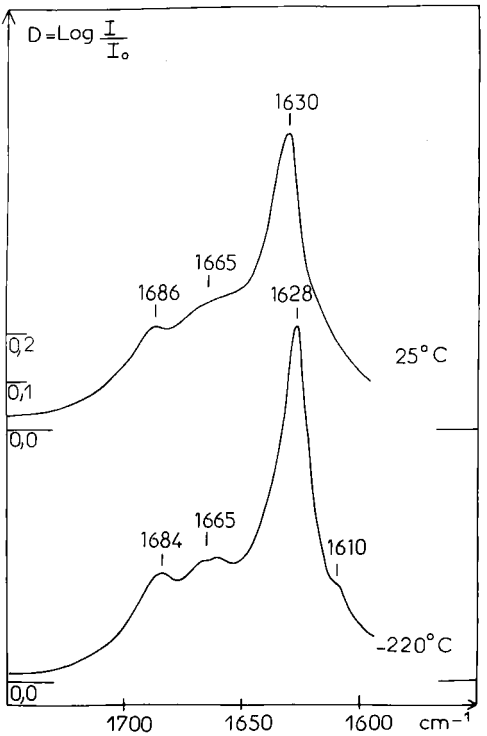

Figure 2: Infrared spectrum of polyglycine I - Amide I Vibration - Temperature effect.

The temperature lowering should bring a decrease of the 1630 cm^{-1} band as in NMA, but this is not observed. Several explanations can be offered: the potential function is more assymmetrical so that the $\nu_{0\to1}$ transition is forbidden and the system is metastable at low temperature (high activation energy required for going from one minimum to the other); the phenomenon occurs only in some parts of the sample.

These results seem to show that in polyglycine I a certain dynamic disorder takes place, due to the possibility of the skew peptide group inverting through tunnel effect. The mid symmetry plane parallel to the sheet is however maintained. This hypothesis is not inconsistent with electron diffraction data.[6,9]

For polyglycine II infrared and Raman spectra do not show any characteristics of tunnel effect in the Amide I region. Theoretical studies have shown that non planar peptide groups may stabilize secondary helicoidal structures.[10] However the helicoidal symmetry leads to two non equivalent skew forms of the peptide group, so that the tunnel effect cannot take place.

Conclusion

These results show that the structure of the peptide group depends on the secondary structure of the polymer; the spectrum in the Amide I region may be characteristic of the state of the peptide group rather than of the polymer conformation.

An inverse hypothesis may be formulated: the interactions of the peptide group with solvents may affect its structure and thereby contribute to induce specific secondary structures. This point is under investigation.

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SECTION III

SYNTHETIC STUDIES

NEW APPROACHES TO PEPTIDE SYNTHESIS

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DURING THE PAST FOUR YEARS we have been engaged in the development of chemical reagents which can be used complementarily to provide a coherent new approach to peptide synthesis. Although many of our projects are still too embryonic to be of general interest, three have progressed to the point at which an assessment of at least a part of their potential utility is possible, and these I would like to discuss today. In doing so, I will also describe the overall strategy which has guided our search.

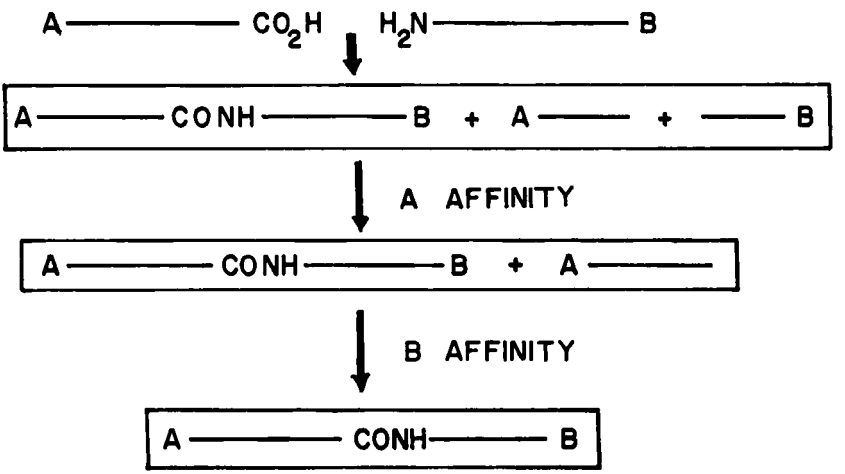
The problems of separation and isolation have dominated much of the recent developments in synthetic methodology. Sparked by Merrifield's path-finding development of polystyrene-based solid-phase C-terminal protection, a range of other polymeric reagents have also been explored. Among the insoluble polymers, Merrifield's original system thus far has held its own. Soluble polymeric supports have been proposed as well, which rely on a variety of techniques including gel and ultrafiltration for isolation,[1,2,3,4] and several of these appear very promising.

A fundamentally different approach is chemical, relying on an affinity between a protective group attached to the peptide, and a group attached to a polymer. The affinity between a protonated amine and a cationic exchange resin has been very successfully exploited by Young and his school with the picolyl ester "handle".[5] In effect, the handle approach is a very simple version of the affinity chromatography principle which has been

employed so successfully for isolation and purification of biological macromolecules.

The practical requirements which a chemical reaction must meet to qualify as a potential "handle" are speed, reversibility, and inertness to the reagents and conditions commonly encountered in peptide synthesis. The groupings which appear as likely candidates include labile disulfides which can be reversibly cleaved by certain nucleophiles, metal chelates, geometrically rigid complementary hydrogen bonded pairs, and boronic acids, which show selective affinity for certain diols. Although we have some experience with each of these possibilities, the boronic acids have proved to be the most successful thus far and we shall report on their properties here.

Each of these functions can meet a further condition--they can be used independently of the others, a prospect which raises the possibility of purification by successive affinity binding. As indicated in Scheme I, the product



Scheme I: Affinity Purification.

from a coupling between a peptide acid bearing affinity grouping A and a peptide amine bearing affinity grouping B could be isolated from starting materials or from products bearing only A or B by successive passage through two

B. If such a procedure could be made convenient and general, it should remove the need for many of the tedious purification procedures which accompany fragment condensation synthesis. Indeed, it may permit development of a partially automated fragment condensation procedure.

Purification by successive affinity binding rests on the assumption that the coupling reagent is perfect to the extent that it cannot yield byproducts which carry both affinity sites. Thus our commitment to the affinity binding strategy has also led us to search for more perfect amide forming reagents. In our search, we have relied entirely on the "safety catch" principle.[6] Yet this principle in turn requires a reexamination of the protective group problem, since one additional independently removable protective group is required whenever a "safety catch" grouping appears in a synthesis.

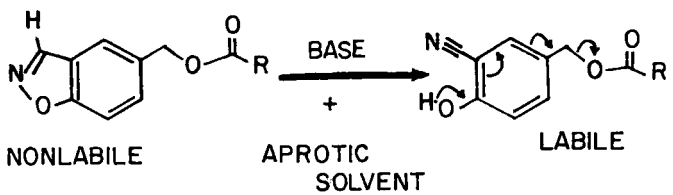
The list of agents which can be used to cleave available protective groups includes acids, reducing agents, nucleophilic agents (*e.g.* hydrazine, or thiolate anions), bases, light, solvent changes, and enzymes. Design of groups labile to acids has reached a very sophisticated level, evidenced by the fact that many modern syntheses rely totally on groups of this class. Yet problems remain. Achievement of selective protective group cleavage through acidity change necessarily requires that the least labile groups are cleaved last, under the most vigorous conditions. Ultimately the field will need protective groups which can resist the conditions used to remove many conventional groupings but which can be cleaved under the mildest of conditions at the end of the synthesis. In effect, the field needs a set of functionalities, resistant to the milder of the conventional cleavage reagents, yet each cleavable at the end of the synthesis at pH 7, 25°, by means of its own selective reagent. Chemical phenomena exist which may be exploitable to produce such functionalities, and nucleophilic and solvation effects seem to us the most likely sources.

Before turning to a working example of the latter effect, I wish to summarize the strategy which would be possible should satisfactory solutions be found to all of the above problems. One could envisage a fragment condensation synthesis which begins with the preparation of a set of pure peptides of the di through tetra size range, using conventional procedures. At this point conventional couplings would be carried out to add to each peptide fragment, amino acids bearing COOH-terminal

"safety catch" activating groups, and amino acids bearing "handle" or affinity protecting groups at NH_2- or side chain termini. Couplings in the medium size range could then be carried out solely via safety catch activation, and isolation and purification could be carried out solely by affinity binding. This is our eventual objective; we are yet some way from achieving it, but it is at least in sight. Let me now turn to three most successful developments we have thus far achieved.

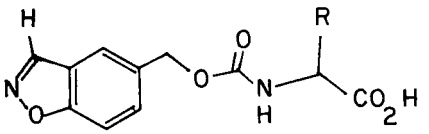
The Bic Group, A Base and Solvent-Labile Functionality

Some years ago,[7] we made the observation that the base catalyzed isomerization of the benzisoxazole function to an *O*-cyanophenol is attended by a very large solvent rate acceleration. For example, the tetraalkyl-ammonium acetate catalyzed isomerization of benzisoxazole itself occurs 10^6 times faster in acetonitrile than in water. Thus the isomerization is expected to be rapid in aprotic solvents in the presence of bases, but slow in protic media in the presence of the same base. Moreover, the available evidence supports the premise that as a substituent the isoxazole moiety of a benzisoxazole acts as a weakly electron-withdrawing group and deactivates the benzene function. Not so the hydroxyl of the isomeric phenol, which is, rather, highly activating, and consequently we envisaged the following reaction sequence:



Synthesis of the required 5-hydroxymethylbenzisoxazole proceeds in two steps in 40-50% yield from salicylaldehyde by chloromethylation, hydrolysis, and *in situ* reaction with hydroxylamine O-sulfonic acid. Reaction with phosgene generates the chloroformate derivative as an oil, stable to storage. The benzisoxazole-5-methyleneoxycarbonyl or Bic group is conveniently introduced as an NH_2-terminal protective group by reaction of the chloroformate with an amino acid in aqueous bicarbonate buffer

The resulting urethanes are often conveniently stored as their dicyclohexylamine salts. The DCA salt of Bic-Gly-OH showed no signs of decomposition after 48 hr in dichloromethane solution. The failure of the carboxylate anion



Bic Amino Acid

of this salt to catalyze isomerization is expected and is attributed to the strong hydrogen bond formed between the ammonium and carboxylate ions of the ion pair in aprotic solvents. It is this feature which is expected to allow use of the Bic grouping under the salt forming conditions of normal peptide synthesis. Further experiments established that relative to the carbobenzoxy group, the Bic group is somewhat more stable to trifluoroacetic acid (TFA).

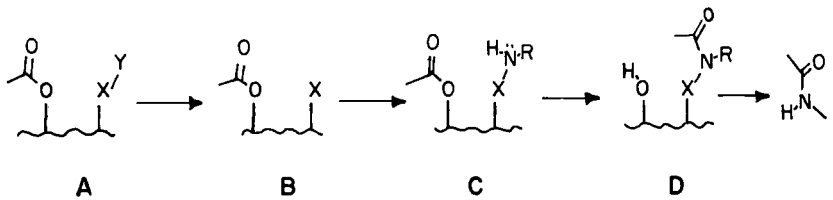
The ready cleavability of the Bic and benzisoxazole-5-methylene ester groupings are established by the following experiments. Treatment of 5-benzoyloxymethylenebenzisoxazole with two equiv. triethylamine in acetonitrile for 30 min quantitatively isomerized the benzisoxazole. Solvolysis in an aqueous TRIS buffer at pH 7.5, 40°, 2 hr resulted in quantitative release of benzoic acid (isotopic dilution). Similarly, treatment of Bic-Valine with NEt_3 followed by TFA for 90 min or with an aqueous ethanolic sulfite buffer, pH 7, 40°, 3 hr led to release of valine in yields of 95 and 92 percent, respectively.

A new protective group must meet the dual tests of conditions for reliable deprotection and compatibility with normal peptide reaction conditions. To examine both issues we carried out a synthesis of a heptapeptide angiotensin analog. (Our yields for the corresponding peptides derived from Z-Arg(NO_2)-OH are cited in parenthesis). Coupling of Bic-Arg(NO_2)-OH with H-Val-Tyr-N_2H_2Boc when carried out by the mixed anhydride procedure using ethyl chloroformate and NEt_3 gave tripeptide in 50-80% yield (60-70%). Reaction with TFA then nitrous acid gave acyl azide which was coupled with H-Ile-His-Pro-Ala-ONb in DMF-EtoAc to yield Bic-Arg(NO_2)-Val-Tyr-Ile-His-Pro-Ala-ONb, 60-70% (65-75%). The Bic group was selectively removed from the

latter in 91% yield by reaction with 3 equiv. NEt_3 in DMF for 30 min, followed by solvolysis in an aqueous pH 7 sulfite buffer at 40° for 3 hr.

Peptide Amide Bond Formation by Prior Amine Capture

For several reasons we have sought a chemical system which is capable of realizing the reaction sequence of Scheme II. Species A bears a relatively unactivated

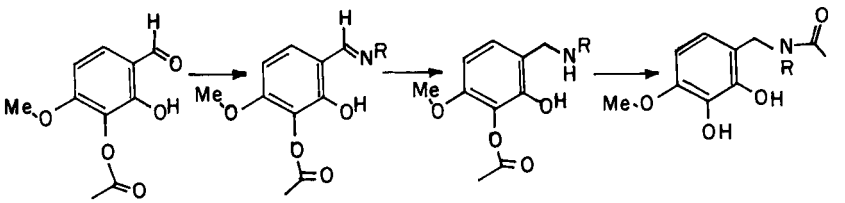


Scheme II.

ester in proximity to a potential electrophilic site X; as long as the X-Y bond is intact, A is a safety catch species in "safety" form. Conversion of A to B releases site X, which can capture an amine to form C. Amide formation, C → D, is an intramolecular process, and the resulting amide bears a NH_2-protecting function until released. Briefly, the theoretical advantages of this novel route to amides are: 1) the ester function, being relatively unactivated, should be less prone to side reactions; 2) the acyl transfer step follows first rather than second order kinetics; 3) provided the amine capture is the slow step, the rate of the overall reaction is determined only the steric bulk of the amine substituent; and 4) the amide protection may offer a means of controlling solubility.

A variety of models were examined[8] and revealed the surprising fact that acyl transfer, C → D, is an extremely slow process ($R\text{-}NH_2 \neq Gly$) even via five or six-ring intermediates, unless the grouping X is very small. For the case to be described, X = CH_2, and our rationalization predicts that this is roughly as large a function as can be tolerated, a finding which severely restricts the choice of candidates for Scheme II.

As expected for salicylaldehydes,[9] 4-methoxy-3-acyloxy-2-hydroxybenzaldehydes react rapidly ($t_{1/2}$ = 3-12 min) with 0.5 *M* solutions of amino acid esters in acetonitrile to form Schiff bases. These can be reduced cleanly with pyridine borane in acetic acid (3-15 min) to benzylamines,[10] which are observed to undergo O → N acyl transfer with half-times in the range of 30-150 min. The resulting N-benzyl amide can be cleaved to the peptide by treatment with HBr/HOAc or with TFA for 1 hr in the presence of resorcinol. For the case of R-CO=Z-Gly,



reaction with the tetramethylguanidine salt of H-Leu-Gly-OH, followed by reduction and rearrangement provided in 92% yield Z-Gly-Leu-Gly-OH bearing a 4-methoxy-2,3-dihydroxybenzyl group on the leucine N. In accord with Weygand's findings for simple benzyl,[11] this tripeptide was freely soluble in chloroform, in marked contrast to Z-Gly-Leu-Gly-OH itself.

The requirement of safety catch activation appears to be met by the 4-methoxy-3-acyloxy-2-benzyloxybenzaldehyde dimethyl acetals, which are cleanly converted to the above salicylaldehydes by treatment with 90% TFA for 1 hr.

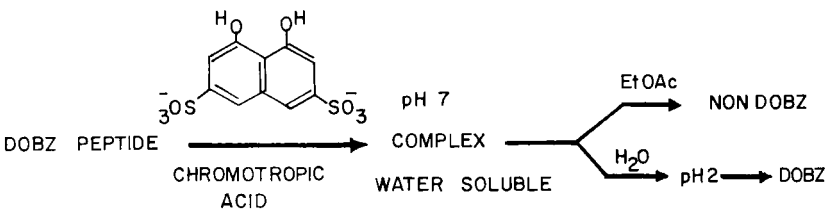
Although the results we have obtained for this system must be regarded as preliminary until it has been tested with realistic synthetic problems and until several unanswered questions have been resolved, they clearly demonstrate the feasibility of the prior amine capture principle.

The Dobz Group, A Boronic Acid Handle

Phenyl boronic acids undergo fast, pH dependent complex formation with certain diols, the highest affinity being shown by 1,8-dihydroxynaphthalene or diethanolamine derivatives. The *p*-dihydroxyboronatobenzyloxycarbonyl group (Dobz) is therefore a highly attractive candidate for an affinity protective group. Reaction of amino acids

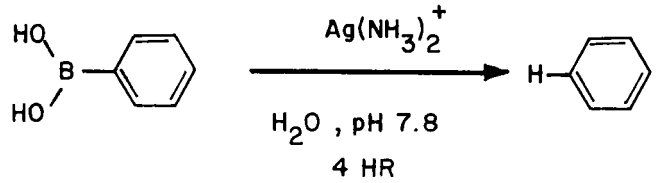
under Schotten-Baumann conditions with catechol blocked Dobz·chloride generates N-Dobz amino acids in 60-80% yields, The Dobz group appears to be more slowly cleaved than the Z group by TFA or by HCl in dioxane. It is stable to bases -- an alkyl ester can be cleanly saponified in its presence. Ready cleavage occurs with hydrogenolysis or by treatment with HBr in acetic acid. Coupling of Dobz bearing peptide acids with amines has been carried out successfully with the ONp ester, mixed anhydride, DCC-HBT, and azide methods, although the latter two appear to give the best results. The Dobz grouping thus behaves as an inert functional group, with one innocuous exception -- under mildly dehydrating conditions the boronic acid functionality trimerizes to form a cyclic six ring anhydride. This reversible reaction results in no new chemical complications and can be useful for characterization since the anhydrides are often highly crystalline.

All of the Dobz bearing peptides so far prepared, including the highly lipophilic Dobz-Lys(Z)-Ala-Val-OMe, react with chromotropic acid in ethanol-water at pH 7 to form water soluble complexes, from which noncomplexed



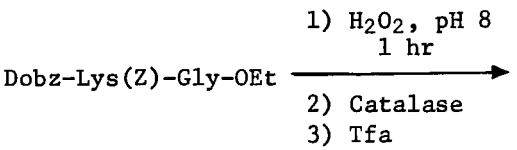
impurities can be extracted. Acidification to pH 2 destroys the complex and allows extraction of the boron-bearing products into organic solvents.

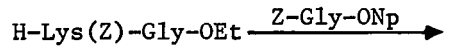
Two further reactions of this versatile function have received some attention. Unlike alkyl boron derivatives, phenyl boronic acids undergo a quantitative hydration which is catalyzed by the diamino silver cation.[12] This reaction permits the conversion of a Dobz into a Z group, thereby facilitating characterization of intermediates.



Dobz-Lys(Z)-Gly-OEt ⟶ Z-Lys(Z)-Gly-OEt 90%

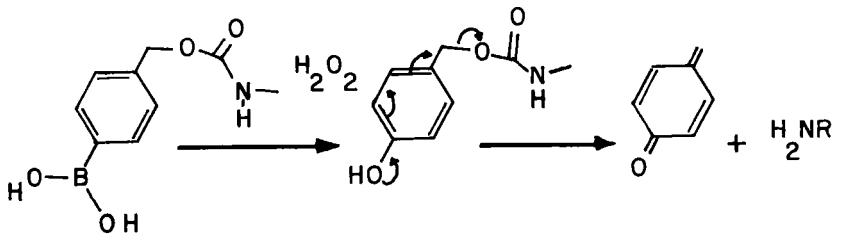
Reaction of phenylboronic acid derivatives with hydrogen peroxide occurs exceedingly rapidly and generates phenols,[13] thus offering hope of an alternative, mild means of deprotection. Two conditions must be met before the results of the accompanying example can be regarded as having practical significance -- the reaction conditions must be shown to be compatible with sulfur containing





Z-Gly-Lys(Z)-Gly-OEt 55% overall

amino acids, and a suitable means must be found for



trapping an intermediary quinonemethide, which has the capability of capturing amine functions at neutral pH.

To date, our most stringent test of the Dobz group has been the synthesis of the heptapeptide: Dobz-Arg(NO_2)-Val-Tyr-Ile-His-Pro-Ala-ONb. Coupling of Dobz-Arg(NO_2)-OH with H-Val-Tyr-N_2H_2Boc by the DCC-HBT procedure

proceeded in 82% yield. Treatment with TFA, diazotization, and coupling with H-Ile-His-Pro-Ala-ONb proceeded in 80% yield. Both yields compare favorably with those realized for the corresponding Z-Arg system. The Dobz tripeptide Boc-hydrazide was converted to the corresponding Z-tripeptide in 70% yield by treatment with $Ag(NH_3)_2^+$ in water, pH 7.5, for 4 hr. Purification of the Dobz tri- and heptapeptides by the chromotropic acid procedure proceeded uneventfully.

Clearly much work remains to be done before the scope of these three reagents can be defined. Further studies with these and with related systems are now in progress and hopefully before long we can report more definitively on their capabilities.

Acknowledgment

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PEPTIDE SYNTHESIS FROM THE PRACTITIONER'S POINT OF VIEW

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I HAVE BEEN ASKED to direct my comments today toward an evaluation of some aspects of current methodology in peptide synthesis from our point of view in the peptide group at Merck. Within this broad subject, I plan to limit my discussion to two areas of interest to us at this time. First, I would like to discuss the growing importance of nmr as a tool for the *synthetic* peptide chemist. Secondly, I would like to discuss our recent studies dealing with the racemization of histidine and the possible implications of these studies in the overall problem of racemization during peptide coupling reactions.

The complexity of the majority of naturally occurring peptides makes most of the analytical methods less precise than is the case for simpler organic compounds. We often hear statements by synthetic peptide chemists that a by-product having the same amino acid analysis as the product was successfully removed. It is often extremely difficult, however, to characterize the by-product. One is, in fact, left with the nagging question as to which is product and which is by-product. The simplest solution is to assume that the dominant product is the desired one.

With this problem in mind, any new structural probe of practical utility is worthy of comment. During the first part of this symposium we have heard a great deal about the theoretical advances that have been attained using nmr spectroscopy. It is worthwhile to further emphasize that nmr, especially utilizing Fourier transform techniques, is of substantial practical value also to the

synthetic peptide chemist. The words Fourier transform should be stressed. It is this technique most particularly which has made the use of nmr a practical tool for us. This technique allows one to obtain useful proton spectra on 1 mg or less and ^{13}C spectra on less than 25 mg of peptides of molecular weight up to at least 1500. Although these spectra are not fully interpretable, a great deal of useful information can be extracted from them.

In order to give an example of the power of nmr, I would like to describe briefly our synthesis of the hypothalamic hormone somatostatin. The synthetic route that we utilized is outlined in Figure 1. A fragment condensation approach was used in which the two protected heptapeptides were prepared sequentially. The protecting groups

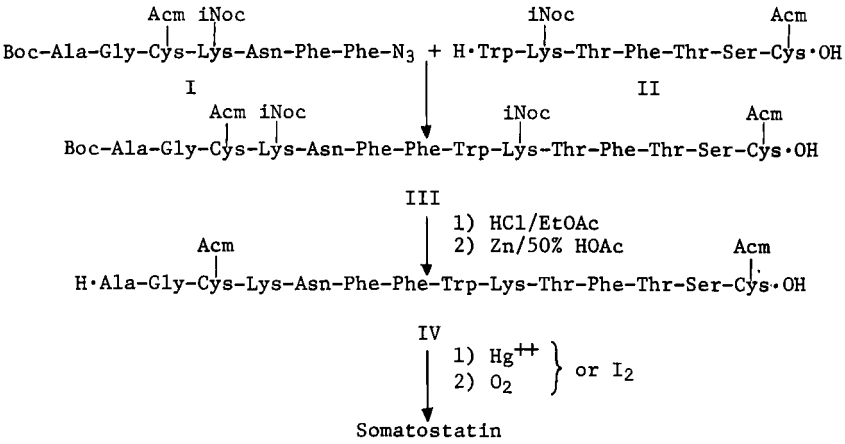


Figure 1: Scheme of somatostatin synthesis.

utilized were Boc for the various terminal amino groups, Acm[1] for the thiol of cysteine, and iNoc[2] for the ε-amino group of lysine. Pure, characterized intermediates were obtained throughout, lending support to the assignment of structure for the final product.

The pmr spectrum of the heptapeptide, II, which was isolated as the crystalline hydrochloride, illustrates the value of 100 MHz pmr when used in conjunction with Fourier transform techniques. The spectrum of a molecule of this size shows peaks associated with each of the seven residues.

Furthermore, there are no peaks that are inconsistent with the structure. This spectrum was obtained using only 1.5 mg of sample.

The two fragments were coupled using the azide method. The Boc and iNoc protecting groups were removed as indicated in Figure 1. After gel filtration on Sephadex G-25 (50% acetic acid), the bis-Acm-dihydrosomatostatin was obtained as a single component. In most experiments the removal of the Acm protecting groups with either Hg^{++} (followed by air oxidation) or I_2 proceeded smoothly to yield a pure product after gel filtration. In one preparation using I_2 for Acm removal, several by-products were formed. Aside from the fact that one product was the major component, most criteria would make it difficult to chose which one had the desired structure. The pmr spectra at 100 MHz of these products provided convincing evidence for the recognition of the desired product.

One of the by-products was recognized as being different from the desired product by the lack of a C-2 proton resonance for the tryptophan residue. Another was recognized to be a mixture of products acetylated at alanine-1 and one or both of the lysine residues. Both ^{13}C and ^{1}H nmr spectra of the dominant product were indeed consistent with the desired structure, adding greatly to our confidence in the assignment of structure. Only 1 mg was required for the proton spectrum and 20 mg for the ^{13}C spectrum. Even more reassuring for the future use of nmr by the synthetic chemist, is the pmr spectrum at 270 MHz (Figure 2). This spectrum is so clear as to be almost conclusive of structure. One is able to recognize and assign normal peaks for 11 of the 14 amino acids. Although the resonances arising from the two 1/2 Cys and the Asn are not specifically assignable, they are clearly present. No other unusual or unassignable peaks are present. In summary, it is becoming apparent that nmr will play an increasingly important role for the synthetic peptide chemist particularly as the new instruments with Fourier transform are becoming more commonly available.

I would like to turn my attention now to a particular chemical problem encountered in the synthesis of some analogs of TRH. One general scheme that we utilized for the synthesis of TRH and related compounds is shown on the following page (Va-VIII).

The reaction of methyl-L-pipecolate(VI)with Va gave two products having the same amino acid composition. Using the method of Manning and Moore[3] and by nmr, we

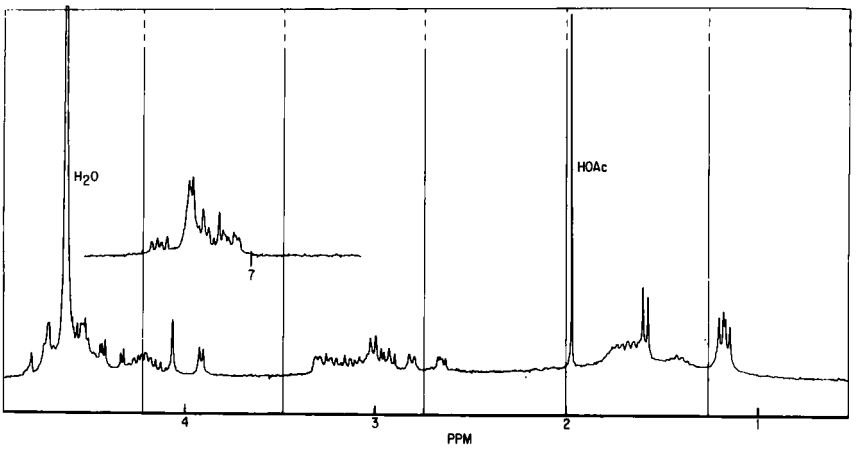
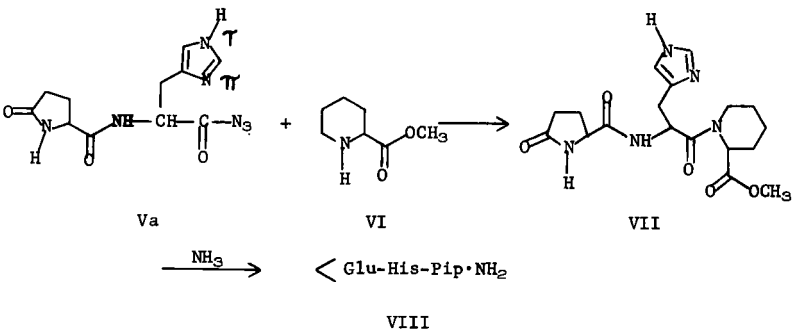


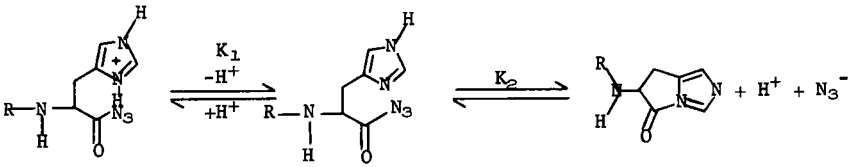
Figure 2: Pmr spectrum of synthetic somatostatin at 270 MHz.



Structures Va-VIII.

were able to demonstrate that these two products differed in the optical configuration at the histidine residue. Racemization at histidine during azide couplings has been reported previously and was enhanced in this instance as a result of the unusually slow rate of reaction of methyl-L-pipecolate. Racemization has been reported for histidine in the protected amino acid, in carboxy terminal histidine peptide azides, and even in urethane protected histidine azide. In fact, histidine appears to be unique among amino acids in a tendency toward racemization.

Three ways in which the imidazole of histidine could catalyze racemization are: (1) intramolecular base catalysis of azlactone formation; (2) base catalysis of the direct abstraction of the α-proton; (3) formation of a cyclic acyl imidazole of the type IX which could have a higher rate of racemization compared to the rate of coupling. An intermediate of this type has been proposed in the preparation of Boc-Ser-His-N_3.[4]



V
a) R = <Glu
b) R = Boc

IX
a) R = <Glu
b) R = Boc
c) R = p-nitroZ

Structures V - IX.

When solutions of either <Glu-His azide·HCl (Va·HCl) or Boc-His azide·HCl (Vb·HCl) in DMF are made neutral or basic by the addition of triethylamine in the absence of acylatable amine, an intermediate is rapidly and reversibly formed. This is followed by a slower racemization at the chiral center of histidine. The ir spectrum of these solutions shows a loss of acyl azide (2110 cm^{-1}) and the generation of azide ion (1980 cm^{-1}) pointing to the formation of a cyclic intermediate. The various spectral properties of the intermediate from both azides, Va and Vb, (ir, nmr, and uv) are comparable to those of IXb. D,L-IXb was prepared by treatment of Boc-L-His with DCCI in a manner analogous to that used by Sheehan[5] for the preparation of IXc. Different spectral properties would be expected for the azlactone (Table I). Each of the spectral changes observed is partially or wholly reversed by the addition of azide ion or acidification of the medium. The existence of an equilibrium between V and IX is also indicated by the fact that the various spectra under some conditions indicate the presence of both compounds and do not change with time.

Table I

Key Spectral Data on Intermediate IXa

Compound	*UV Max mμ*	*IR (C=O) cm*$^{-1}$	*NMR (IMIDAZOLE)* τ
Intermediate (IXa)	261	1770	1.92, 3.21
IXb	262	1770	1.93, 3.20
Azlactone	248[a]	1823[a]	2.32, 3.05[b]

a) Data from the azlactone of Bz-Phe[6].
b) Chemical shift of imidazole protons of Boc-His-methyl ester.

The cyclization to give IX is dependent on the presence of a free imidazole and is promoted by the addition of triethylamine in anhydrous medium. Acidification reverses the reaction. The reaction of IX with azide ion generates a strong base, the anion of imidazole. It is, therefore, possible to follow the establishment of this equilibrium by measurement of the amount of acid required to maintain a fixed pH on mixing IXb and NaN_3 in 20% DMF/H_2O. The rate of hydrolysis of IXb between pH 6 and pH 7 is sufficiently slow so as not to interfere with the measurement of the rapid (< 30 sec) establishment of equilibrium between Vb and IXb. The equilibrium constant (K_2) was found to be about .05 *M* if the pKa of V is assumed to be 6.5 (Boc-His-OMe pKa = 6.4). N-Hydroxysuccinimide also reacts with IXb at pH 6.5 but the equilibrium is more to the right. N-Hydroxybenzotriazole, acetate, and p-nitrophenol do not react to give the active ester to any measurable degree at pH 6-7. It is noteworthy that the rate of coupling of proline amide with IXb in DMF is relatively slow (K = .047 M^{-1} min^{-1}). This reaction is acid catalyzed, as has been shown to be true for the hydrolysis of acyl amidazoles.[7] Thus, in 0.012 *M* $Et_3N \cdot HOAc$ in DMF, the rate of coupling increases substantially (k = .47 M^{-1} min^{-1}). In addition, we have observed catalysis of peptide bond formation from IXb by azide ion. The addition of 1 equivalent of NaN_3 to a mixture of proline amide and IXb in DMF leads to about a 14-fold increase in the rate of coupling. Second order rate constants for the coupling in .012 *M* Et_3NHOAc/DMF with added NaN_3 at various concentrations are: with 0.5 equivalent NaN_3, k = 1.13 M^{-1} min^{-1};

with 1.0 equivalent NaN_3, k = 1.45 M^{-1} min^{-1}; and 2.0 equivalent NaN_3, k = 1.89 M^{-1} min^{-1}.

Although the catalysis by triethylammonium acetate is substantial, further catalysis by azide ion is observed even at .12 *M* Et_3NHOAc. Under no condition was mixed anhydride formation with acetate observed. Under the reaction conditions, a small amount of acyl azide is observed in the ir spectrum. It has not proven possible to shift the equilibrium to more than a few percent acyl azide in anhydrous medium without addition of acid. It is clear, though, that the small amount of acyl azide reacts with an amine at a substantially greater rate than IXa or b. Under more acidic conditions, a shift in the equilibrium toward acyl azide is demonstrated by ir along with an increase in rate of reaction with amine, leading to an overall decrease in racemization. Thus, in a reaction of Ia with methyl pepecolate run at a basicity* comparable to that used for the measurement of the reaction rates, 50% D-histidine was observed in the product. At lower "pH"** a substantial shift in the equilibrium toward Va is indicated by ir, the rate of the coupling reaction increases, and D-histidine in the product is decreased to about 25%.

It has not been possible to sort out the individual contributions of acid and azide ion catalysis due to the complexity of ionic equilibria in DMF. All we can say is that both contribute substantially.

Discussions of the properties of the leaving group which affect the formation of a racemizing intermediate such as an azlactone have centered on factors such as electronegativity which affect only the rate of formation of that intermediate. Selective effects on the rate of peptide bond formation by such mechanisms as internal catalysis by the leaving group have also been cited by others. Such discussions have always failed to satisfactorily explain the uniquely low rate of racemization of acyl azides as compared with peptide bond formation.

We are reminded by our studies with carboxy-terminal histidine azides that one must also consider those factors which affect the rate of the reverse reaction. Ritchie has pointed out that the rate of reaction of a given electrophilic center with various nucleophiles is dependent

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* The basicity was measured as "pH 8 - 8.5" as indicated on moist narrow range indicator paper.

** Moist indicator paper showed "pH 7 - 7.5".

only on the nucleophilicity or N+ value of those nucleophiles.[8] Nucleophilicity can in general be directly related to electronegativity. As long as this relationship holds, the rate of formation of a racemizing cyclic intermediate and the rate of peptide bond formation should remain essentially parallel for all active esters in the general case. There are a number of exceptions to the parallelism of electronegativity and nucleophilicity, particularly the so-called α-effect nucleophiles such as derivatives of hydrazine and hydroxylamine and azide ion among others. Thus, azide ion, in spite of a lower basicity, reacts more rapidly than an amine with the cyclic racemizing intermediate observed in our studies. This high reactivity of azide ion is consistent with the reported nucleophilicity of azide ion being about 10^3 greater than that of gly-gly.[9]

It is expected that further studies planned in our laboratories on the effect of azide ion on the rate of peptide bond formation from an azlactone will show a rate enhancement similar to that observed for the cyclic histidine derivatives. The existence of an equilibrium between a nitrophenyl ester and azlactone has already been demonstrated[5,10] as well as the intermediacy of azlactone in at least some azide couplings.[11]

It should be noted that in cases where the equilibrium is such that substantial cyclic intermediate forms, as we have reported here, excess azide ion has only a limited capacity to suppress racemization. Thus, "over-activation" relative to a potential racemizing intermediate would appear to play an important role in the amount of racemization observed. The observed effectiveness of N-methylimidazole as an acyl transfer catalyst would suggest that simple substitution in the τ position of the imidazole does not necessarily prevent cyclization. Thus, it is not surprising that im-benzyl histidine derivatives are also prone to racemize.[12] Electron withdrawing substituents in the same position (Boc and Tosyl) should and do suppress racemization.[13] Since racemization of histidine by any of the previously mentioned mechanisms is not prevented by τ substitution, the safest derivative might prove to be any protecting group in the π position which has not, to my knowledge, when specifically studied. (The position of protection has not been established for many of the derivatives of histidine. However, the greater reactivity of the τ position makes substitution there more likely.)

External factors which would shift an equilibrium toward dissociation of active ester such as increased ionic strength, dilution, increased temperature, and increased solvent polarity are already known to promote racemization. Such factors have not previously been rationalized on the basis of an equilibrium with a racemizing species, however. Our studies on the racemization of histidine would also suggest an additional factor, nucleophilicity of the leaving group, to be considered in the design of novel active esters having reduced racemization potential. Thus, greater nucleophilicity of a leaving group relative to electronegativity should be expected to reduce racemization.

Acknowledgment

I would like to acknowledge the substantial contributions of my collaborators in this work: Dr. Frederick W. Holly, Mrs. Ruth F. Nutt, Mr. William J. Paleveda, Mr. Robert G. Strachan, and Dr. Sandor L. Varga; and the assistance of Drs. Byron H. Arison and David W. Cochran in obtaining and interpreting the nmr spectra. I would also like to acknowledge the encouragement and guidance given by Dr. Ralph F. Hirschmann.

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APPLICATION OF THE ADDITIVITY PRINCIPLE FOR PREDICTION OF RATE CONSTANTS IN PEPTIDE CHEMISTRY. FURTHER STUDIES ON THE PROBLEM OF RACEMIZATION OF PEPTIDE ACTIVE ESTERS

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THE THEORY WHICH IS USED HERE to predict rate constants is based upon the additivity principle.[1] It is postulated that log k is the unweighted sum of contributions from each reactant and each component of each reactant and from the solvent as indicated by Scheme I.

$$\log k = \log f_1 + \log f_2 + \cdots \log f_i \qquad [1]$$

$$k = f_1 \cdot f_2 \cdot\cdot f_i \qquad [2]$$

Scheme I.

where k is the experimental rate constant and f_i is the contribution of the solvent, or the reactants or their substituents.

The arbitrary standard chosen for racemization reactions is the racemization of Z-Ala-OPcp in the presence of NEt_3 in tetrahydrofuran (THF) at 23° (k_r = 0.825 x $10^{-6} M^{-1} sec^{-1}$); for coupling, the reaction of Z-Ala-OPcp with H-Val-OMe in THF at 23° was selected (k_c = 0.506 x $10^{-2} M^{-1} sec^{-1}$).[6]

The relative contribution of the side chain of Phe to racemization, for example, can be calculated from the ratio of the racemization rate constants of Z-Phe-OPcp and Z-Ala-OPcp since, from Equation [2] all the contributory factors would cancel out of the ratio except those due to the Phe and Ala side chains (Scheme II).

$$\frac{k_{Z\text{-}Phe\text{-}OPcp}}{k_{Z\text{-}Ala\text{-}OPcp}} = \frac{f_{Phe}}{f_{Ala}} = F_{Phe}$$

Scheme II.

In this way relative contributions (F) of N-protecting groups, amino acid side chains and residue, active ester groups and solvents for both racemization and coupling were calculated using data from our own and other laboratories.[2-9] These factors are "best" average values obtained by pooling the available estimates. In general, the percent average deviation in the factor is ±25% ranging from ±7 - 50%; in some dipeptide cases this deviation is higher.

Table I

Predictive Factors

Activating Ester	F_r	F_c	F_c/F_r	Solvent	F_r	F_c	F_c/F_r
OPfp	15	37	2.5	DMSO	23	42	1.8
OTcp(2,4,5)	2.5	0.53	0.21	DMF	10	15	1.5
ONp	1.6	0.22	0.14	Dioxane	0.43	0.54	1.3
OPcp	1.0	1.0	1.0	THF	1.0	1.0	1.0
				$CHCl_3$	3.0	0.14	0.047
N-Protecting Group				Amino Acid Residue			
Boc	0.23	0.94	4.1	-Gly-	105	8.5	0.081
Z	1.0	1.0	1.0				
Amino Acid Side Chain				Amino Acid Side Chain			
Ala	1.0	1.0	1.0	Ser	29	1.3	0.045
Phe	7.1	0.70	0.099	Asp(OMe)	20	0.90	0.045
Try	0.89	0.60	0.67	Asp(Bzl)	22	1.4	0.064
Cys(Bzl)	280	1.3	0.0046	Glu(OMe)	2.5	0.47	0.19

The following two schemes illustrate the calculation of the racemization rate constant of Z-Asp(OMe)-OPfp with NEt_3 in THF and the coupling rate constant for the reaction of Z-Glu(OMe)-ONp with H-Val-OMe in THF respectively.

$$k_r = F_Z F_{Asp(OMe)} F_{OPfp} F_{THF} (0.825 \times 10^{-6} M^{-1} sec^{-1})$$

$$k_r = (1.0)\ (20)\ (15)\ (1.0)\ (0.825 \times 10^{-6} M^{-1} sec^{-1})$$

calculated $k_r = 2.48 \times 10^{-4} M^{-1} sec^{-1}$

experimental $k_r = 2.44 \times 10^{-4} M^{-1} sec^{-1}$

Scheme III.

$$k_c = F_Z F_{Glu(OMe)} F_{ONp} (0.506 \times 10^{-2} M^{-1} sec^{-1}$$

$$k_c = (1)\ (0.47)\ (0.22)\ (0.506 \times 10^{-2} M^{-1} sec^{-1})$$

calculated $k_c = 0.052 \times 10^{-2} M^{-1} sec^{-1}$

experimental $k_c = 0.045 \times 10^{-1} M^{-1} sec^{-1}$

Scheme IV.

Related calculations for coupling of N-protected amino acid *p*-nitrophenyl esters with amino acid ethyl or *t*-butyl esters in DMF have been reported previously by Kemp.[2] Our formulation differs and permits the isolation of factors for each reactant, solvent, and side chain that are contributory to reaction rate.

Table II indicates the experimental and calculated racemization rate constants of Z- and Boc-amino acid active esters with NEt_3 in THF. The racemization of these active esters proceeds by α-hydrogen abstraction and, in the case of Z-Cys(Bzl)-OPcp and Z-Phe-OPcp, the mechanism of racemization is isoracemization in non-polar solvents.[10] This was reported earlier based on deuterium exchange studies. Data in Table II show good agreement between the experimental and calculated rate constants. It is interesting to compare the effect of the two most frequently used N-protecting groups, Z and Boc on the rate of racemization by α-hydrogen abstraction. In general, the Boc-amino acid active esters racemize about 2 to 10 times slower than the corresponding Z-derivatives. A surprising finding is the significantly greater protecting effect of the Boc-group

Table II

Experimental and Calculated Racemization Rate Constants for the Reaction of Z- and Boc-L-Amino Acid Active Esters with NEt_3 in THF at 23°[a]

Compound	k_{r1} x 10^6		Compound	k_{r2} x 10^6		k_{r1}/k_{r2}
Z-Phe-OPcp	3.3	(5.86)	Boc-Phe-OPcp	0.91	(1.1)	3.6
Z-Phe-OTcp	12.0	(14.6)	Boc-Phe-OTcp	3.07	(2.65)	3.9
Z-Phe-ONp	9.2	(9.37)	Boc-Phe-ONp	4.8	(1.87)	1.9
Z-Phe-OPfp	82.0	(87.9)	Boc-Phe-OPfp	20.0	(15.6)	4.1
Z-Phe-ONSu	139.0	(146.)	Boc-Phe-ONSu	20.5	(27)	6.8
Z-Cys(Bzl)-OPcp	414	(231)	Boc-Cys(Bzl)-OPcp	38.9	(53.1)	10.6
Z-Cys(Bzl)-ONp	394	(370)	Boc-Cys(Bzl)-ONp	39.9	(73.9)	9.9
Z-Cys(Bzl)-OPfp	3,300	(3,470)	Boc-Cys(Bzl)-OPfp	379.5	(617)	8.7
Z-Glu(Bzl)-OPfp	34	30.9)	Boc-Glu(OBzl)-OPfp	18.8	(7.1)	1.8

[a] k_r values are $\underline{M}^{-1}\,sec^{-1}$ in most cases two or three different concentrations of NEt_3 were used. Calculated values are in parenthesis.

on the rate of racemization of cysteine active esters which are known to racemize unusually rapidly. This suggests that in Cys coupling the Z protecting group should be replaced by Boc whenever it is possible. Sakakibara[11] reported 95.4% optical purity of the product formed by coupling of Z-Cys(Bzl)-ONp with HCl·H-Gly-OEt in DMF in the presence of 1 equivalent of NEt_3. Our data indicate that by replacing Z with Boc N-protecting groups, about 8 to 10 times less racemization would occur.

Table III shows the experimental and calculated racemization rate constants for the reaction of Boc-Cys(Bzl)-OPcp and Z and Boc protected Gly-Phe active esters with NEt_3 in different solvents. Again good agreement is seen between the experimental and calculated k_r values. In the case of the dipeptides the racemization was followed by polarimeter and IR, which permitted the detection of the presence of oxazolone in addition to the active ester as the racemization proceeded. It is understandable that the rate measured in these experiments is primarily the rate of oxazolone formation, since the racemization of oxazolone is the fast step in the overall reaction as established by previous workers.[12] Furthermore, the equilibrium for oxazolone -- Z-Gly-Phe-OPcp in the

Table III

Experimental and Calculated Racemization Rate Constants for the Reaction of Boc-Cys(Bzl)-OPcp and N-Protected Gly-Phe Active Esters in Different Solvents[a]

Boc-Cys(Bzl)-OPcp				Boc-Gly-Phe-ONp			
Base	Solvent	$k_r \times 10^6$		Base	Solvent	$k_r \times 10^6$	
NEt_3	THF	38.9	(43.5)	NEt_3	EtOAc	242	(498)
NEt_3	Dioxane	17.9	(36.7)	NEt_3	DMSO	1,310	(5,450)
NEt_3	$CHCl_3$	80.1	(135)	NEt_3	Dioxane	515	(120)
NEt_3	DMF	238.	(325)				
Z-Gly-Phe-OPcp				Boc-Gly-Phe-OPcp			
NEt_3	THF	431.	(644)	NEt_3	THF	301	(198)
NEt_3	DMF	4,870.	(6,440)	NEt_3	DMF	1,480	(1,480)
				NEt_3	EtoAc	385	(131)

[a] k_r values are $\underline{M}^{-1}\,sec^{-1}$

presence of NEt_3 in THF was reported earlier from this laboratory.[5] The increase of racemization rate with solvent polarity is in agreement with observations of others and is valid for the amino acid and dipeptide active ester derivatives. Also noticeable is the greater protecting effect of the Boc-group over the Z-group against racemization of dipeptides, as well.

Table IV summarizes the experimental and calculated racemization and coupling rate constants for Z-amino acid active esters and benzyloxycarbonylglycyl dipeptide active esters in THF solution. The racemizations were carried out with NEt_3. The coupling reactions were studied with H-Val-OMe since the considerably slower coupling rate (than that with H-Gly-OEt) permits the use of IR. In addition, coupling with H-Val-OMe decreases the k_c/k_r values since the H-Val-OMe is expected to couple at least an order of magnitude slower than H-Gly-OMe. This table also shows the important k_c/k_r values which indicate the relative extent of racemization which can be expected during coupling. The larger this ratio, the more optically pure is the product to be expected during coupling. The k_{r2}/k_{r1} and k_{c2}/k_{c1} values which are also included in the table indicate how much faster a dipeptide active ester

Table IV

Experimental and Calculated Racemization and Coupling Rate Constants of Z-Amino Acid and Benzyloxycarbonylglycyl Dipeptide Active Esters[a]

Compound	$k_r \times 10^6$		$k_c \times 10^2$		k_c/k_r	k_{r2}/k_{r1}	k_{c2}/k_{c1}
Z-Ala-ONp	1.38	(1.32)	0.152	(0.111)	1100	82	8.4
Z-Gly-Ala-ONp	113	(139	1.28	(0.95)	113		
Z-Ala-OPcp	0.82	(0.83)	0.51	(0.506)	6220	140	2.2
Z-Gly-Ala-OPcp	112	(87)	1.1	(4.30)	98.2		
Z-Glu(OMe)-OPcp	2	(2.06)	0.164	(0.238)	820	155	13.8
Z-Gly-Glu(OMe)-OPcp	309	(217)	2.26	(2.02)	73.1		
Z-Glu(OMe)-ONp	3.0	(3.30)	0.045	(0.052)	150	96.7	17.3
Z-Gly-Glu(OMe)-ONp	290	(347)	0.78	(0.445)	26.9		
Z-Cys(Bzl)-OPcp	414	(231)	1.72	(0.658)	41.5	2.7	20.9
Z-Gly-Cys(Bzl)-OPcp	1101	(24,300)	36.0	(5.59)	327		
Z-Cys(Bzl)-ONp	394	(370)	0.105	(0.145)	2.66	1.42	7.8
Z-Gly-Cys(Bzl)-ONp	562	(38,800)	0.82	(1.23)	14.6		
Z-Asp(OMe)-OPcp	17.6	(16.5)	0.74	(0.455)	420	93	1.0
Z-Gly-Asp(OMe)-OPcp	1640	(1,730)	0.77	(3.87)	4.70		
Z-Ser-OPcp	26.3	(23.9)	0.74	(0.658)	281	29	
Z-Gly-Ser-OPcp	782	(2,500)		(5.59)			
Z-Try-OPcp	0.72	(0.734)	0.32	(0.304)	4440	377	14.
Z-Gly-Try-OPcp	272	(77.1)	4.5	(2.58)	165		
Z-Phe-OPcp	3.3	(5.86)	0.29	(0.354)	879	130	7.3
Z-Gly-Phe-OPcp	431	(615)	2.12	(3.01)	49		
Z-Phe-ONp	9.2	(9.37)	0.032	(0.0779)	35	20	9.7
Z-Gly-Phe-ONp	187	(984)	0.31	(0.662)	17		

[a]Racemization with NEt_3 in THF solution. Coupling with H-Val-OMe in THF solution. k_r and k_c in $\underline{M}^{-1}\,sec^{-1}$.

racemizes or couples than the corresponding Z-amino acid active ester.

The results show good agreement between the experimental and calculated k_r and k_c values with the exception of Z-Cys(Bzl)-ONp which racemizes about as fast as the corresponding dipeptide ONp ester; similar behavior was observed for the two OPcp esters. The IR did not indicate the presence of any detectable oxazolone during racemization.

Since the theory behind the rate constant calculations is a form of linear free energy relationship[13], large deviation from the calculated rate constant values indicate deviation from the constant reaction mechanism. From the kinetic and IR data it could be concluded that the Z-Gly-Cys(Bzl)-ONp and Z-Gly-Cys(Bzl)-OPcp most probably racemize mainly through direct α-hydrogen abstraction. Furthermore, k_c/k_r values predict that these Cys dipeptide active esters probably will give optically purer products than the corresponding simple cysteine active esters. Another unusual finding is that Z-Gly-Asp(OMe)-OPcp couples only as fast as Z-Asp(OMe)-OPcp.

Besides the cysteine "specific cases", generally, whenever the additivity principle is demonstrably operable, a large amount of peptide kinetic data can be surveyed for relatively small effort, *e. g.*, large numbers of racemization and coupling rate constants can be estimated when only few rate constants are determined experimentally.

It was shown in a previous publication[3] that for a practical coupling reaction the ratio of the concentrations of the optically pure peptide, P, and the undesired "racemized" product, P', is

$$(C_P/C_{P'})_\infty \approx \left(\frac{k_c}{k_r}\right)$$

It follows that the optical purity of the product can be optimized by maximizing k_c/k_r.

The k_c/k_r values for dipeptide active esters for Ala, Glu(OMe), Cys(Bzl), Asp(OMe), Try and Phe indicate, for example, that in a fragment condensation reaction one would select Try or Ala rather than Asp(OMe) or Phe since they seem to be prone to less racemization. This is valid for the ONp and OPcp active esters under the conditions reported here; we feel this is probably applicable for other COOH-activating reagents as well. Selection of COOH-activating group to minimize racemization is well established for most coupling reagents on model peptides. However, the suggestion of selecting a cysteine dipeptide instead of a simple cysteine active ester derivative is a surprising result of these kinetic studies.

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ENZYMES AS REAGENTS IN PEPTIDE SYNTHESIS

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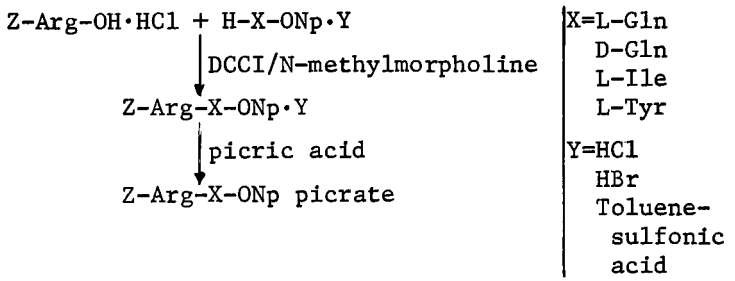
EARLIER COMMUNICATIONS FROM THIS LABORATORY[1,2] proposed the use of N^{α}-protected arginyl moieties as trypsin-labile protecting groups for the α-amino function during stepwise peptide synthesis. Standardized procedures were developed for the preparation of several Z-arginyl amino acid nitrophenyl esters (Z-Arg-X-ONp's) as crystalline picrate salts:



These intermediates reacted with amino compounds to yield the expected peptide derivatives. A pair of diastereomeric peptides prepared in this way featured Z-Arg-*D*-Gln- and Z-Arg-*L*-Gln- sequences, respectively. Trypsin specifically catalyzed hydrolytic cleavage of the Z-Arg protecting group from each of the diastereomers at pH 8 in aqueous solution; however, the peptide containing *L*-Gln was cleaved

much faster than the diastereomer containing *D*-Gln. These results were considered sufficiently encouraging to warrant further studies.

In order to evaluate the effectiveness of the Z-Arg-X-ONp method for the successive introductions of several different amino acid residues into a peptide sequence and to demonstrate the utility of technical modifications in the experimental format, we undertook the synthesis of deamino-oxytocin,[3-5] beginning with H-Asn-Cys(Acm)-Pro-Leu-GlyNH_2, and proceeding stepwise to Mpr(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-GlyNH_2.[6] The Gln, Ile, and Tyr residues were introduced *via* the respective Z-Arg-X-ONp picrates, and the removal of Z-Arg protecting groups was in each case accomplished by tryptic hydrolysis. Routine ion exchange methods were introduced for the conversion of water-insoluble Z-Arg-peptide picrate salts to the corresponding acetate salts and for the separation of the alternating series of ammonium (N-deprotected) and guanidinium (Z-arginyl) intermediates generated by the synthetic method. Of particular interest was the use of the enzymatic deprotection step to stereochemically purify an intermediate which had been subjected to racemizing conditions during a coupling reaction (Figure 1). The S-protected

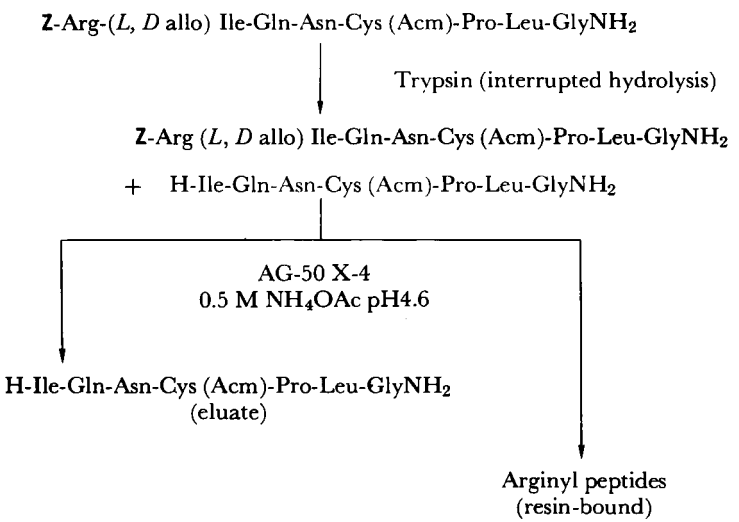


Figure 1: Stereochemical rectification of a partially racemized peptide intermediate.

deaminooxytoceine intermediate was converted to deamino-oxytocin by treatment with I_2 in aqueous MeOH.[6] After purification by partition chromatography[7] the synthetic deamino-oxytocin appeared homogenous by tlc, and it compared favorably with amorphous deamino-oxytocin prepared by other methods[3-5,13] both in specific optical rotation and in specific avian vasodepressor activity. We are presently attempting to crystallize the synthetic product for final characterization.

Experimental

S-Acetamidomethyl-3-mercaptopropionyl-p-nitrophenylate

Mpr(Acm)-OH was treated with a 25% molar excess of TFA-ONp[8] in dry pyridine for 30 min at room temp. The product was precipitated with water and recrystallized from EtOAc. Elemental analyses (C,H,N) agreed with calculated values within 0.2%; yield; 63% mp, 119-121.

Coupling of Z-Arg-X-ONp Picrates with Peptide Intermediates

Peptides as free bases, or as acid salts neutralized with N-methylmorpholine, were treated with 20% molar excesses of the appropriate Z-Arg-X-ONp picrates in minimal volumes of DMF for 3-5 days. Crude peptide picrates were precipitated and triturated with ether to produce yellow powders. The crude picrate salts were converted to either hydrochloride or acetate salts by the standard procedures and applied to a column of BioRad AG50W-X4 in a 0.5*M* acetate buffer adjusted to pH 4.6 as the ammonium salt (pH 4.6 buffer). The columns were washed with pH 4.6 buffer and water. The products were then eluted in 30:4:66, pyridine: HOAc: H_2O [9,10] and purified by gel filtration on Sephadex G-10 or G-15 in 0.2*M* HOAc.

Conversion of Peptide Picrates to Acetate or Hydrochloride Salts

Before tryptic digestions to remove Z-Arg protecting groups, sparingly soluble peptide picrates were converted either to hydrochlorides by repeated treatment with aqueous DCHA·HCl at 45° followed by removal of precipitated DCHA picrate on a filter, or preferably to acetate salts by passage through short columns of Amberlyst A-21 resin in 1:1, DMF:0.2*M* HOAc.

Tryptic Removal of Amine-Protecting Groups

Z-Arg-protected peptides were dissolved (15mg/ml) in 0.02 *M* $CaCl_2$,[11] pH 8.0 For each ml of substrate solution, 0.1ml of a stock trypsin solution (either 1mg or 10mg of thrice crystallized trypsin per ml of 0.02 *M* $CaCl_2$ in 10^{-3} *M* HCl) was added and the pH was maintained at 8.0 by automatic additions of aqueous NaOH. Unless otherwise noted, the reaction mixtures were adjusted to pH 4.6 with HOAc and applied to columns of AG50W-X4 in pH 4.6 buffer. The deprotected peptides passed through the column in the buffer and the Z-Arg-OH released in the digestion, along with any unchanged Z-Arg-peptide, remained on the column. The deprotected products were purified by gel filtration on Sephadex G-10 or G-15 in 0.2 *M* HOAc.

Thin-Layer Chromatography

Chromatographic analyses of peptide derivatives were carried out on silica gel G layers in 4:1:1, BuOH: HOAc: H_2O unless otherwise noted. Products were visualized by the Cl_2/starch-KI method,[12] either directly or after development with ninhydrin.

Z-Arg-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2

H-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2·HCl, neutralized with N-methylmorpholine, was coupled with Z-Arg-Gln-ONp picrate and the product isolated according to the standard procedure to yield 83% of the theoretical amount of chromatographically homogeneous (tlc) product. The material exhibited $[\alpha]_D^{25}$-77 (c=0.5 in 0.2 N HOAc) and an acid hydrolysate contained amino acids in the following ratios* Arg, 0.9: Asp, 1.0: Glu, 1.1: Pro, 1.1: Gly, 1.0: Leu, 0.9

Z-Arg-(*L*, *D*-allo)Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2

H-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2 was prepared from the corresponding Z-Arg derivative by tryptic hydrolysis using the 10mg/ml stock solution of trypsin. The peptide, obtained as a crude powder by lyophilization of the frozen reaction mixture, was treated with a 20% molar excess of Z-Arg-Ile-ONp picrate in a minimal volume of DMF. The product was isolated according to the standard procedure

in 74% yield. An acid hydrolyzed sample gave the following ratios of amino acids: *Arg, 0.9: Asp, 1.0: Glu, 1.0: Pro, 1.1: Gly, 1.0: *a*Ile, 0.2: Ile, 0.7: Leu, 1.0.

H-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2

Tryptic deprotection of the preceding, partially racemized intermediate was accomplished with the 1mg/ml stock solution of trypsin. The deprotected intermediate was isolated according to the standard method to yield 52% of the theoretical amount of chromatographically homogeneous, ninhydrin positive product. An acid hydrolyzed sample of the peptide yielded amino acids in the following ratios:* Asp, 1.0: Glu, 1.0: Pro, 1.1: Gly, 1.1: Ile, 1.0: Leu, 1.0. No trace of either *a*Ile or Arg was detected.

Mpr(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2

H-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2 prepared from the preceding intermediate by the standard methods in 80% yield, was allowed to react with Mpr(Acm)-ONp in DMF. The product was obtained in 67% yield using the isolation procedure reported by Marbach *et al.*[6]

Deamino-Oxytocin

The S-protected derivative prepared above was converted to deamino-oxytocin with I_2 in aqueous MeOH as reported by Marbach *et al.*[6] The hormone analog was also isolated according to the directions of those authors except that partition chromatography on Sephadex G-25 in the system BuOH: benzene: HOAc: pyridine: H_2O, 500: 500: 35: 15: 950[5] was substituted for their CCD step. The purified product was isolated in 45% yield as a chromatographically homogeneous material exhibiting $[\alpha]_D^{25}$-94 (c=0.33, 1*M* HOAc) [Lit.[13] $[\alpha]_D^{25}$-95.1 (c=0.5, 1*M* HOAc) for the crystalline material] and an avian vasodepressor potency[14,15] of approximately 880 U/mg. Values of approximately 733 U/mg for amorphous deamino-oxytocin[3] and 900 U/mg[5] (970 U/mg corrected to anhydrous peptide) have been reported for the crystalline material.[13]

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* Cyst(e)ine values were not determined from hydrolysates of S-Acm-protected peptide derivatives.

Discussion

The preparation of crystalline Z-Arg-X-ONp picrates is conveniently accomplished by the "backing off" method[16] which affords activated esters of peptide fragments while avoiding the racemization-prone step of activating preformed peptide derivatives. The synthesis of deamino-oxytocin demonstrates the application of these intermediates in a short series of peptide elongation cycles.

Both the capabilities and the limitations of the synthetic scheme are brought into focus in the series of steps leading from Z-Arg-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2 to H-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2. Because of the danger of spontaneous pyroglutamyl-peptide formation, we chose not to purify the deprotected glutaminyl compound, but rather to couple the crude, salty product with Z-Arg-Ile-ONp picrate directly. Incorporation of Z-Arg-Ile- into the peptide chain was accompanied by partial racemization of the Ile residue. On the one hand, this result served notice that abused Z-Arg-X-ONp derivatives may racemize*: on the other hand, it presented an opportunity to put the stereoselectivity of the enzymatic deprotection reaction to a practical test. Resolution of the racemate was accomplished cleanly and without deviation from the normal routine of the synthesis.

While the model system demonstrated in this study may not be competitive with established techniques for routine synthetic preparations, it is encouraging to note that the most promising features of the tryptic deprotection scheme (steric specificity, mild aqueous conditions, ionic properties of intermediates, etc.) are intrinsic to the trypsin-catalyzed reaction, whereas the more obvious limitations of the method are confined to the "organic chemical" side of the operation which is subject to modification. As systematic efforts continue to refine the chemical details of the method and as the unique attributes of the enzymatic step come into demand for synthesis or semi-synthesis of delicate peptide derivatives, the use of enzymatic N-deprotection and other applications of enzymatic reagents to peptide synthesis[17-20] will surely merit serious consideration.

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* Under more favorable coupling conditions Z-Arg-Ile-ONp has been used without detectable racemization.

Acknowledgment

This work was supported by US Public Health Service Grants GM 18752 and AM-10080 and by the Energy Research and Development Administration.

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NEW URETHANE PROTECTING GROUPS: THE OPTICALLY ACTIVE 1-ARYLETHOXYCARBONYL GROUP

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IT HAS BECOME INCREASINGLY APPARENT[1] that as longer and more complex peptides are being prepared by the Merrifield solid phase method, the Boc α-amino protecting group must be replaced by a more acid-labile group if the present benzyl-related side chain protecting groups and resin are to be retained. Toward this goal many efforts have been directed. In the present work we have attempted to evaluate some of the existing very acid labile protecting groups[2] and report a new class of 1-arylethoxycarbonyl protecting groups: $Ar-\underset{\displaystyle O-CO-}{\overset{}{CH}}-CH_3$. Inspection of this chemical structure indicates that chemical reactivity can be modified by aromatic-ring substitutions as with the Z-system. Furthermore, the asymmetric carbon in the protecting group would result in the formation of diastereomeric compounds if an optically pure amino acid were derivatized. We hope to take advantage of this situation by using one or the other optically active form of the protecting group with L-amino acids. It would follow that physical purity of the diastereomeric protected amino acids under ideal conditions will provide optical purity of the derivatized amino acids. It is conceivable that one of the two configurations of the urethane protecting group might offer some advantage in peptide synthesis.

In the preparation of the parent racemic 1-arylethanols, the aromatic hydrocarbons were first acylated using a reversed-Perrier procedure of the Friedel-Crafts reaction[3]

to provide the arylmethylketones in good yields (70-95%). The ketones were reduced with sodium borohydride in ethanol to give the racemic 1-arylethanols. It was necessary to avoid excessive exposure of certain 1-arylethanols to the hydrochloric acid used to decompose the excess sodium borohydride. The 1-arylethoxycarbonyl glycine ethyl esters were prepared according to the procedure used by Sieber and Iselin.[4]

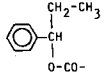
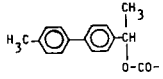
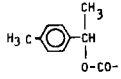
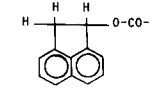
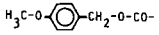
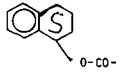
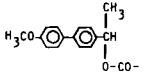
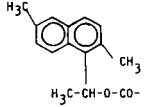
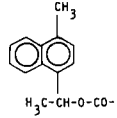
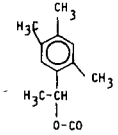
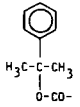
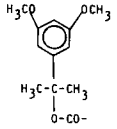
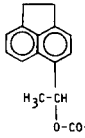
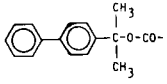
Because of our primary interest in solid phase peptide synthesis, we have focused on deprotection by acidolysis with chloroform or dichloromethane solutions of TFA. Chloroform has been used routinely in this laboratory since altitude problems (1.6 km) have been encountered when dichloromethane was used in an automatic synthesizer equipped with a metering pump. A typical deprotection reaction of an 1-arylethoxycarbonylglycine ethyl ester was started by adding a TFA solution to provide a final concentration of 3% (v/v) and 135-fold excess over substrate. During the deprotection reaction aliquots were taken and neutralized with triethylamine. After removing the chloroform with a gentle air stream, 5 ml water was added and an appropriate aliquot was used in a fluorescamine procedure[5] for determination of liberated free amine (linear 0-20 nanomoles glycine ethyl ester). Under ideal conditions when a plateau value of glycine ethyl ester was obtained, the half-life calculated from the pseudo-first order rate coefficient could be graphically confirmed. This was not the case for the cleavage of protecting groups with rate coefficients similar to Boc, but it was necessary to use the same conditions to permit strict comparisons. The relative rates shown in Table I are referred to the rate of the α, 2,4,5-tetramethylbenzyloxycarbonyl group (TmZ).

The TmZ group was selected for further study on the basis of its chemistry (rate of deprotection approximately 4000 x Boc and 1/30 x Bpoc) its physical properties (readily crystallizable derivatives) and its ease of preparation (molar synthesis of acetylpseudocumene gave 95% yield with greater than 99.5% purity by GLC, starting with reagent grade pseudocumene).

The effect of different solvents on the rate of deprotection of TmZ-Gly-OEt was studied by a different experimental protocol. Aliquots of TmZ-Gly-OEt were treated for exactly 60 sec. with varying concentrations of TFA in the solvent under consideration. The neutralized samples were worked up as described above for the fluorescamine

Table I

Rate of Deprotection of 1-Arylethoxycarbonyl- and Related Urethane Protected Glycine Ethylesters

Structure of Urethane Protecting Group	Half-Life	Rate Relative to "TmZ"
	100 hrs[a]	1/8400
	50 hrs[a]	1/4200
	50 hrs[a]	1/4200
	2.7 hrs[a]	1/220
	18 min[a]	1/25
	17 min[a]	1/23
	12.5 min[a]	1/17
	8 min[a]	1/11.4
	4.4 min[a]	1/5.9
	3.0 min[a]	1/4.2
	2.7 min[a]	1/3.8
	TmZ	42 sec[a] 420 sec[b]	[1]
		120 sec[b]	3.4
	DdZ	75 sec[b]	5.4
		~30 sec[b,c]	~10
		~ 10 sec[b,c]	~30

[a] deprotection with 3% (v/v) TFA in chloroform

[b] deprotection with 0.5% (v/v) TFA in chloroform

[c] calculated by assuming plateau time approximates $t_{99.9\%}$

assay. The results are shown in Figure 1. Assuming the pseudo-first order rate law can be applied, it can be shown that the percent deprotection (%D) observed at

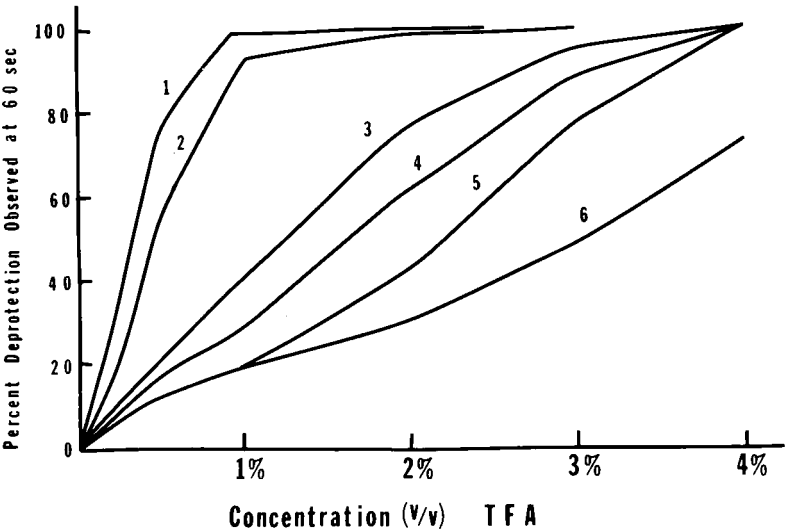


Figure 1: Solvent effects on the extent of deprotection of TmZ-Gly-OEt by TFA in: 1) dichloromethane, 2) 1,2-dichloroethane, 3) chloroform (hydrocarbon stabilized), 4) benzene, 5) chloroform (EtOH stabilized) and 6) carbon tetrachloride.

any time, t, is related to the rate coefficient, k, by the expression:

$$k = \frac{\ln \left[\frac{100}{(100 - \%D)}\right]}{t}$$

It would follow, therefore, that the rate of deprotection depends on solvent and acid strength as shown in Figure 1. The acceleration of deprotection in dichloromethane is impressive.

Certain derivatives of TmZ-Gly were prepared in order to determine the effect of substitution on the rate of deprotection. Very little difference was observed when TmZ-Gly-OH, TmZ-Gly-NH_2 and TmZ-Gly-OEt were compared in

a deprotection reaction in solution, as shown in Figure 2. Furthermore, it was gratifying to find that TmZ-Gly coupled to a benzhydrylamine polymer was deprotected at a rate comparable to TmZ-Gly in solution, as indicated in

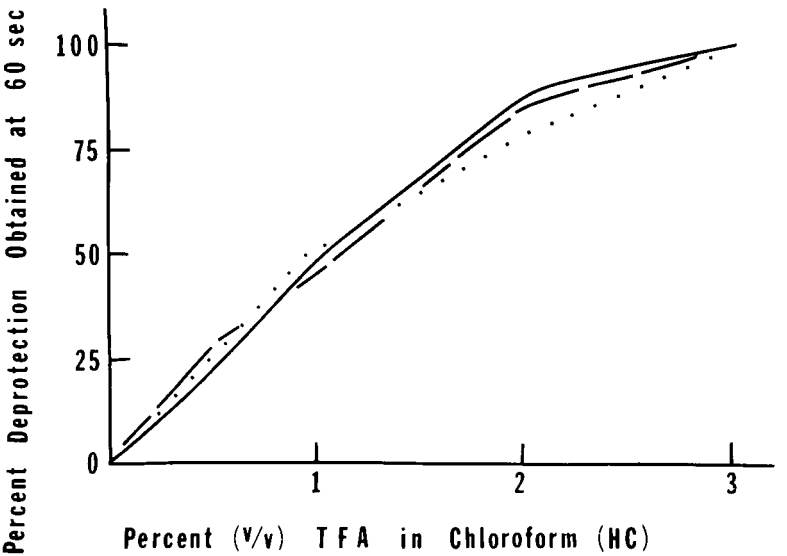


Figure 2: The extent of deprotection of TmZ-Gly derivatives by TFA: (...) TmZ-Gly-OH, (— — —) TmZ-Gly-NH_2 and (————) TmZ-Gly-OEt.

Figure 3. Also in Figure 3 the accelerating effect of dichloromethane relative to chloroform was again observed during deprotection from the solid phase. These deprotections were performed by monitoring a stirred suspension of resin in 3% (v/v) TFA in $CHCl_3$. Only the absorbance of the acidolyzed TmZ group in solution outside of the resin beads contributed to the observed data. When the deprotection was completed, the resin was permitted to float clear of the light path for an absorbance measurement of the solution. This value permitted calculation of an extinction coefficient which was within experimental error of the determination made in solution (e_{271} = 840 ± 40). TmZ derivatives of Arg(Tos), Pro, Phe and Ser(Bzl) have been prepared and used successfully in a solid phase synthesis of bradykinin. This synthesis, which should

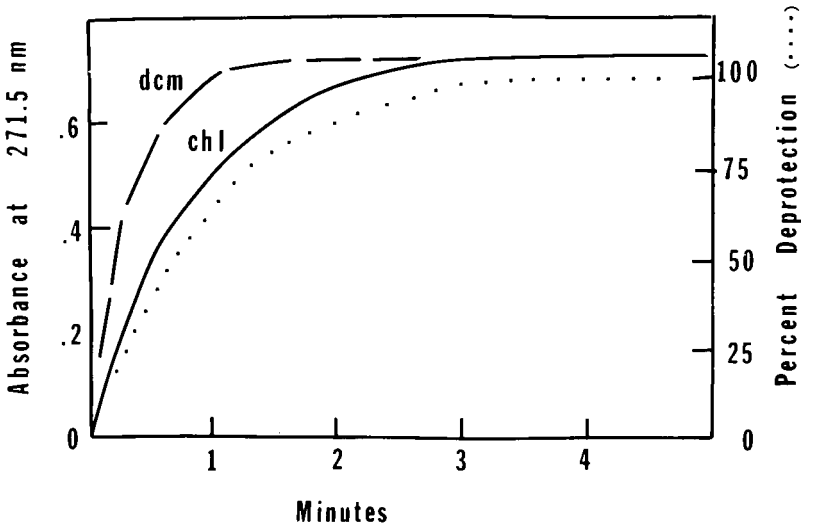


Figure 3: Comparison of TmZ-Gly-OEt deprotection in Chloroform solution (....) vs. TmZ-Gly-NH-BHA resin deprotection from the solid phase in chloroform (———) and in dichloromethane (— — —). 3% (v/v) TFA was used in each deprotection.

have abrogated any deprotection failures, did not noticeably improve the crude product over that obtained by use of Boc amino acids, and still contained truncated peptides. Presumably failure to couple completely must have caused the observed problems. The TmZ group is currently being evaluated in other sequences where failure of deprotection is known to cause problems.

As a preview of the contribution of the asymmetric protecting group, it was noticed that during the preparation of TmZ-Pro DCHA salt, two crystal fractions were collected with melting ranges 122-131 and 152-165. It is anticipated that any benefits provided by either the L or D form of the TmZ group can be tested with the purified preparations of L-TmZ-L-Pro and D-TmZ-L-Pro.

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THE USE OF BORON TRISTRIFLUORO ACETATE (BTFA) IN THE SYNTHESIS OF BIOLOGICALLY ACTIVE PEPTIDES

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EXPERIMENTS WITH SIMPLE MODEL COMPOUNDS showed that BTFA* can be used to remove protective groups from peptides and and that its properties run parallel with those of HF.[1] In the course of further investigations, we extended our studies to biologically active polypeptides. Our purpose was to establish the optimum conditions combining complete removal of the protecting groups with the preservation of maximum biological activity. We succeeded in replacing the dangerous HF with BTFA and in this summary we report the isolation of oxytocin, LH-RH and somatostatin of high purity in excellent yield.

To investigate the removal of protective groups from the cysteine residues of polypeptides, we first studied protected bis-S-methyloxybenzyl (Mbzl) *oxytocin*

$$\begin{array}{c} \text{Mbzl} \qquad\qquad\qquad\qquad \text{Mbzl} \\ | \qquad\qquad\qquad\qquad\quad | \\ \text{Boc-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH}_2 \end{array}$$

which was specially synthesised for the purpose by the conventional route. We obtained chromatographic proof that the S-Mbzl was quantitatively removed, as we had expected, but the presence of the tyrosine residue, which is sensitive to alkylation, prompted us to study the use of BTFA in conjunction with various scavengers that are frequently employed. The best results were obtained by adding methyl ethyl sulphide in the following ratio:

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* Commercially available from Aldrich & Co., or Pierce & Co.

protected oxytocin: Me-S-Et BTFA = 1:100:100.

In this case, the protecting groups were completely removed and after oxidation and purification by countercurrent distribution we obtained the usual yield of the hormone, which showed full activity. Methyl ethyl sulphide also proved a useful scavenger in the cleavage of model peptides containing methionine residues, as

Z-Ser-Met-OMe.

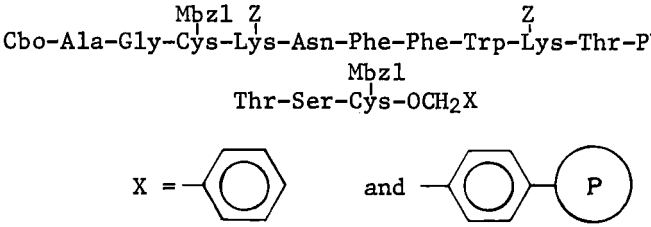
For the examination of *LH-RH* and its analogs we synthesised the protected precursor

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2

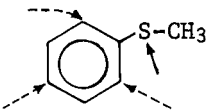
by the usual methods.[2] This peptide provided an opportunity for studying the effect of BTFA on the nitro-arginine. Tlc furnished evidence that the guanido nitro group could be completely removed, but the presence of the sensitive Trp residue in the hormone made it necessary to add a suitable scavenger. We have tried indole, diethyl phosphite, methyl ethyl sulphide, anisole and phenol. Anisole could not be used successfully in conjunction with BTFA because it underwent competitive dealkylation, with the result that not enough BTFA remained for the removal of all the protective groups. By using phenol in the ratio, LH-RH: phenol: BTFA = 1:10:50 we succeeded in isolating LH-RH that was, after purification, active and chromatographically pure.

Before proceeding to the synthesis of *somatostatin* and its analogs, we wished to establish a general procedure, embracing the type of scavenger and its ratio to BTFA and the protected peptide. We wished to determine time and temperature, and other experimental conditions and we attached particular importance to the elimination of excess boron. A final point of practical importance was to find out if different experimental conditions are required for the removal of protective groups, when the peptide is bound to a polymer (P).

To solve all these problems, we synthesised the protected somatostatin tetradecapeptide by two routes, *viz.* by the classical method of fragment condensation and by the Merrifield method, ending up with



Amino groups were protected with Z, and cysteine with S-Mbzl, threonine and serine remaining unprotected. Preliminary experiments with model peptides showed that *thioanisole* is a very useful new scavenger which is generally adaptable and which, in contrast with anisole, provides at least 2 nucleophilic centres for cationic attack, namely the sulphur atom and the aromatic nucleus.



On the other hand, BTFA does not react with thioethers, so that none is lost by this route.

By using the same protected somatostatin molecule, we were able to compare the effect of different cleavage mixtures on the yield and the purity of the resultant hormone. From Table I, it can be seen that thioanisole proves to be the best scavenger (Nr.1) and that an advantage in yield is gained by adding Cys or Trp (Nr.2). Thioanisole is not only the best scavenger in conjunction with BTFA, but is also a useful scavenger in conjunction with HF (Nr.5). We did not succeed in obtaining satisfactory results with trifluoromethylsulphonic acid,[3] nor with sodium in liquid ammonia, which destroyed part of the peptide chain.

The following typical example will serve to illustrate the *optimum experimental conditions for the removal of protecting groups with BTFA:* 2 mMol of protected somatostatin and 100 mMol of thioanisole were mixed with 50 ml of CF_3COOH. 100 ml of a 2-molar solution of BTFA in trifluoroacetic acid were added at 0° (the same quantity is also used for peptides built up by the solid-phase method) and the mixture was shaken for one hour at room temperature.* Following evaporation under reduced

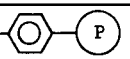
*Ratio: Protected somatostatin: thioanisole: BTFA = 1:50:100.

Table I

Cleavage of Protected Somatostatin with Different Reagents

Z-Ala-Gly-Cys(Bzl(OMe))-Lys(Z)-Asn-Phe-Phe-Trp-Lyz(Z)-Thr-Ser-Cys(Bzl(OMe))-OCH_2X

Nr.	Reagent	Temp.	Scavenger	Yield*	Remark
1	BTFA	24°C	Thioanisole	∿50%	pure
2	BTFA	24°C	Thioanisole Trp Cys	∿70%	pure
3	BTFA	24°C	Anisole Trp Cys	∿50%	not homogeneous
4	BTFA	24°C	Thiophenol Trp Cys	∿25%	pure
5	HF	0°C	Thioanisole Trp Cys	∿40%	pure
6	HF	0°C	Met Trp Cys	∿60%	not homogeneous
7	HF	0°C	Anisole Trp Cys	∿30%	pure
8	HF	0°C	Thiophenol Trp Cys	∿20%	pure
9	CF_3SO_3H	24°C	Thiophenol Trp Cys	10%	not homogeneous
10	CF_3SO_3H	24°C	Thioanisole Trp Cys	60%	not homogeneous
11	Na/NH_3			20%	peptide chain cleavage

X =  and 

*Yield: After purification on Sephadex G-25.

pressure**, the oily residue was dissolved in dilute acetic acid and thioanisole was extracted with ethylacetate. Elimination of boron and simultaneous purification of somatostatin was achieved by gel filtration on Sephadex G-25, or

**In absence of carboxyl groups in the molecule, a four-fold addition of 50 ml methanol followed by evaporation under reduced pressure can be introduced at this step.

ion-exchange chromatography on Bio-Rex 70. This resin does not retain boron so that it can be washed out and somatostatin subsequently eluted in an acetic acid gradient. The average yield from different lots of homogeneous pure somatostatin with full biological activity ranges from 50% to 80%.

In conclusion, BTFA has proved to be at least as effective as liquid HF for the removal of protecting groups from biologically-active peptides. This has been demonstrated with oxytocin, LH-RH and somatostatin. -- It can also be used to remove protecting groups from peptides bound to polymer (Merrifield-Resin). -- BTFA is easily prepared on a large scale in the laboratory and it is also commercially available. No special apparatus or security precautions are required with this reagent. -- Exact experimental conditions have been established specifying the ratios of the reagents and a method of removing excess boron. -- Thioanisole has been introduced as a new and superior scavenger that can be used not only in combination with BTFA but also with liquid HF.

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COMPARISON OF *p*-NITROBENZYL AND *p*-NITROPHENYL RESIDUES AS CARBOXYL PROTECTING GROUPS IN THE SYNTHESIS OF OLIGOPEPTIDES

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IN OUR EXPERIMENTS[1-5] on the synthesis of an active center peptide of the streptococcae proteinase (EC 3.4.22.10) with the sequence Val-Lys-Pro-Gly-Glu-Gln-Ser-Phe-Val-Gly-Gln-Ala-Ala-Thr-Gly-His-Cys-Val-Ala-Thr-Ala-Thr-Ala-Gln-Ile-Met-Lys[6] by classical fragment condensation we have tried to start the synthesis of the fragments with the *p*-nitrophenyl esters of the COOH-terminal amino acids. The optical rotation and yields of the resulting acyl peptide *p*-nitrophenyl esters were compared with the same peptide derivatives prepared by starting the synthesis with the *p*-nitrobenzyl esters, subsequent hydrogenolysis and formation of the *p*-nitrophenyl esters from the acyl-peptides according to the usual procedure. The advantages of starting the synthesis with amino acid *p*-nitrophenyl esters are evident: Protection and activation of the carboxyl groups of the fragments are accomplished by the same step.

Starting with the amino acid *p*-nitrophenyl ester salts the coupling reactions of the synthesis were performed in chloroform using N-methylmorpholine as base and dicyclohexylcarbodiimide (DCC) as coupling reagent (procedure A). The use of triethylamine as base or another aprotic solvent, for instance tetrahydrofuran, effects formation of diketopiperazines from dipeptide *p*-nitrophenyl esters or polymerisation. Starting with *p*-nitrobenzyl esters the solvent was dimethylformamide, the base was triethylamine and the coupling reagent DCC and 1-hydroxybenzotriazole (procedure B). In the case of the peptide His-Cys-Val procedure B was modified by starting with free valine.

Cysteine and histidine were coupled in this case as *p*-nitrophenyl esters with 1-hydroxybenzotriazole. The synthesis of the peptide Val-Lys-Pro-Gly in procedure B was started with the methyl ester for carboxyl protection which was removed by hydrolysis with aqueous sodium hydroxide in methanol.[1]

The application of procedure A is limited by the solubility of higher peptide derivatives in chloroform. We have further observed that the *p*-nitrophenyl esters of differently S-protected cysteine derivatives (Boc-Cys(X)-ONp; X = *t*-butylmercapto [StBu], tetrahydropyranyl-(2) [Thp], *p*-methoxybenzyl) were hydrolyzed under conditions of deblocking the *t*-butyloxycarbonyl [Boc] amino protecting group with 4 N hydrogen chloride in dioxane. Peptides with COOH-terminal cysteine could therefore not be synthesized by procedure A. With methionine however only small side reactions were observed. The results of our experiments are summarized in Table I.

Table I

Optical Rotation and Yields of Different Acylpeptide *p*-Nitrophenyl Esters Synthesized According to Procedures A and B (see text)

Procedure	*A*	*B*	*A*	*B*
Peptide	$[\alpha]_D^{24}$	*(c=1, DMF)*	*%*	*Yield*§
Z-Val-Lys(Z)-Pro-Gly-ONp [1]	-43,7	-45,2	53	10
Boc-Ala-Ala-Thr-Gly-ONp [7]	-21,4	-22,2	28	13
Boc-His(Boc)-Cys(StBu)-Val-ONp [7]	-62,6	-59,2	60	14
Boc-His(Boc)-Cys(Thp)-Val-ONp [7]	-37,1	-37,2	42	3
Boc-Ala-Thr-Ala-Thr-Ala-ONp [7]	-20,4	-14,2	13	23

§ Calculated on the basis of the first amino acid

The advantages of procedure A are especially evident with regard to the yield of the acylpeptide *p*-nitrophenyl esters. The poor yields for the peptide Boc-Ala-Thr-Ala-Thr-Ala-ONp synthesized according to A is a consequence of the low solubility of intermediates in chloroform. Similar experiments are described by Goodman *et al.*[8], Iselin *et al.*[9] and Wittinghofer[10].

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α,β-UNSATURATED AND THIOETHER AMINO ACIDS IN PEPTIDE SYNTHESIS

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SELECTIVITY IS OF FOREMOST IMPORTANCE in reactions applied to the nonenzymatic structural elucidation of peptides and proteins.[1] What is true analytically, is of like significance to the synthesis of peptides and proteins.

Certain reactions, well established in peptide[2] and protein[1] fragmentation, have been extended to peptide synthesis and proved their applicability (a) to the removal of protecting groups, (b) as links between the growing peptide chain and solid supports,[3] (c) with regard to the flexibility in generating at α- and ω-carbon atoms of amino acids either amide functions or free COOH-groups.

The Application of the Cyanogen Bromide Reaction to Peptide Synthesis

The cyanogen bromide reaction[1] is applicable to peptide synthesis when acetylmethionine (AM) is employed as protection of the H_2N-group[4,5] (Figure 1).

The 4-Methylthiobutyryl (MTB) group, $H_3C\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}$, lacking the acetamino group of acetylmethionine offers also suitable protection of α- and/or ω-amino groups in amino acids. Deprotection is quantitative under the *mild* and *selective* conditions of the cyanogen bromide reaction.[1]

$^{\alpha}$N-tert-Butyloxycarbonyl-$^{\varepsilon}$N-4-methylthiobutyryl-L-lysine [Boc-Lys(MTB)-OH] was prepared from Boc-Lys-OH and 4-methylthiobutyric acid via activation of the latter as N-hydroxysuccinimide ester[6] (yield 74%; tlc[12]: R_fA 0.52;

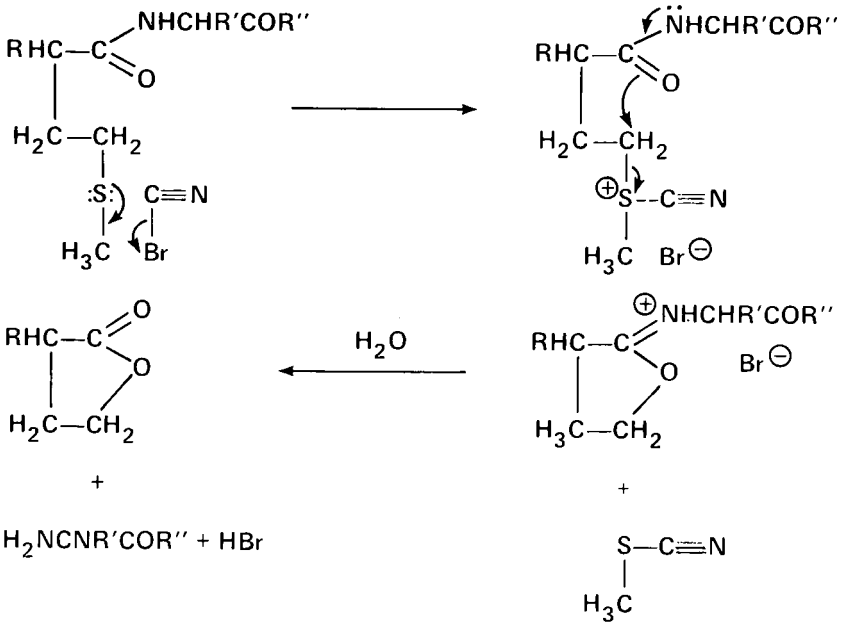


Figure 1: The reaction mechanisms for the removal of the 4-methylthiobutyryl and the acetyl methionyl protections from amino groups. R = H: 4-Methylthiobutyryl-(MTB); R = $HNCOCH_3$: Acetyl-L-methionyl (AM); R' = Amino acid side chain; R'' = COOH-protection; amino acid(s).

R_fB 0.77; oil $[\alpha]_D^{20}$ + 3.2 (c = 1; methanol); calculated for C_{16} H_{30} O_5 N_2 S: C 53.02, H 8.34, N 7.73%; Found: C 53.35; H 8.53 N 7.94%.

$^{\alpha}$N-tert-Butyloxycarbonyl-$^{\varepsilon}$N-acetylmethionyl-L-lysine [Boc-Lys(AM)-OH] was also prepared via N-hydroxysuccinimide ester activation (yield 80%; tlc[12]: R_fA 0.41; R_fB 0.77; mp 143-144° $[\alpha]_D^{20}$ + 9.20° (c = 1, methanol); calculated for C_{18} H_{33} N_3 O_6 S: C 51.54, H 7.93, N 10.02%; Found: C 51.77, H 8.12, N 10.34%.

Treatment of Boc-Lys(MTB)-OH or Boc-Lys(AM)-OH with 10 equivalents of *cyanogen bromide* in 0.1 N hydrochloric acid (room temperature; 5 hr) removes the MTB and AM-group, respectively, from the $^{\varepsilon}NH_2$-groups and also almost quantitatively the Boc-protection of the $^{\alpha}NH_2$-groups (Figure 2: frames A and B). Treatment of Boc-Lys(MTB)-OH

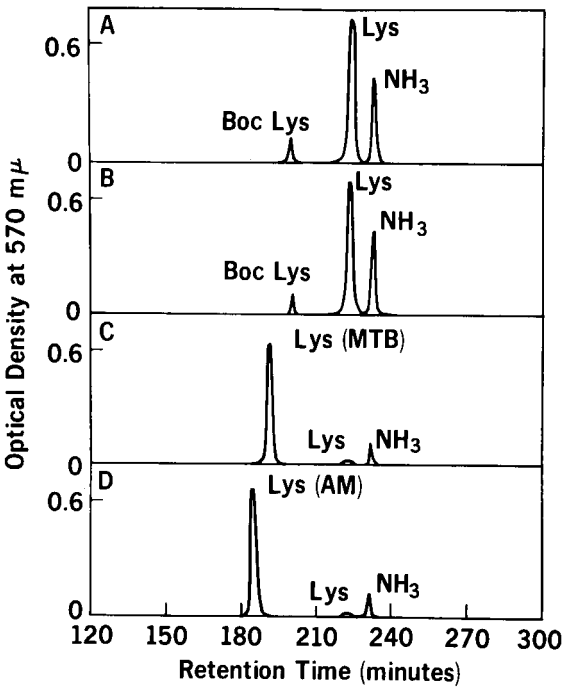
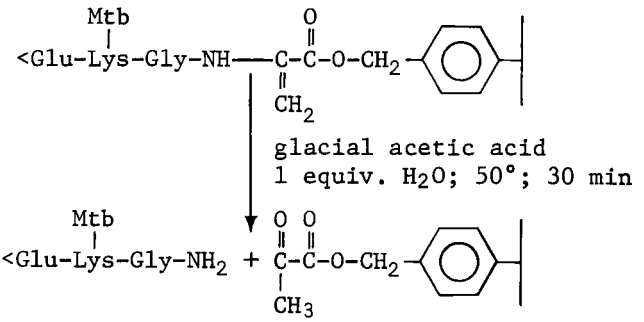


Figure 2: Amino acid analysis after treatment of: A. $^{\alpha}$N-t-Boc-$^{\varepsilon}$N-MTB-lysine with 10 eq. of cyanogen bromide (BrCN); 0.1 N HCl; room temperature (r.t.); 5 h. B. $^{\alpha}$N-t-Boc-$^{\varepsilon}$N-AM-lysine with 10 eq. of BrCN; 0.1 N HCl; r.t.; 5h. C. $^{\alpha}$N-t-Boc-$^{\varepsilon}$N-MTB-lysine with 25% trifluoracetic acid (TFA) in methylene chloride (CH_2Cl_2 r.t.; 30'. D. $^{\alpha}$N-t-Boc-$^{\varepsilon}$N-AM-lysine with 25% TFA/CH_2Cl_2; r.t.; 30'. MTB = 4-methylthiobutyryl; AM = acetylmethionyl.

or Boc-Lys(AM)-OH with 25% trifluoroacetic acid in methylene chloride removes only the Boc-protection of the $^{\alpha}NH_2$-groups (Figure 2; frames C and D).

Pyro-L-Glutamyl-L-lysylglycine amide (<Glu-Lys-Gly-NH_2) was synthesized via the Dehydroalanine Method for the solid phase synthesis of peptide amides.[3] Following attachment of Boc-glycyldehydroalanine[7] to a 1% cross-linked chloromethyl resin (0.26 mmoles of peptide/g resin), Boc-Lys (MTB) -OH and Boc-<Glu-OH were coupled under the standard conditions of solid phase peptide synthesis.[8] <Glu-Lys(MTB)-Gly-NH_2 was readily removed from the

Dehydroalanine Resin by treatment with 1 equiv. water in 1 N HCl in glacial acetic acid at 50°C for 30 minutes (Scheme 1).



Scheme 1.

Purification by countercurrent distribution (Figure 3A) was followed by the quantitative removal of the MTB group by treatment with cyanogen bromide and renewed

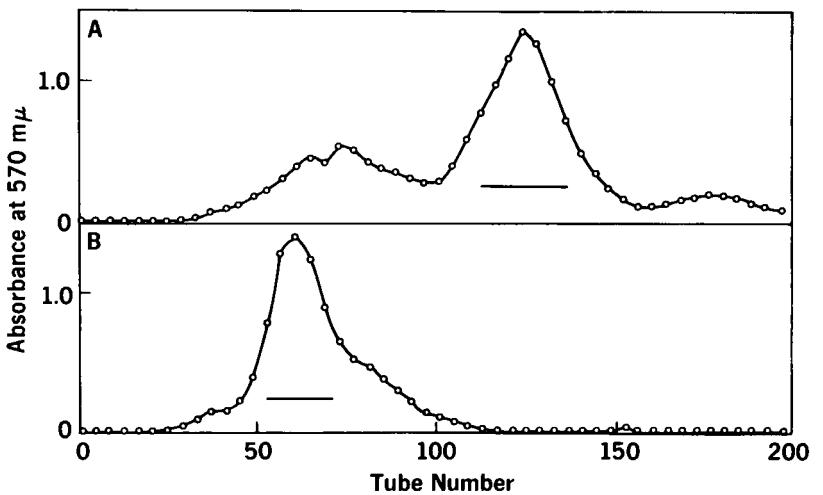


Figure 3: Countercurrent distribution of A: <Glu-Lys(MTB)-Gly-NH_2 and B: <Glu-Lys-Gly-NH_2. Solvent System: n-butanol:acetic acid:water (3:1:4). The bars mark fractions combined.

countercurrent distribution to give in 61% yield a product (Figure 3B) showing this amino acid analysis: Glu 0.98, Gly 1.00, Lys 0.99, and (NH_3 1.03) residues. Single spots in thin layer chromatography[12] were seen in several solvent systems: R_fB 0.05, R_fC 0.12, R_fD 0.16, R_fE 0.10.

<Glu-Lys-Gly-NH_2 is presently under investigation for possible hormone releasing and/or hormone release inhibiting activity.

Discussion

α,β-Unsaturated amino acids occur naturally in relatively greatest proportions in nisin[2] from *Streptococcus lactis* and subtilin[9] from *Bacillus subtilis*. The structural elucidation of the two peptides[2,9] provided much opportunity for the extensive study of the chemistry of α,β-unsaturated amino acids. Aspects of the chemistry of α,β-unsaturated amino acids have since been proved applicable to peptide synthesis. Peptides with COOH-terminal amides are accessible via *Dehydroalanine Resins*[3] and amides in endo-positions (asparagine and glutamine) are incorporated via attachment of dehydroalanine alkylamides to the ω-carboxyl groups.[10]

The high degree of selectivity of the cyanogen bromide reaction[1] invited extension of this potential to peptide synthesis. Earlier explored with acetylmethionine,[4] a novel and most promising protecting group is now being offered in the form of the 4-methylthiobutyryl group (MTB-group).

The MTB-group is applicable even to peptides with methionine residues as demonstrated by the synthesis of $^{\alpha}$N-MTB-tryptophyl-methionyl-aspartyl-phenylalanine amide (COOH-terminal tetrapeptide of gastrin) via MTB-Trp-Met(O)-Asp(OBzl)-Phe-NH_2 using the *Dehydroalanine Resin* method. The sulfoxide provides adequate protection for methionine while the MTB-group of trytophan is removed by treatment with cyanogen bromide. Subsequent reduction with mercaptan restores methionine to the molecule.

The utility of the cyanogen bromide reaction in peptide synthesis is not restricted to methionine derivatives and/or thioethers (*cf.* the MTB-group) capable of undergoing neighboring group assisted 1,5-interactions in the same unit. The extension of the cyanogen bromide reaction to S-methylcysteine[11] offers alternatives in that the carboxyl group of the amino acid preceeding S-methylcysteine may (1) either be secured as such or (2) it may be converted to a carboxamido group.

(1). Glycylalanyl-S-methyl-cysteinylphenylalanine (H-Gly-Ala-Cys(Me)-Phe-OH) reacts with 100 equiv. of cyanogen bromide (50% trifluoroacetic acid; 35°; 48 h) to give in 65% yield (Figure 4) glycylalanine and

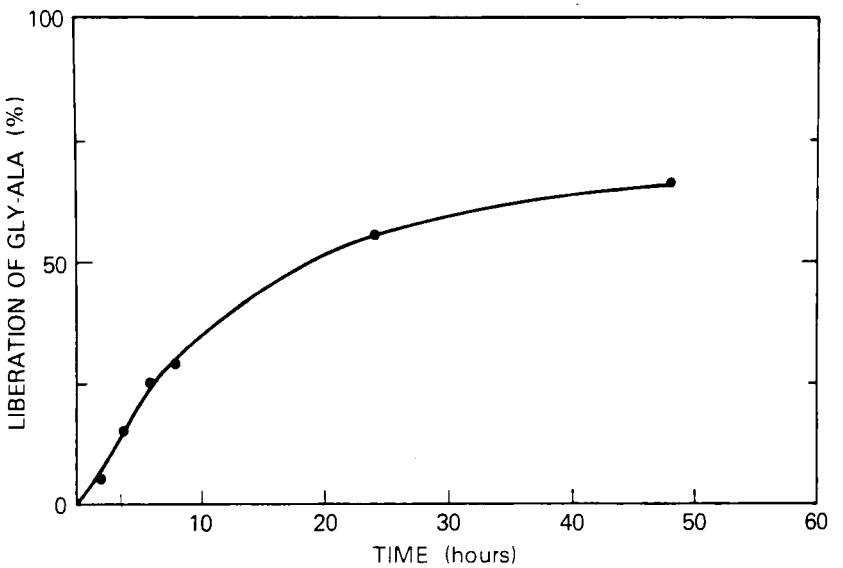


Figure 4: Treatment of glycylalanyl-S-methylcysteinyl-phenylalanine with cyanogen bromide (50% trifluoroacetic acid; 35°).

serylphenylalanine (cf. Figure 5 for the ion exchange chromatography of the reaction products). Execution of the same reaction in dilute acid and at 0-5°C sets the stage for the O → N-acyl shift in the first reaction product, O-(glycylalanyl)-serylphenylalanine, and the formation of a peptide now containing serine in place of S-methylcysteine (glycylalanylserylphenylalanine).

(2). Oxidation of S-methylcysteine to the sulfone, followed by β-elimination and cleavage of the resulting *dehydroalanine* under acidic conditions gives glyclyalanine amide and pyruvylphenylalanine.

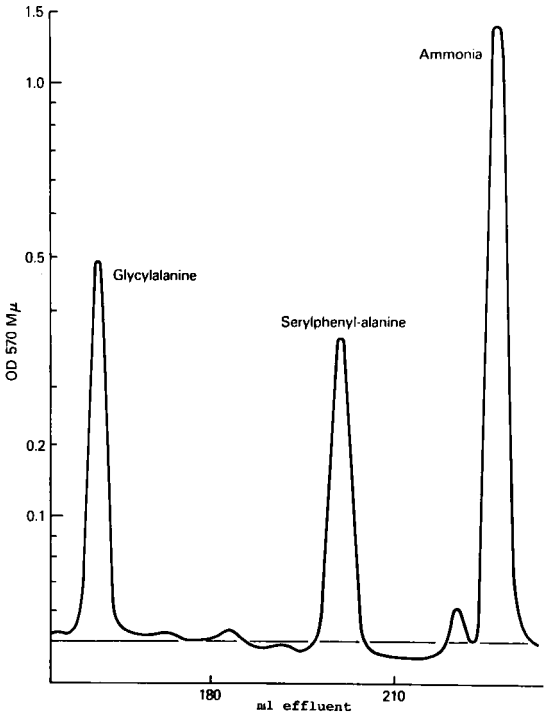


Figure 5: Ion exchange chromatography of the products of the reaction of glycylalanyl-S-methylcysteinylphenylalanine with cyanogen bromide.

The limited number of examples given here for applications of α,β-unsaturated and thioether amino acids demonstrates their utility in peptide synthesis. The broader use of both classes of compounds as protecting groups and vehicles of attachment in solid phase synthesis continues to be the subject of further explorations. The semisynthesis of peptides and proteins is likely to benefit from the selective reactions and mild conditions under which compounds with the α,β-unsaturation and thioethers are applicable.

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12. Tlc (silica gel) solvent systems: A, chloroform: methanol:acetic acid = 85:10:5; B, n-butanol:acetic acid:water = 4:1:1; C, n-butanol:acetic acid: pyridine:water = 15:3:10:12; D, n-butanol:acetic acid:pyridine:water = 4:1:1:2; E, chloroform: methanol = 1:1. Optical rotations were determined in a Perkin-Elmer 141 polarimeter.

AN APPROACH TO THE SYNTHESIS OF DEHYDROPEPTIDES

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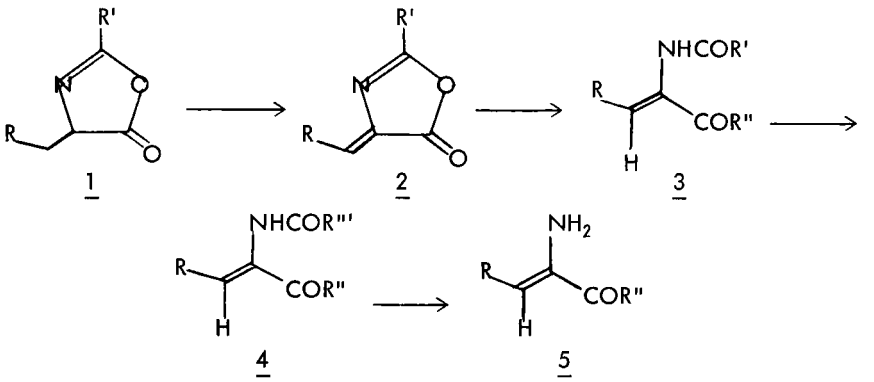
IN RECENT WORK toward the development of a synthetic route to α,β-dehydro amino acids (DHAA) and dehydropeptides (DHP) we had occasion to investigate some azlactone chemistry. If we envision a route to DHP using DHAA as intermediates, a possible sequence is shown in Scheme I. An

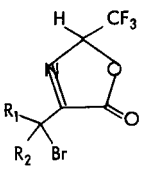


Scheme I.

amino acid is converted into an azlactone (1) which is oxidized to the unsaturated azlactone (2) and 2 is condensed with another amino acid derivative giving 3 (R" = amino acid moiety). Removal of the acyl group from 3 by non-hydrolytic means is mandatory since the enamine 4 is

extremely susceptible to hydrolysis. The coupling of 4 with an activated amino acid derivative then completes the DHP (5) synthesis. In order for this approach to be feasible, R' must be so constituted as to allow azlactone formation (R' ≠ alkoxy group), facilitate non-hydrolytic removal of the acyl function, and be compatible with the 1⟶2 oxidation. We have recently described a method[1] for the introduction of the double bond (1⟶2) using R' = 1-methylstryryl, but non-hydrolytic removal of the resulting α-methylcinnamoyl group from 3 was not possible. This report describes the use of R' = CF_3 and the removal of the trifluoroacetyl function from 3.

Some years ago, Weygand and coworkers reported[2] that amino acids could be converted directly into 2-trifluoromethyl pseudoazlactones and that these could be readily brominated giving 6. We found that 6 were readily dehydrobrominated (Et_3N/Et_2O) to form the unsaturated azlactones (7) (See Table I) and that these would react *in situ* with



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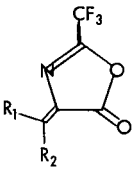

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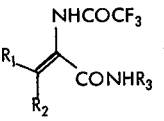

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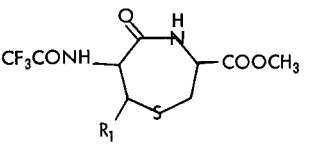

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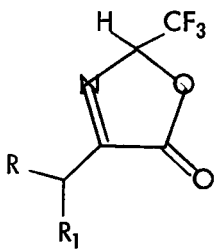
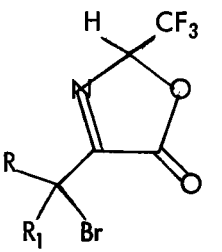
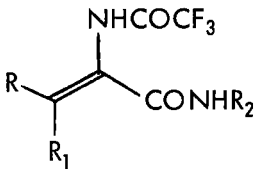
Table I

Amino Acids from which Pseudo Azlactones Were Prepared

	
Phe, Val, Leu Ile, Ala, Abu	Phe, Val, Leu Ile, Ala, Abu

various amino acid esters giving N-trifluoroacetyl dehydropeptide esters (8). (See Table II). The formation of 7 was uneventful except in the cases of alanine and butyrine

Table II
TFA Dehydro Peptides



TFA-DHPhe-Phe·OCH_3

TFA-DHPhe-Leu·OCH_3

TFA-DHVal-Phe·OCH_3

TFA-DHVal-Leu·OCH_3

TFA-DHLeu-Phe·OCH_3

TFA-DHLeu-Leu·OCH_3

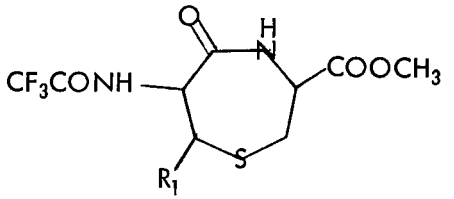
TFA-DHPhe-Gly-NH_2

TFA-DHPhe-Gly-Gly·OEt

when the bromo compounds (6) had to be fractionally distilled in order to remove a dibromo contaminant. In most cases 7 was not isolated but was converted *in situ* into the peptide (8). In every case where R_2 = H, only one double bond isomer was obtained, presumably of the Z-configuration[1].

Interestingly, when 7, (R_2 = H) was treated with cysteine methyl ester, thiazepinones (9) were obtained, but if R_1 and R_2 were both alkyl groups only cysteine peptides 8 (R_3 = Cys residue) were obtained (See Table III). A strong steric hindrance to ring closure was obviously in effect.

Table III
1,4-Thiazepinones



from

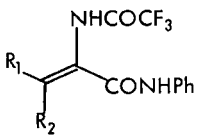
TFA-DHPhe-Cys·OMe

TFA-DHLeu-Cys·OMe

TFA-DHAla-Cys·OMe

TFA-DHAbu-Cys·OMe

When 7 was treated with aniline the expected anilides (10) were obtained. The anilide of dehydrophenylalanine was chosen to study the removal of the trifluoroacetyl



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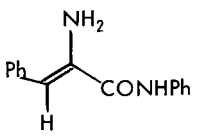

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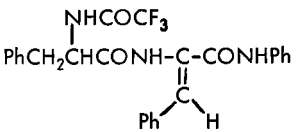
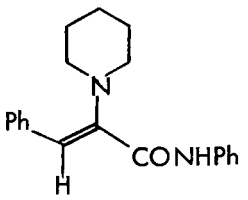


12

(TFA) group. We found that most of the basic reagents tried did not react with 10 (R = Ph, R_1 = H) but NH_3/THF smoothly removed the TFA group giving the enamine (11) in 74% yield. The structure of 11 was secured by its hydrolysis to phenylpyruvanilide and its benzoylation to give N-benzoyl dehydrophenylalanine anilide, which we prepared from N-benzoyl DHPhe. The enamine structure (11) was confirmed by the presence of a vinyl proton at δ 6.12 and an exchangeable two-proton singlet (NH_2) at δ 5.06 ppm in its pmr spectrum. There was no spectral evidence for the presence of even small amounts of the alternative imine structure. The enamine (11) was surprisingly difficult to hydrolyze (0.1 N HCl for 24 hr) and to acylate.

Indeed, we were unable to couple it with benzoic acid using dicyclohexylcarbodiimide or a standard mixed anhydride procedure. We did prepare N-trifluoroacetylphenylalanyldehydrophenylalanine anilide (12) in 44% yield by treatment of 11 with N-trifluoroacetylphenylalanyl chloride. The reduced nucleophilicity of the enamine nitrogen atom was particularly severe in this case since the enamine nitrogen was conjugated with the benzene ring. The NH_3/THF reagent was also used successfully to deblock two dehydrophenylalanine peptides, DHPhe-Gly-NH_2 and DHPhe-Gly-Gly-OEt, to the enamino compounds in 76% and 69% yields, respectively.

Surprisingly, when ammonia was bubbled through a solution of N-trifluoroacetylphenylalanine anilide, no reaction occurred in 15 hr. An explanation for the fact that the dehydro compound 10 (R_1 = Ph, R_2 = H) reacted with ammonia, while its saturated analog did not, became apparent when we found that 10 (R_1 = Ph, R_2 = H) also reacted with piperidine and N-methylpiperazine to form 13 and 14, respectively. These amines had displaced the trifluoroacetyl group



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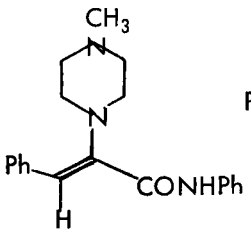

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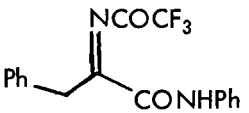

15

in toto. We might speculate that, under the basic conditions of these experiments, 10 is equilibrated with its imine form (15) which then adds the amine (or NH_3) and eliminates trifluoroacetamide to form the enamines 11, 13, and 14.

Acknowledgment

We would like to thank the Office of the Vice President for Research of the University of Georgia for its generous support of this work.

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APPLICATION OF A NEW REPETITIVE METHOD TO THE SYNTHESIS OF A PENTAPEPTIDE SEQUENCE FROM ANGIOTENSIN II

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SYNTHESIS OF A PROTECTED PENTAPEPTIDE from angiotensin II has been effected in solution by a new repetitive method. The carboxyl terminal residue is esterified to a bulky protecting group which permits the growing peptide, after addition of each residue, to be purified by chromatography as the free amine on Sephadex LH-20.

Peptides prepared by nonclassical methods are purified at intermediate stages by means of a "handle" which other components of the synthetic procedure lack. Usually this is a polystyrene resin which distinguishes the growing peptide from side products by rendering it insoluble.[1] Research with facilitated methods[2] has, however, demonstrated the importance of a chemically homogeneous handle when stepwise techniques are employed. The intermediate peptides are distinct chemical entities and may be characterized by standard analytical methods. This repeated analysis represents the best proof of the homogeneity of the final product.

Because of high yields and the gentle conditions employed, gel filtration is particularly suitable for the purification of a growing peptide at each stage of a stepwise synthesis. Use of this technique for the purification of protected peptides has been reported,[3] but lack of a suitable carrier has limited application. The homogeneous handle reported here overcomes some of the problems of soluble heterogeneous carriers[4] and confers the

additional advantages of crystallizability and resistance to diketopiperazine (DKP) formation on the peptide.

Protected amino acids are added in a stepwise fashion to a growing peptide chain esterified to cholestan-3β-ol. The bulky carboxyl protecting group (Figure 1) causes the

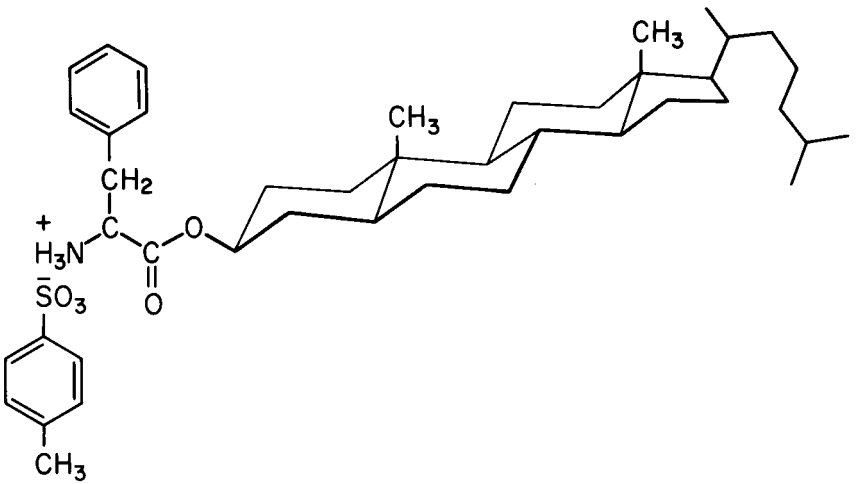
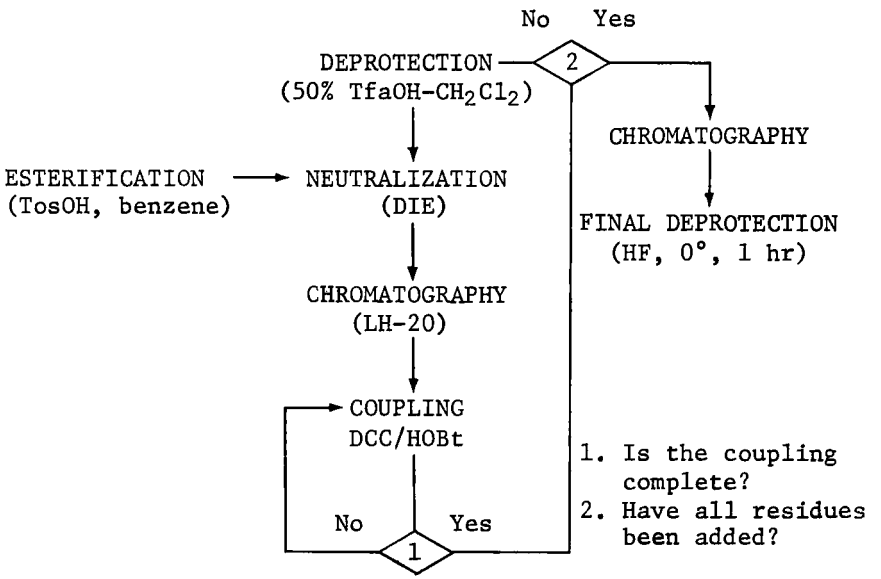


Figure 1: Phenylalanine cholestanyl ester toluene sulfonic acid salt.

desired peptide free amine to elute before other components of the reaction mixture when chromatographed on Sephadex LH-20 in dimethylformamide (DMF). Evaporation yields the peptide cholestanyl ester (OCh) as the free amine for subsequent elongation by repetition of the synthetic cycle, as outlined in the following scheme. Each step is discussed within the scheme.

Esterification

The synthesis of Boc-Tyr(Bzl)-Val-His(Tos)-Pro-Phe-OCh was initiated by esterification of the C-terminal phenylalanine residue to commercially available cholestanol (5β-dihydrocholesterol). The method outlined for benzyl esters[5] is modified to permit synthesis on a reduced scale by using a soxhlet apparatus packed with indicating Drierite. Water generated by the esterification is



separated by azeotropic distillation and is absorbed by the desiccant. Esterification is complete within a few hours. Phenylalanine cholestanyl ester toluene sulfonic acid salt (TosOH x H-Phe-OCh) precipitates on cooling and is crystallized from 1-propanol. Yield is 43%. R_F (1-butanol: 3% NH_3::3:1) 0.76, R_F ($CHCl_3$:CH_3OH::9:1) 0.69; m.p. 229 - 232° . $[\alpha]_D^{23°}$ + 17.6° (0.38%, $CHCl_3$) *analyzed* for $C_{43}H_{65}NO_5S$: C, 72.9; H, 9.3; N, 2.0. Found: C, 72.8: H, 9.3; N, 1.9%.

Amino acid cholestanyl ester hydrochlorides have been synthesized by passing HCl gas through a semimolten mixture of cholestanol and the amino acid.[6]

Neutralization

The peptide ester is suspended in a minimum quantity of DMF-1 *M* in toluene and neutralized with diisopropylethylamine (DIE). Aliquots applied to moist pH paper are used to estimate the neutralization end point. The salts which toluenesulfonic acid, hydrogen chloride, and trifluoroacetic acid form with DIE are more soluble in DMF than those formed with Et_3N. Phenylalanine cholestanyl ester is soluble at a concentration of at least 0.1 *M* in the chromatographic solvent. Both interchain and intrachain (DKP) aminolyses of the cholestanyl esters are slow

as judged by the appearance of cholestanol in the neutralization mixture. Less than 1% of neutralized H-Pro-Phe-OCh is cleaved in 24 hours. H-Phe-OCh is stable for at least one week.

Chromatography

The sample (less than 5% of the column volume) is applied to LH-20 Sephadex in DMF-1 *M* toluene. Toluene suppresses tailing of the cholestanyl esters. The column (1.2 X 95 cm) is pumped at 50 ml/hr, and the desired amino acid cholestanyl ester elutes within 90 minutes. Elution volumes are given in Table I. Composition of the

Table I

Data from the Preparation of the C-Terminal Pentapeptide of Angiotensin II

	Yield %	*Elution Volume (max), ml*	*Amino Acid Analysis*
H-Phe-OCh	98	71	
H-Pro-Phe-OCh	93	67	Phe,1.00; Pro,1.00
H-His(Tos)-Pro-Phe-OCh	99	59	Phe,0.96; Pro,1.07; His,0.96
H-Ile-His(Tos)-Pro-Phe-OCh	94	52	Phe,1.08; Pro,1.08; His,0.95; Ile,0.88
H-Tyr(Bzl)-Ile-His(Tos)-Pro-Phe-OCh	--	52	Phe,0.91; Pro, 1.05; His,1.04; Ile,0.83; Tyr,0.98

column effluent is determined by thin layer chromatography, amino acid analysis, radioactivity, and differential refractometry. Identification is made by tlc and radioactivity. Ninhydrin reagent indicates which fractions contain free amine; subsequent development with H_2SO_4:CH_3OH::1:1 at 110° for 15 minutes[7] chars spots containing cholestanol to

confirm presence of the peptidyl-steroid. Yield from the chromatography varied between 95-98%. Elution volume for the peptide decreased in proportion to molecular weight as reported previously.[8]

Coupling

Residues are added to the growing peptide chain using standard DCC/HOBt couplings. A small aliquot of the peptidyl-steroid free amine taken directly from the column eluant serves as the standard with which to judge completeness of coupling by tlc.

Deprotection

On completion of the coupling the reaction mixture is filtered, evaporated, and the residue dissolved in 50% (v/v) trifluoroacetic acid-dichloromethane for 30 minutes at room temperature to remove the t-butoxycarbonyl protecting group. Under these conditions the cholestanol ester bond is stable for at least 24 hours. Evaporation of the reaction mixture yields the peptidyl ester trifluoroacetate. Completeness of deprotection is judged by tlc.

Final Deprotection

After repetition of the synthetic cycle until a peptide of the desired length is produced, cleavage of the cholestanyl ester is effected by treatment for one hour at 0° with HF. Conditions are similar to those developed for removal of the related isopropyl esters.[8]

Application of the above method to the synthesis of the protected C-terminal sequence from angiotensin II has been completed. Use of the technique to prepare larger, biologically active peptides is in progress.

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THE MECHANISM OF THE CARBODIIMIDE REACTION. IV. NEW PROBES FOR THE STUDY OF ACYLATION REACTIONS

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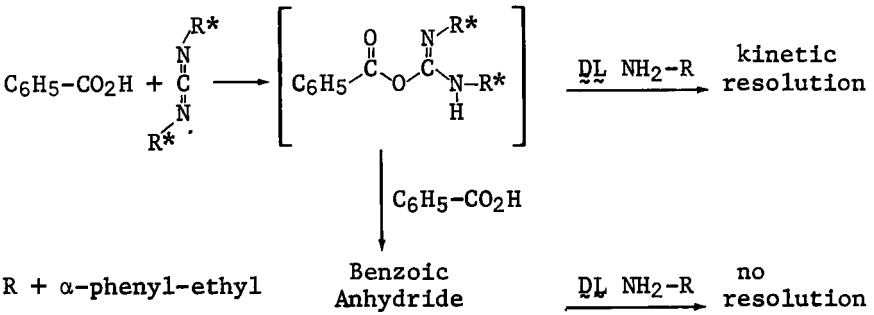
Department of Chemistry, University of California
Los Angeles, California 90024

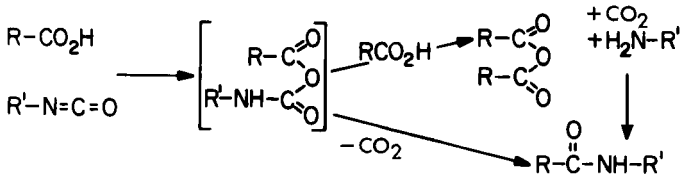
DURING OUR CONTINUED STUDY of the mechanism of the carbodiimide reaction[1] we have developed two new probes for detecting intermediates under the conditions of amide synthesis. The first of these involves the use of optically active dehydrating agents and may be used to distinguish between symmetrical anhydrides and other carboxyl-activated species. Application of this method is shown in Scheme 1 for the carbodiimide reaction. An achiral carboxylic acid, racemic amine and a limiting amount of optically active carbodiimide are allowed to react; kinetic resolution of the amine can occur only if the actual acylating agent is optically active (*i.e.*, the O-benzoyl-isourea).



Scheme 1.

The actual acylating agent was found to be dependent on the reaction conditions. Using excess acid, as prescribed for routine solid phase synthesis, no resolution occurred indicating that the anhydride was the acylating agent.[2] When the acid was added slowly to the amine and carbodiimide (a procedure recommended for solid phase couplings in which diketopiperazine formation is a side reaction[3]) kinetic resolution occurred, giving benzamide of 60% optical purity. The O-benzoyl-isourea reacts more rapidly with the amine of the same absolute configuration with a selectivity of at least 4 to 1. When acid and amine were present in equal concentration, benzamide of 25% optical purity was obtained revealing the presence of both pathways. Using the selectivity factor (4:1), 60% of this final reaction proceeds *via* the symmetrical anhydride.

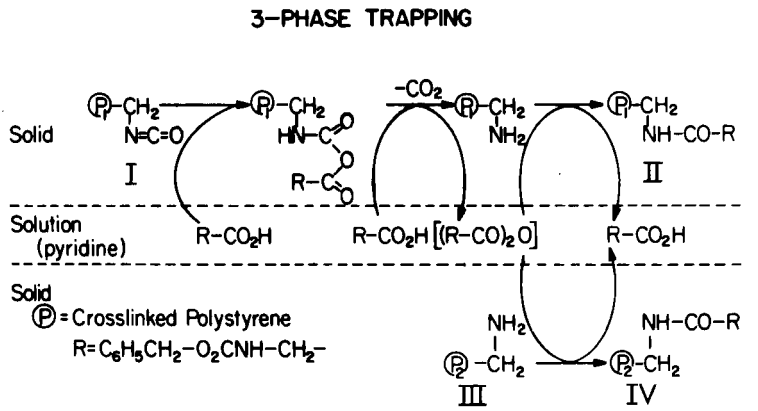
The second probe uses polymer-bound reagents to trap symmetrical anhydrides when they are present in peptide coupling reactions. For the specific case of the reaction of acids with isocyanates[4] (Scheme 2), the product could



Scheme 2.

arise from the initially-formed mixed anhydride by either intramolecular rearrangement or by disproportionation to the symmetrical anhydride and amine.

The use of a polymer-bound isocyanate permits the selective trapping of the anhydride by a second solid phase reagent. Thus, when the polymer-bound isocyanate (I, Scheme 3) was treated with a solution of Z-Gly-OH in warm pyridine, smooth acylation resulted giving the glycine derivative II. However, when this reaction was performed in the presence of the polymer-bound amine, III, 80% of the acylation occurred on this second polymer to give IV.[5]



Scheme 3.

The acylation, III→IV, requires the existence of an acylating agent which is free and in solution. Therefore, the symmetrical anhydride (or the acylpyridinium salt) must be the major acylating agent duirng the reaction of acids with isocyanates. The alternative pathway involving intramolecular rearrangement of the mixed carboxylic-carbamic anhydride can occur to only a minor extent, if at all.

Acknowledgment

This research was supported financially by the National Institutes of Health (GM 22044).

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5. Resins of differing mesh size were used and separated after reaction by screening.

PREFERENTIAL ACYLATION OF 'NON-HINDERED' PEPTIDE BONDS. ACYLATION AND METHANOLYSIS AS A POSSIBLE MEANS OF PRODUCING FRAGMENT PEPTIDES

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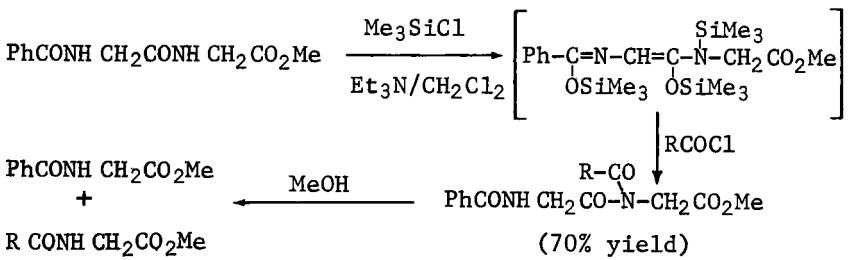
IN RECENT STUDIES[1] on the *N*-acylation of amide bonds using trimethylsilylation followed by acylation, we have found that *N*-acylated amides undergo cleavage in methanol to give *N*-acylated amino-acid esters as shown by the sequence:



(+ starting material)

This information provided us with the opportunity of investigating the potential of the method as a means of interchanging peptide fragments through the *N*-acylation of a specific peptide bond (*i.e.* in the above example the RCO- component can be seen as having replaced the original N-terminal acyl fragment). The eventual success of the method of course depends on, (a) achieving *N*-acylation at specific amide bonds, and (b) developing an efficient means of separating the products. The present paper summarises

our progress, using model systems, in assessing the specificity of the reaction as required by condition (a). Table II summarises the effect of side chains on the yield of *N*-acylated product from the reaction [1].

$$PhCH_2CH_2CONHCHCONEt_2 \xrightarrow[(ii)ZNH(CH_2)_2COCl]{(i)Me_3SiCl/Et_3N} PhCH_2CH_2CON(ZNH(CH_2)_2CO\,R)CHCONEt_2 \quad [1]$$

Table I

	R = H	*Me*	*$PhCH_2$*	*Me_2CH*
Silylation	100	90	50	20
Acylation (Isolated % yield)	60	40	0	0

The *N*-acylated compounds are fairly labile but can be purified by chromatography through a short column of Kieselgel G (tlc grade) using methylene chloride/ethyl acetate for elution.

Repeating the same sequence of reactions with compounds of the general structure (II) provided us with information concerning selectivity on an amide group at each

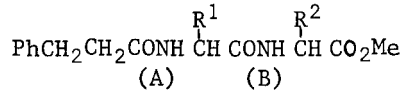
$$PhCH_2CH_2CONH\underset{(A)}{CH}\underset{(B)}{CONHEt}$$

(II)

side of the side chain group with R = H, Me, $PhCH_2$ or Me_2CH. Only when R = H was there any significant amount of N-acylation detected. In this glycine derivative 50% *N*-acylation occurred at amide (A) together with 15% of the diacylated product. The only other compound obtained in an isolatable amount (10%) was the product of *N*-acylation at amide (B) when R = $PhCH_2$. Proof of the structure of the N-acylated products has been obtained from the analysis of the products after methanolysis and from nmr spectroscopy. On *N*-acylation protons attached to the carbon next to the carbonyl group of the amide are deshielded ($\Delta\tau \approx 0.4$) while the protons on carbon attached to the nitrogen are similarly deshielded and change their multiplicity.

Similar analyses have been carried out on *N*-acyl products resulting from acylation in the dipeptide series (III) where R^1 and R^2 were chosen with various combinations of

of the side chains, H, Me, Me_2CH and $PhCH_2$. When R^1 and R^2 were both bulky groups no acylation occurs. However when R^1 = H and R^2 = $PhCH_2$ or Me_2CH the yields of *N*-acylated product at amide (A) were 50 and 60% respectively. (About 10% acylation at amide (A) occurred when R^1 = Me



(III)

and R^2 = $PhCH_2$ or Me_2CH). It appears therefore that *N*-acylation, via trimethylsilylation and acylation, can be readily achieved at 'non-hindered' peptide bonds.

Mass spectrometric and high pressure liquid chromatographic analyses of the methanolysis products of *N*-acylated amide derivatives (produced by silylation/acylation) of the tripeptides, Ac-Val-Gly-Gly-OMe, Ac-Phe-Gly-Gly-OMe and Z-Ile-Gly-Gly-OMe confirm that *N*-acylation takes place readily at the Gly-Gly bond.

Acknowledgment

We gratefully acknowledge financial support from the Science Research Council.

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SYNTHESIS OF SERINE-CONTAINING GLYCOTRIPEPTIDES*

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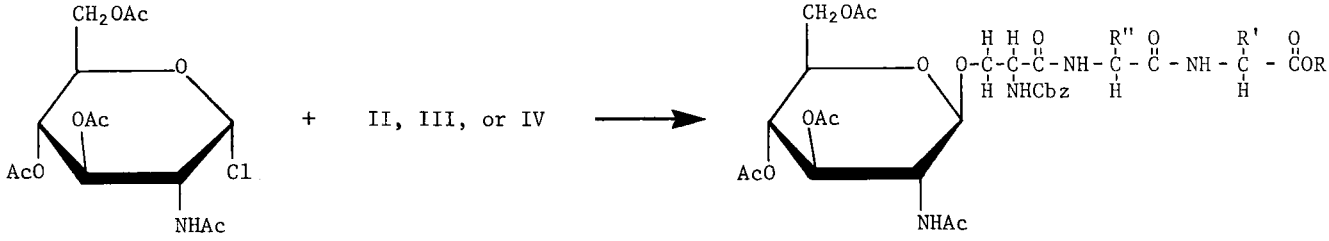
SYNTHETIC MODEL COMPOUNDS CONTAINING a 2-acetamido-2-deoxy-*D*-glucose residue linked to the hydroxyl group of serine are of great interest for the study of the base-catalyzed β-elimination of the carbohydrate chains of some glycoproteins of animal origin (*e.g.* submaxillary mucins, blood-group substances) and proteoglycans.[1] In addition, these derivatives may provide information on the mechanism of glycosylation of glycoproteins that contain a 2-acetamido-2-deoxy-*D*-glucose residue linked to the Asn residue, since the sequence Asn-X-Ser (Thr) is required.

We herewith report the synthesis of [*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-β-*D*-glycopyranosyl)-*N*-(benzyloxycarbonyl)-*L*-seryl]-glycyl-*L*-alanine methyl ester (V), [*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-β-*D*-glucopyranosyl)-*N*-(benzyloxycarbonyl)-*L*-seryl]-*L*-alanyl-glycine ethyl ester (VI), and [*O*-(2- acetamido-3,4,6-tri-*O*-acetyl-β-*D*-glucopyranosyl)-*N*-(benzyloxycarbonyl)-*L*-seryl]-*L*-alanyl-*L*-alanine methyl ester (VII). As shown in Figure 1, the synthesis of glycopeptides (V-VII) was achieved essentially by Koenigs-Knorr condensation of 2-acetamido-3,4,6-tri-*O*-acetyl-α-*D*-glucopyranosyl chloride[2] (I) with *N*-(benzyloxycarbonyl)-*L*-seryl dipeptide esters (II-IV) in the presence of mercuric

* Amino Sugars IC. This is publication No. 667 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School and the Massachusetts General Hospital, Boston, MA. 02114.



I

V R = Me, R' = Me, R'' = H

VI R = Et, R' = H, R'' = Me

VII R = Me, R' = Me, R'' = Me

Figure 1: [*O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-β-*D*-glucopyranosyl)-*N*-benzyloxycarbonyl) seryl] dipeptide esters.

cyanide.[3] The schemes of synthesis of *N*-(benzyloxycarbonyl)-*L*-seryl-glycyl-*L*-alanine methyl ester (II), *N*-(benzyloxycarbonyl)-*L*-seryl-*L*-alanyl-glycine ethyl ester (III), and *N*-(benzyloxycarbonyl)-*L*-alanyl-*L*-alanine methyl ester (IV) are shown in Figure 2 and Figure 3. The physical properties of the fully protected peptides and glycopeptides are given in Table I.

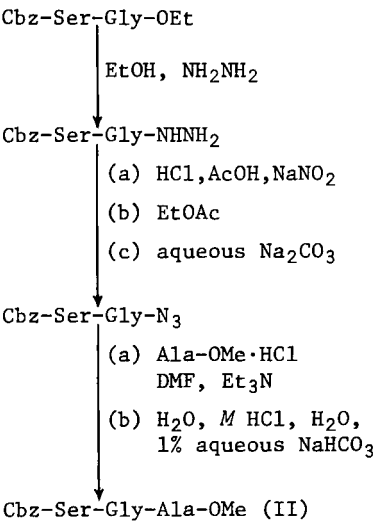


Figure 2: Synthesis of Cbz-Ser-Gly-Ala-OMe (II).

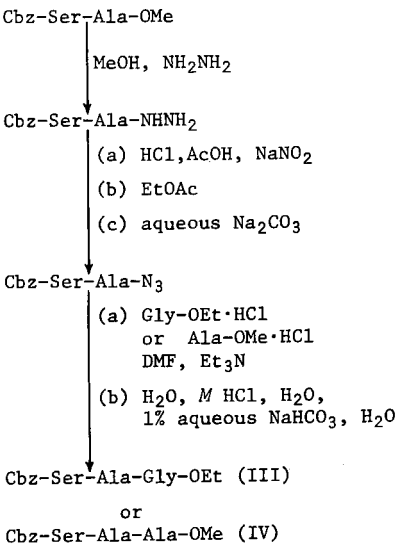


Figure 3: Synthesis of Cbz-Ser-Ala-Gly-OEt (III) or Cbz-Ser-Ala-Ala-OMe (IV).

Table I

Physical Characteristics of the Protected Peptides and Glycopeptides*

Compound		*Melting Point* § *(° Solvent)*	*tlc*† *Rf*	$[\alpha]_D^{20}$‡
Cbz-Ser-Gly-Ala-OMe	II	118-120 ($EtOAc-Et_2O$)	0.29	+4.6° (*c* 2.3, $CHCl_3$)
Cbz-Ser-Ala-Gly-OEt	III	142-144 (EtOAc)	0.35	-29.0° (*c* 0.9, $CHCl_3$)
Cbz-Ser-Ala-Ala-OMe	IV	172-175 (EtOAc)	0.33	-17.0° (*c* 2.3, $CHCl_3$)
Cbz-Ser(OGlcNAc)-Gly-Ala-OMe	V	193-194 (EtOH)	0.33	-6.9° (*c* 1.9, $CHCl_3$)
Cbz-Ser(OGlcNAc)-Ala-Gly-OEt	VI	195.5-196 (EtOH)	0.50	-13.2° (*c* 1.5, $CHCl_3$)
Cbz-Ser(OGlcNAc)-Ala-Ala-OMe	VII	213-214 (dec) (EtOH)	0.45	-16.0° (*c* 2.2, $CHCl_3$)

* The microanalyses were performed by Dr. W. Manser, Zürich, Switzerland. All compounds reported gave satisfactory elemental and amino acid analyses. After hydrolysis with 5.8*M* HCl for 24 hr at 108°, followed by evaporation, the residue was heated with 3*M* HCl in BuOH (5 ml) for 1 hr at 100°, followed by treatment with a 25% solution of trifluoroacetic anhydride in dichloromethane (0.1 ml) for 1 hr at 100°. Glc of these *N*-trifluoroacetyl butyl esters was performed on a column of Tabsorb (Regis Chemical Co., Chicago, Ill. 60610) programmed for a rise of 4° per min from 75° to 225° to determine the composition of amino acids.

§ Melting points were determined with a Mettler FP-2 apparatus and correspond to "corrected melting points".

† The homogeneity of all the compounds was verified by tlc on precoated plates of Silica Gel (Merck); the solvent used was chloroform-methanol, 14:1 (v/v).

‡ Rotations were determined for solutions in 1-dm semimicro tubes with a Perkin-Elmer No. 141 polarimeter.

Acknowledgment

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LINEAR AND CROSS-LINKED POLYMERS AS CHEMICAL REAGENTS

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WHILE THE DEVELOPMENT of solid-state peptide synthesis by Merrifield has revolutionized the possibilities of synthesizing peptides with long sequences of amino acid residues, the fact remains that the polymeric support which was originally selected merely because of its easy commercial availability is still most widely used for this purpose. There is no reason to expect cross-linked polystyrene to be particularly suitable for this specific application and in a recent review of the field Marshall and Merrifield[1] complain that "the role of the physical nature of the support and its effects on the synthetic process have been sadly neglected". This paper is an initial attempt to define some of the general problems encountered in syntheses using polymeric supports from the point of view of the physical polymer chemist.

Reactivity of Functional Groups Attached to Soluble Polymers

Before considering the special problems posed by the reactivity of a two-phase system composed of a solution and a gel phase, it is appropriate to consider the principles which affect the reactivity of functional groups appended to a soluble flexible polymer chain. Many years ago, Flory[2] formulated the general principle that the reactivity of a functional group should not be affected by its attachment to a polymer of high molecular weight. In particular, it should be emphasized that the viscosity increase, which results from the dissolution of a polymer in a given solvent, should not affect the rates of reactions which require a substantial activation energy. This is so since a large

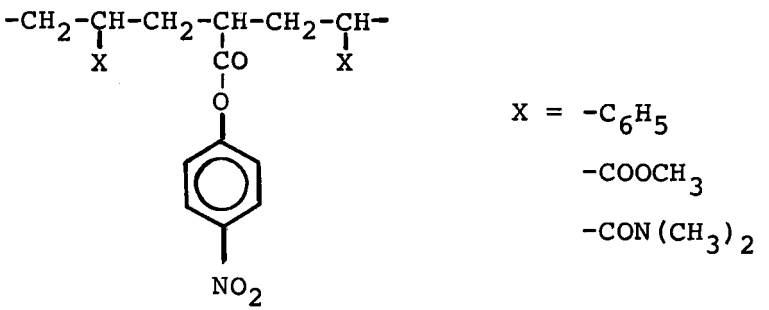
number of collisions is required for the reaction to occur and an increase in the viscosity will reduce equally the diffusion of interacting species towards each other and away from each other, so that the number of "productive" collisions will remain unchanged. However, three factors may modify the application of Flory's principle:

(a) Steric hindrance effects operative in polymeric reagents are often not easily simulated in their low molecular weight analogs.

(b) Forces operating between a polymer and a low molecular weight reagent (involving parts of the polymeric chain which may be quite distant from the reaction site) may either favor or oppose the accumulation of the small reagent in the polymer domain. This may lead to large kinetic effects, since the reaction rate will depend on the "local effective concentration" of the reagent in the neighborhood of the polymer.[3]

(c) If the reaction is sensitive to the properties of the solvent medium, then the difference between the "local effective medium" in the vicinity of the polymer and the pure solvent has to be taken into account. This difference has been demonstrated by the dependence of the photochromism of spyropyran groups on the nature of polymers to which they were attached[4] and also by the change in the *cis-trans* isomerization rates of azobenzene groups when they were appended to polymer chains.[5]

We have studied this effect on the aminolysis of p-nitrophenyl acrylate copolymers with a large excess of styrene, methyl acrylate or N,N-dimethylacrylamide:[6]



Structure I.

The reaction was carried out in dioxane solution at 50° and the apparent second-order rate constant, k_2, was compared with the corresponding rate constant, k_2°, characterizing the aminolysis of p-nitrophenyl acetate. The results, listed in Table I, show that k_2/k_2° is close to

Table I

Ratio of the Rate Constant for the Aminolysis of p-Nitrophenyl Acrylate Copolymer (k_2) and the Aminolysis of p-Nitrophenyl Acetate (k_2°)

(Dioxane Solution, 50°)

Comonomer	k_2/k_2°			
	n-Butylamine	*n-Decylamine*	*Benzylamine*	*Ethanolamine*
Styrene	0.0056	0.0055	0.0036	0.0074
Methyl Acrylate	0.047	0.058	0.046	0.069
N,N-Dimethyl-acrylamide	0.96	0.89	0.66	0.97

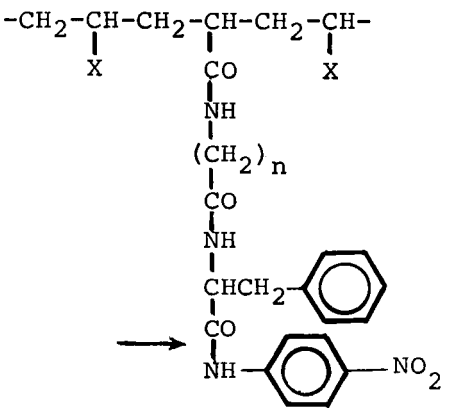
unity for the N,N-dimethylacrylamide copolymer, but drops off by one and two orders of magnitude in copolymers of methyl acrylate and of styrene, respectively. It is striking that k_2/k_2° is virtually independent of the nature of the amine reagent, from which it amy be inferred that energetic interactions between the various copolymers and the amines are not the cause of the large difference in the copolymer reactivities. These reflect the properties of the local medium and, in the case of the N,N-dimethylacrylamide copolymer, the catalytic action of the dimethylamide group as demonstrated by Su and Watson.[7] It is of interest, in this context, that the "effective local concentration" of a group attached to every residue of a vinyl polymer has been shown to be in the range of 2M-4M.[8]

The above study is of interest because of the use of the aminolysis of active esters in polypeptide synthesis and also because of the development by Bayer and Mutter[9-10] of procedures in which a soluble polymeric carrier was employed. Recently, Bayer *et al.*[11] studied the reaction of Boc-glycine p-nitrophenyl ester with glycine esters of

low molecular weight alcohols and of the terminal hydroxyls of poly(ethylene oxide) of high molecular weight in acetonitrile solution. It was found that glycine attached to the high polymer was about 60% more reactive than the low molecular weight analog $CH_3OCH_2CH_2OCOCH_2NH_2$. I believe that this result reflects the favorable "effective solvent medium" in the polymer domain.

Enzymatic Attack on Side Chains of Linear Polymers

We have also studied[12] the chymotrypsin-catalyzed hydrolysis of a specific substrate group carried in the side chains of an acrylic acid or acrylamide copolymer:



X = $-CONH_2$

$-COOH$

n = 1,3,5

Structure II.

It was found that the acrylamide copolymer in which ε-amino-caproic acid (n = 5) was used as a spacer between the chain backbone and the phenylalanyl derivative had a susceptibility to enzymatic attack on the p-nitroanilide group quite similar to that of an analogous small molecule. When the acrylic acid polymer was the substrate, the pH-activity curve was shifted to more acid media, where the electrostatic attraction of the cationic enzyme and the polymeric acid contributes to the stability of the enzyme-substrate complex. Shortening of the side chain from n = 5 to n = 3 and n = 1 led to a twenty-fold reduction of the reaction rate, although even with n = 1 the susceptible bond is separated by seven bonds from the chain backbone. This behavior, indicative of the

substantial steric requirements of the enzyme, is analogous to the requirement of an "anchoring arm" for the attachment of a substrate analog to a resin in affinity chromatography.[13]

Effect of Network Topology on the Reactivity of Groups Attached to a Cross-Linked Polymer

At any given cross-link density, a polymer network may exhibit a variety of topological structures. Consider, for instance, a mixture of a vinyl monomer and a cross-linking agent which is polymerized in the absence of a diluent. The cross-linked gel may now be expanded in a given solvent to an equilibrium swelling volume in which the cross-links are distributed, more or less, at random. On the other hand, if the monomer is diluted with an inert solvent and polymerized with a high concentration of the cross-linking agent, we arrive at a rigid polymer gel which has only a limited ability to swell further. Moreover, the swelling is now very insensitive to the polymer-solvent interaction, in contrast with the randomly cross-linked structure, which expands strongly in good solvents and contracts in poorly solvating media.[14]

We have investigated, on a model system, the importance of the topological structure of a cross-linked network on the reactivity of groups attached to it.[15] Columns packed with cross-linked acrylamide copolymer beads containing 10% by weight of 4-acrylamidopyridine residues were evaluated as to their efficiency to catalyze the hydrolysis of p-nitrophenyl acetate (NPA) or butyrate (NPB). A "catalytic efficiency" was defined as the ratio of rate constants observed in the column and in a homogeneous solution with an equivalent concentration of 4-acetamidopyridine. The results, listed in Table II, lead to the following conclusions:

(a) When the monomer dilution ratio (MDR) during polymerization is held at 1:1, the catalytic efficiency first increases and then declines as the swelling of the resin is increased by reducing the cross-link density (CD).

(b) The catalytic efficiency is higher with NPB than with NPA. This reflects the more favorable partitioning of the less polar ester between the solution and the gel phase. Characteristically, this efficiency is reduced when 10% methanol is added to the water(with the single exception of PNB with resin batch #81, which is not considered significant).

Table II

Swelling Ratio and Catalytic Efficiency of Polymer Beads as a Function of the Degree of Cross-linking (DC) and the Monomer Dilution Ratio (MDR) during Polymerization

Batch No.	DC %	MDR	Swelling Ratio H_2O	Swelling Ratio 10%MeOH	Catalytic Efficiency H_2O NPA	Catalytic Efficiency H_2O NPB	Catalytic Efficiency 10% MeOH NPA	Catalytic Efficiency 10% MeOH NPB
74	4	1	5.0	4.0	0.21	0.91	0.19	0.44
75	3	1	5.6	4.3	0.66	2.8	0.50	1.6
71	2	1	7.1	5.3	1.00	4.5	0.23	0.62
72	1	1	12.4	9.6	0.74	1.5	0.22	0.35
73	0.5	1	17.0	15.3	0.33	0.59	0.077	0.14
76	2	2	13.1	8.8	1.8	4.3	0.82	1.5
77	2	3	33	18	1.3	5.1	1.04	3.4
81	2	4	42	23	4.2	6.8	4.1	9.6
79	3	3	11.5	10.7	2.6	7.2	1.7	2.9
80	4	3	10.5	8.8	3.5	5.9	1.2	2.4

(c) When both the MDR and the CD are increased so as to keep the swelling ratio of the gel constant, the catalytic efficiency increases. Compare, *e.g.*, the results for batch #72, #76 and #79, which have water swelling ratios of 12,4. 13.1 and 11.5 and for which the catalytic efficiencies for NPA hydrolysis are 0.74, 1.8 and 2.6. It is striking that the catalytic efficiency reaches values much larger than unity, suggesting that the reaction medium is highly favorable for the reaction.

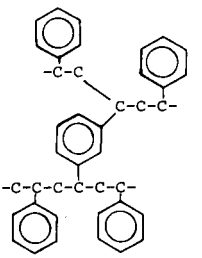
We are at present unable to define the principles which would account for these data. Obviously, several factors are involved. Increased swelling changes the local solvent medium, the gel-solution distribution coefficient and the density of the reactive groups within the gel, which may be important if two of the groups carried by the polymer play a role in the reaction mechanism. The evidence that the topology of the network is an important variable should stimulate further studies of this factor.

Evaluation of Cross-Linked Resins for their Suitability as Supports in Polypeptide Synthesis

It has often been pointed out that the practical utility of the use of cross-linked polymer beads as a support in polypeptide synthesis depends on an unusually high requirement for the completion of each reaction step. The intrinsic rates of the reaction to be carried out are generally adequate and the problem which is encountered is only due to the non-equivalence of the reaction sites.

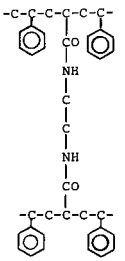
These would be expected to be related to the distance between a reaction site and a cross-link. It is instructive to see what the distance between neighboring cross-links would be in a polystyrene resin containing 2% cross-links if they were regularly distributed in a tetrahedral diamond lattice. Assigning half of the four connecting chains to each cross-link, the "molecular volume" of a cross-link would be about 10,000 ml as against 3.42 ml for diamond. Thus, the distance between cross-linkages would be $(10,000/3.4)^{1/3} \times 1.53$ Å = 21Å. If the resin undergoes eightfold increase in volume on swelling, this distance will be doubled. In actual fact, the structure is of course not regular and some cross-links may be much closer to each other. This may easily lead to significant restraints on chemical reactions.

It would seem that the usual manner of preparing cross-linked polystyrene, in which a mixture of *para* and *meta* divinylbenzene is used as the bifunctional monomer, is rather unfavorable for this particular purpose, since it leads to very tight cross-links, *i.e.*, in the case of the *meta* isomer, structures of the type:



Structure III.

It would seem that use of a bifunctional monomer in which the vinyl groups are well separated from one another would be much preferable. Cross-linking agents such as diacrylyl ethylenediamine, for instance, would form cross-links represented by:



Structure IV.

In any case, it seems desirable to develop a testing procedure by which the chemical equivalence of reactive sites in a cross-linked resin can be characterized. Past efforts to study the course of a reaction on a resin support suffered from the following limitations:

(a) Most of the procedures used for following the progress of the reaction were discontinuous. This is the case, for instance, with the titration techniques developed by Brunfeldt *et al.*[16], by Dorman[17] and by Beyerman *et al.*[18] and the monitoring of the reaction with the use of fluorescamine.[19]

(b) While Gut and Rudinger[20] developed a continuous method for following the release of nitrophenol from its ester by resin-bound amine groups, their procedure was sensitive to the amount of the amine which *had reacted*, rather than the amount which was left *unreacted*. As a result, while the method was valuable in demonstrating deviations from first-order kinetics in the early stages of the reaction, it was unsuitable for the characterization of the final stage, *i.e.*, conversions exceeding 95%. Yet, it is precisely these final stages that we are most interested in.

We are presently engaged in the development of a method designed to overcome both of these limitations. Gabel *et al.*[21] have demonstrated that it is possible to make precise spectroscopic measurements on resin-bound protein by utilizing reflection fluorescence from a packed resin bed*. We are exploring the use of this method for the evaluation of the heterogeneity of reactive sites on a polystyrene resin by attaching to it the carboxyl of p-aminobenzoic acid which produces a fluorescence at 335 nm. This fluorescence is almost completely quenched when the amino group is coupled with an amino acid derivative. We hope that we shall be able to follow the remaining fluorescence as a function of time and determine, up to high conversion, the deviation of the reaction from first-order kinetics when using a large excess of the acylating reagent. The technique should then be exploited by a systematic study of the dependence of such heterogeneities of reaction sites on the manner in which the resin is prepared.

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* I am greatly indebted to Dr. M. Shinitzky of the Weizmann Institute of Science for drawing this technique to my attention.

Acknowledgment

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POLYMERIC REAGENTS IN PEPTIDE SYNTHESIS: RECENT DEVELOPMENTS

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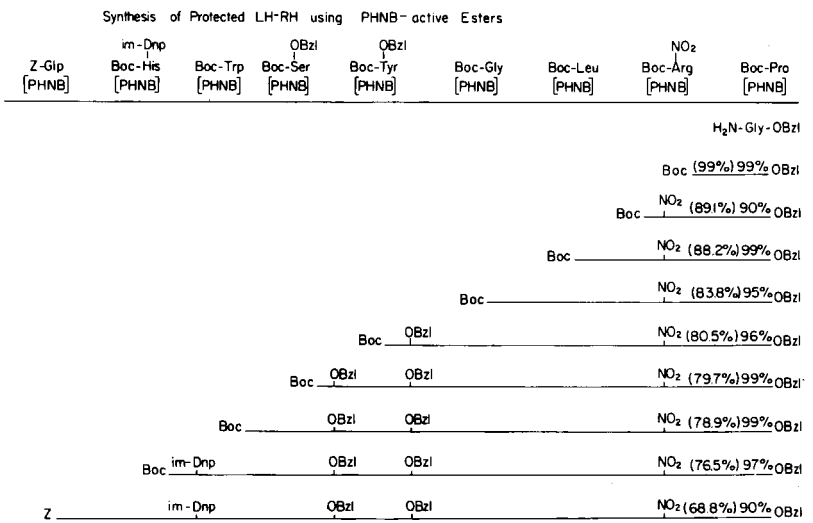
THE USE OF LARGE EXCESS of acylating agents, namely activated N-blocked amino acids, enhances markedly the course and efficiency of peptide synthesis. The excess reagent however, may complicate the ability to purify the desired products.

The polymeric-reagents approach,[1,2] as well as several other techniques, *i.e.*,[3-5] have been designed and developed, to overcome this difficulty. The essence of the former method is the transfer of an N-blocked amino acid residue from an insoluble donor (polymeric active ester) to a soluble acceptor (the free amino terminus of a growing peptide chain) in an acylation reaction. An elongated, soluble, N-blocked peptide is thus obtained. This synthesis may be carried further through selective removal of the N-terminal protecting group and acylation of the newly exposed free amino group by a suitable polymer-activated N-blocked amino acid. Nearly quantitative yields, convenient technical procedures and high purity of products are advantages associated with such syntheses. These features are a result of the ability to use an excess of polymeric active esters and the ready removal of polymer by filtration from the desired products at the end of the reaction.

Several types of polymeric active esters have been prepared and used by us for the successful synthesis of various peptides.[7-9] In view of the challenging demands for the synthesis of more complicated peptides we have reinvestigated the polymers physical and chemical properties using the following criteria: (a) mechanical stability and integrity of reagents during peptide synthesis;

(b) good swelling in solvents commonly employed in such synthesis; (c) accessibility of insoluble activated amino acid moieties to incoming amino components; (d) minimization of the effect of steric factors on efficiency of synthesis; and (e) increased reactivity of the reagents. These studies led to the development of two new series of highly efficient polymeric reagents derived from (4-hydroxy-3-nitro)-benzylated polystyrene (PHNB),[9] and from 1-hydroxybenzotriazole-bound polystyrene (PHBT).

PHNB[9] was prepared by reacting 4-hydroxy-3-nitrobenzyl chloride with commercially available polystyrene in the presence of aluminum trichloride. PHNB-amino acid active esters, obtained by binding of PHNB to various N-blocked amino acid derivatives using dicyclohexylcarbodiimide, were successfully utilized in the synthesis of many relatively simple peptides.[9] A protected derivative of the luteinizing hormone-releasing hormone (LH-RH) was recently prepared using PHNB-esters in an over-all-yield of 68.8% (see scheme I).

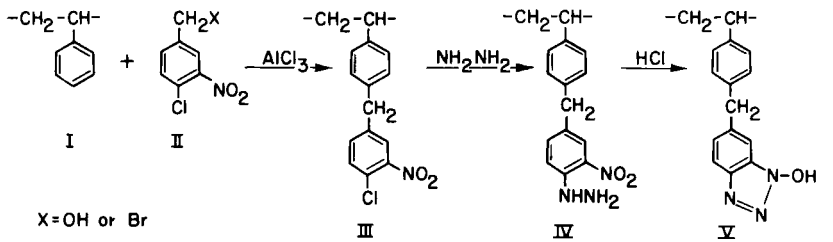


Scheme I.

Our experimental data show that requirements (a) through (c) are successfully met with PHNB-esters. A marked reductions of coupling rates, however, were sometimes observed when bulky amino acids like valine or leucine

were situated at the amino terminus of the growing peptide chain or used as the polymer-activated carboxylic components.[9]

Because of the well-known high reactivity of the 1-hydroxybenzotriazole esters of N-blocked amino acids and the consequent remarkable facilitation of peptide synthesis when these derivatives are used as coupling agents,[10,11] similar properties for the corresponding polymeric esters were anticipated. Insoluble polystyrene bound 1-hydroxybenzotriazole (PHBT) was prepared through a series of chemical modifications of polystyrene (see Scheme II).



Preparation of Polystyrene-Bound 1-Hydroxybenzotriazole

Scheme II.

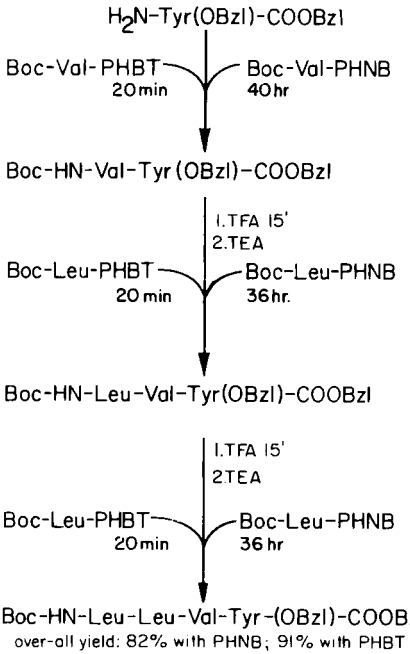
Reaction of 3-nitro-4-chlorobenzyl alcohol (II) or 3-nitro-4-chlorcbenzyl bromide (II) with commercial beads of macroporous polystyrene or with copoly-styrene-2% divinylbenzene (I), in nitrobenzene using aluminum trichloride as a catalyst at 65-70°, yielded (3-nitro-4-chloro)benzylated polystyrene (III). III was treated with hydrazine to yield (3-nitro-4-hydrazine)-benzylated polystyrene (IV) which was converted into polystyrene-bound 1-hydroxybenzotriazole (V) upon cyclization with hydrochloric acid. The amount of reactive hydroxyls present in V (0.8-2.2 mmol/g) was estimated by two different assays: the polymer was either reacted with an excess of sodium methylate in benzene-methanol and back-titrated with $HClO_4$,[12] or PHBT was exhaustively acetylated with acetic anhydride or acetyl chloride, followed by reaction of the ensuing acetyl-PHBT with excess benzylamine and back-titration with $HClO_4$.[9]

The PHBT derived from copolystyrene-2% divinylbenzene swells well in dimethylformamide, dichloromethane or chloroform, solvents commonly used for peptide synthesis,

and can be readily filtered and washed with these solvents. The rigid macroporous PHBT permits efficient peptide synthesis in the above organic solvents and in mixtures of water and solvents as dimethylformamide, dioxane or acetonitrile. PHBT-esters of various N-Boc and N-Z derivatives of amino acids were prepared by coupling, with dicyclohexylcarbodiimide, the appropriate component to PHBT in dimethylformamide or methylenechloride. Active ester formation was usually complete within 15-20 minutes at 4°. The amount of the amino acid derivative bound to the polymer (0.6-1.3 mmol/g) was estimated as described previously for other polymeric active esters.[7-9] The PHBT-esters were stable for at least two months when stored as dry powders in a desicator.

Peptide synthesis with these insoluble reagents usually proceeds by addition of excess (2 equiv) of the polymeric powder to an organic solution of the neutralized amino component (1 equiv) and the mixture is gently shaken at room temperature. Synthesis is followed by tlc and usually terminates within 10-30 min. The polymer is filtered, washed with dichloromethane or chloroform and the combined organic solution is washed with water to remove trialkylammonium salts. Evaporation of the organic solvent generally yielded pure products. Many peptides including thyrotropin releasing hormone (TRH; 80% over-all yield) have been prepared in our laboratory by this procedure. Small scale preparations, of as little as 0.01 mmol peptide, and large preparations, 10-20 mmol peptide, were possible. Simple synthetic manipulations, high purity, high product yields and fast coupling reactions were features common to all the synthesis performed. Of special interest is the synthesis of the tetrapeptide: Boc-L-Leu-L-Leu-L-Val-L-Tyr(Bzl)-OBzl in which bulky amino acids were linked in a stepwise manner. The peptide has been previously prepared in our laboratory using PHNB-active esters.[9] The synthesis was characterized by high efficiency (82% over-all-yield) but also required prolonged (36-40 h) coupling steps. When the PHBT-amino acid esters were utilized as the acylating agents, under similar conditions, all coupling reactions were completed within 20 min. In addition the over-all yield of the synthesis was 91%. The synthesis of the tetrapeptide by the two types of polymeric reagents is compared is Scheme 3.

Comparative Preparation of a "Problematic" Tetrapeptide



Scheme III.

Further demonstration of the acceleration of acylations using PHBT-active esters is provided by comparing the synthesis of Boc-L-Ala-L-Ala-OBzl(NO_2) from H-L-Ala-OBzl(NO_2) using Boc-L-Ala-PHBT and Boc-L-Ala-PHNB as acylators. This experiment (see Figure 1) reveals the dramatic difference in the reactivities of the two polymeric reagents.

In conclusion, our findings indicate that N-blocked amino acid esters of 1-hydroxybenzotriazole-bound polystyrene fulfil the criteria of a good polymeric active ester: (a) mechanical stability; (b) good swelling properties; (c) accessibility of activated amino acids; (d) reduced effect of steric hindrance and (e) high reactivity. These excellent properties coupled with the advantages of polymers as reagents in peptide synthesis lead us to believe that 1-hydroxybenzotriazole-bound polystyrene will in the future be commonly found on the shelf of the peptide chemist.

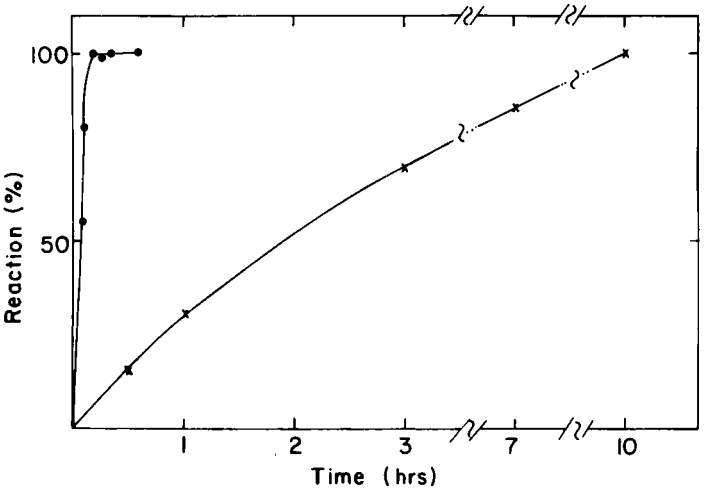


Figure 1: Comparative acylation of H-Ala-OBzl(NO_2) with Boc-Ala-PHBT and with Boc-Ala-PHNB. Aminolysis was initiated by mixing 1 equiv H-Ala-OBzl(NO_2) (0.04M in ethanol free chloroform) with beads of Boc-Ala-PHBT (•) and Boc-Ala-PHNB (x)(containing 2 equiv activated Boc-Ala; concentration of Boc-Ala in the suspension is 0.08M). Reactions were followed spectrophotometrically (at 280 nm) with formation of the neutral dipeptide.

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SOLID PHASE PEPTIDE SYNTHESIS BY OXIDATION-REDUCTION CONDENSATION

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THE OXIDATION-REDUCTION CONDENSATION REACTION forming peptide linkage with use of triphenylphosphine(Ph_3P) and 2,2'-dipyridyl disulfide [$(PyS)_2$] proceeds under mild conditions without the need for basic or acidic catalysts and has the advantages of minimizing the racemization of a carboxyl component and the side reactions of amino acids.[1] This condensation reaction has been successfully employed not only to syntheses of peptides[2] but also to phosphorylation as to syntheses of nucleotides[3] and Coenzymes[4] and syntheses of various macrolides.[5] The active dehydrating agent in the reaction, phosphorane, is generated by mixing the carboxyl component with Ph_3P and $(PyS)_2$ whose melting points are 80° and 58° respectively and peptide bond formation would proceed through a cyclic transition state.[1] The high reactivity with the high selectivity of this reaction depends upon the stability of the intermediate of acyloxyphosphonium salt wherein the phosphorous atom exists largely in the pentacovalent form. This being the case, some modification is possible by changing the nature of the trivalent phosphorous compounds. For example, the

use of tris(p-bromophenyl)phosphite in place of the triphenylphosphine increases the stability of the intermediate in polar solvents such as dimethylformamide. On the other hand, tri-*n*-butyl-phosphine decreases the stability of the intermediate which results in the decrease of selectivity in spite of the increase of reactivity.[7]

We have shown recently that the oxidation-reduction condensation can be successfully applied to the synthesis of LH-RH via fragment condensation on solid support by employing the chain elongation from N-terminal amino acid to C-terminal amino acid.[8] This approach was further extended to a synthesis of ACTH(1-24)[9] employing a new technique to monitor the amount of amino component in solution based on high pressure liquid chromatography (HPLC). General procedures for this approach are shown in Figure 1.

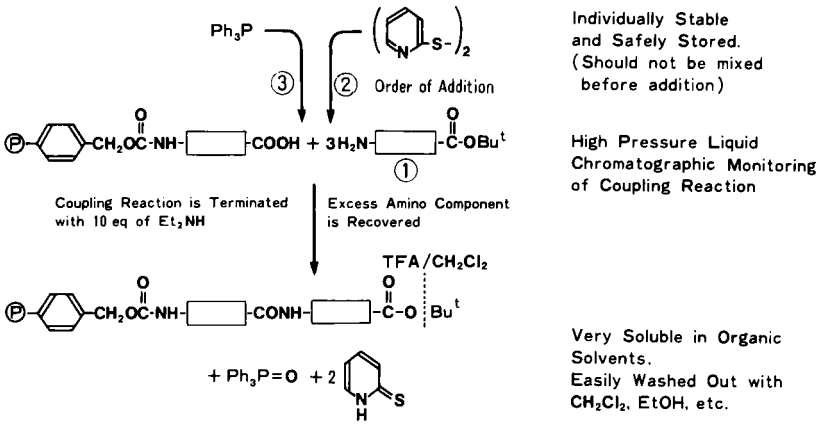

Figure 1: General scheme of solid phase peptide synthesis by oxidation-reduction condensation. Ⓟ-, Polymer support; Ph, phenyl.

Care should be taken to add $(PyS)_2$ and Ph_3P separately, in this order, to the mixture of amino and carboxyl components with exclusion of moisture. The amino component is used as free form. When the phosphorane generated from Ph_3P and $(PyS)_2$ becomes inadvertently partially hydrolyzed

the dehydrating activity is lost.

The synthesis of ACTH(1-24) [9] was carried out by this method according to the following scheme:

resin-Ser(Bzl)-Tyr + Ser(Bzl)-Met-Glu(OBzl)-His-Phe +
Frag.(I) Frag.(II)

Arg(Tos)-Trp-Gly + Lys(Z)-Pro-Val-Gly + Lys(Z)-Lys(Z)-Arg(Tos)-
Frag.(III) Frag.(IV) Frag.(V)

Pro + Val-Lys(Z)-Val-Tyr(But)-Pro-OBut.
Frag.(VI)

In this strategy, each fragment was designed to incorporate a UV-absorbing moiety in order to detect any remaining fragment by way of a highly sensitive UV detector.

During the course of the synthesis, coupling rates were easily monitored by measuring the amount of fragment in solution by HPLC and the remaining amino component was plotted against reaction time. Typical examples of monitoring results are shown in Figure 2. One is the example for the coupling of Frag.(II) and the other for that of Frag.(IV), wherein the coupling was carried out at -15° and room temperature respectively. Reaction completion was principally determined by noting the time at which the curve indicated no further consumption of the fragment. In addition, coupling was repeated until no fragment uptake was detected and it was also confirmed by determination of the amino acid ratios of the resulting peptide resin.

The resin-tetracosapeptide thus prepared was treated with HF[10], and chromatographed on carboxymethylcellulose.[11] The pure ACTH(1-24)·7CH_3COOH·9H_2O with 96(83-128) IU/mg of ascorbic acid depletion activity[12] was obtained in 25% yield from the initial resin-Ser(Bzl)-Tyr-OH by this analytically controlled synthesis.

Thus, this new approach demonstrated that an oligopeptide as ACTH(1-24) is prepared in good yield by simple purification procedures since contamination with failure sequences is eliminated.

The merits of this method are: 1) unreacted amino components can be recovered intact and accordingly can be recycled in the chain elongation procedure: 2) the number of protecting groups of the side chains can be reduced;

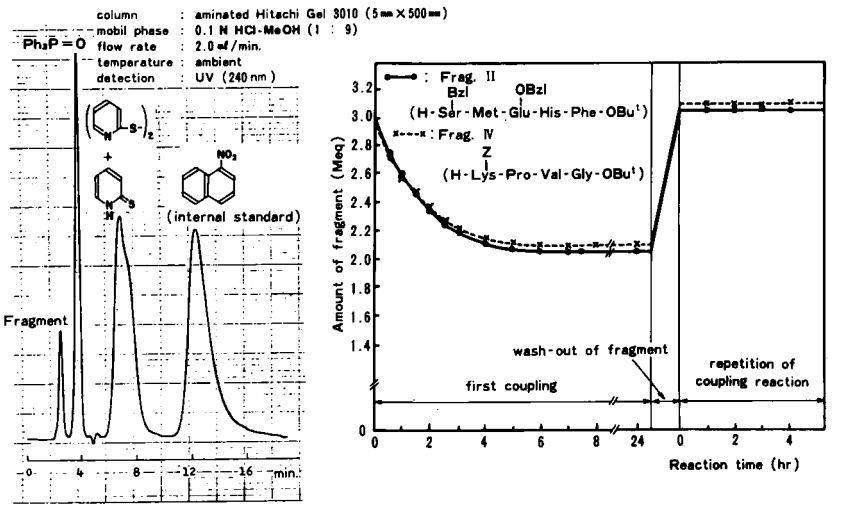

Figure 2: Monitoring of coupling reaction by high pressure liquid chromatography. (a) Elution pattern of reaction mixture, (b) Plot of amounts of fragments consumed with time (•—• , fragment II; x---x, fragment IV).

3) fragments are synthesized by classical method from readily available Z-amino acid; 4) diacylations are avoided since excess amino components are used in solution; 5) the end peptide is homogeneous since failure sequence contaminations are eliminated: 6) the number of acidolytic steps is reduced, an advantage in the presence of acid sensitive tryptophan residue; 7) the progress and completeness of each coupling reaction are easily analyzed by high pressure liquid chromatography.

The versatility of this approach, along with the combination of classical synthesis in solution, promises new possibilities for preparation of peptides with long sequence and significantly expands the scope of the usual solid phase peptide synthesis.

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QUANTITATIVE MONITORING OF THE REDOX CONDENSATION OF Ddz-AMINO ACIDS IN THE MERRIFIELD SYNTHESIS OF SECRETIN SEQUENCES

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THE REDOX CONDENSATION METHOD of Mukaiyama, Ueki, Maruyama and Matsueda[1] in its 2,2'-dipyridyl disulfide-modified form[2] was improved further by Ueki and Ikeda utilizing trianisyl phosphine as the reducing agent,[3] which Ueki[4] recommended particularly for activating urethane-protected Arg(NO_2) in the stepwise Merrifield synthesis.[5] We therefore chose this method to study its versatility in the solid phase peptide synthesis of the sequences 18-27 and 12-17 of secretin, which contain four Arg residues. In this contribuition some general and analytical details of this synthesis are described.

Starting from 1% cross-linked benzhydrylaminepolystyrene[6,7] we synthetized the sequence Ddz-Arg(NO_2)-Leu-Gln(Mbh)-Arg(NO_2)-Leu-Leu-Gln(Mbh)-Gly-Leu-Val-NH-benzhydrylpolystyrene* with the exclusive use of Ddz-protected amino acid derivatives.[10] The redox reagents 2,2'-dipyridyl disulfide (A) and trianisylphosphine (B) as well as the specific Ddz-amino acid derivative (C) were introduced into the support-charged centrifugal reactor[11] in the order A, C, B as controlled by the punched tape program of an automatic peptide synthesizer[12] (Schwarz BioResearch, N.Y.). A, B and C were each used in a 10-fold excess in a total volumn of 80 ml dichloromethane.

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* Ddz-, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl;[8] Mbh-, 4,4'-dimethoxybenzhydryl.[9]

The 1 hour reaction time in each peptide elongation step was repeated once with fresh reagents.

All operations of the synthesis were monitored continuously at 280 and 225 nm (absorption maxima of the Ddz-protecting group and of its fission products) by the aid of an uv flow cell photometer with reference technique (Uvicord III, LKB, Sweden) connected to the centrifugal reactor (LKB Instruments, Munich) and a 3 channel continuous line recorder with 15-times automatic scale expansion and simultaneous integration (Linseis, Germany), as presented at the 13th European Peptide Symposium in Israel.[13] By its mode of action the centrifugal reactor perfuses the uv flow cell with reaction liquids or washing solvents. The varying absorptions of the circulating liquids in the proceeding Merrifield synthesis are recorded as demonstrated in Figure 1 showing a typical course of the peptide

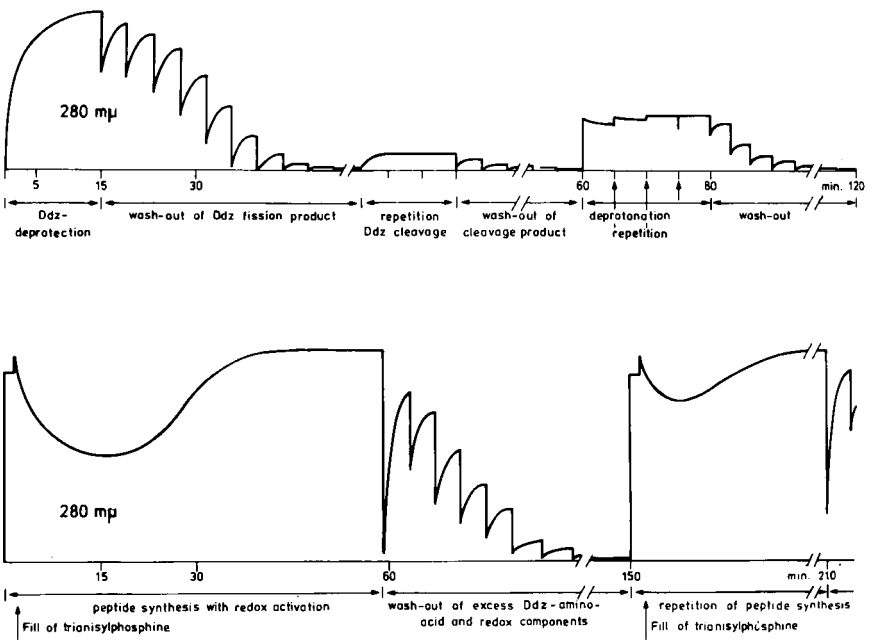

Figure 1: Course of the continuously monitored Redox condensation peptide synthesis on solid phase.

synthesis with redox condensation. The end of the cleavage of the Ddz-protecting group by 5% trifluoroacetic acid in dichloromethane is indicated by the constant course of the cleavage reaction curve and is re-examined by repetition of the deprotection process, which in the case of completeness shows zero on the recorder or reaches immediately a constant small uv absorption level. All washing operations between the different reaction steps are repeated until the recorder attains zero. The complete deprotonation of the amino functions on polymer with triethylamine in dichloromethane is ensured when the optical density of the reaction liquid, renewed several times, is no longer altered by salt formation and is seen on the recording as a constant line. In several recordings of the uv absorption of the reaction mixture during the redox condensation peptide synthesis we did not obtain the monotone curve resulting from incorporation of the specific Dcz-amino acid into the growing peptide on polymer - as is the case in the usual Merrifield synthesis[13] because of the consumption of the uv absorbing component in the circulating reaction liquid - but rather a superimposed sigmoid one, as demonstrated in the lower half of Figure 1.

In the initial phase of the redox activation the reactive intermediate is formed immediately, probably of the type revealed in Figure 2, consisting of the specific Ddz-amino acid and of the two redox components 2.2'-dipyridyl disulfide and trianisylphosphine showing the additive uv absorption of all three compounds at 280 and 225 nm. During the peptide reaction period this complex intermediate at first becomes associated to the amino functions on polymer resulting in lower concentrations of all three uv absorbing compounds in the circulating liquid, observed as a decreasing absorption on the recording. In the continuing peptide reaction, however, only the Ddz-aminoacyl becomes bound covalently to the growing peptide on polymer, whereas trianisylphosphinoxide and 2-mercaptopyridine are released into the circulating liquid causing an increasing uv absorption curve on the recorder. Since in addition the reduced mercaptan tautomerizes mainly to pyridine-2-thione,[2] which exhibits both a bathochromic shift and in increased absorption coefficient, the total uv absorption of the redox reaction mixture at the end of the peptide reaction period shows a higher value than at the beginning. With a continuously perfused ir flow cell we tried to detect typical signals of the redox intermediate by recording the whole ir spectrum every 5 min during

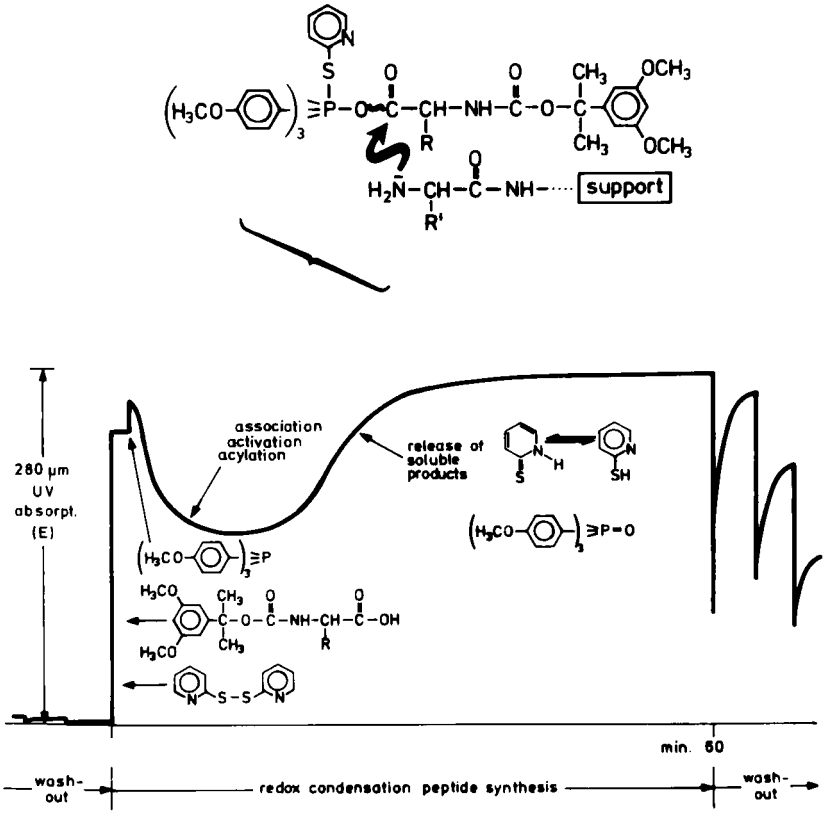

Figure 2: Interpretation of the course of the Redox condensation peptide synthesis on solid phase using continuous monitoring by flow photometry.

the programmed time of the peptide condensation, as documented in Figure 3. Only slightly decreasing ir absorptions are detectable in the region of the ester carbonyl band (1745 cm^{-1}) and of a band at 1045 - 1065 cm^{-1}, resulting from dipyridyl disulfide.

During the synthesis of the secretin sequence 18-27 the incorporation of the Ddz-amino acids was measured quantitatively by photometry of the deprotections and their respective washings.[14] Within the tolerance of the photometric determination and by reference to the continual monitoring of the completion of all synthetic operations

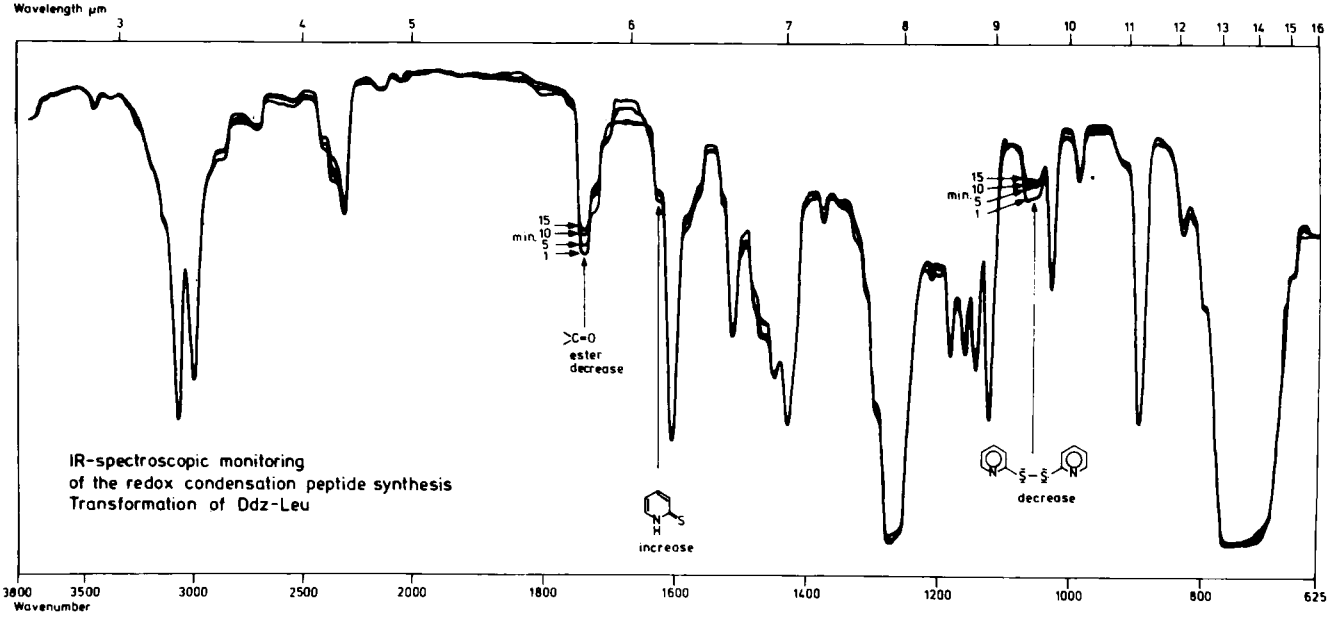

Figure 3: Ir-spectroscopic monitoring of the Redox condensation peptide synthesis on solid phase.

quantitative peptide couplings were established for all stages of the synthesis. This particularly is worth mentioning in the case of Arg(NO_2) in positions 21 and 18.

Because of the sometimes misleading results from amino acid analyses after total hydrolysis of protected peptides on polymeric supports, we reexamined the course of the synthesis by Edman degradation[15] of the peptide on polymer by the Laursen method,[16] which we improved for direct automatic formation[17] and quantitative determination[18] of the phenylthiohydantoins (PTHs). In Figure 4 the pattern of the PTHs are shown, obtained from ten stages of degradation of the synthetic sequence on polymer after separation and quantitative identification by high pressure liquid chromatography. Because of the Leu residue besides Arg(NO_2) in the sequence only the evaluations of the HPL chromatograms from stage 1-5 are representative for the identification of sequential inhomogeneities, whereas in the stages 6-10 the PTH peaks from sub-sequences could be superimposed by those resulting from incomplete degradations on preceding stages.

The results of the quantitative evaluation are summarized in Figure 5. We established the desired peptide to be 96% homogeneous containing seven distinguishable most probably sub-sequences yielding each < 1% of the total material. From the 85% average efficiency of the degradation/stage and from a yield of 180µMol PTH-Arg(NO_2)/g support in the first degradation stage it was detected that the incorporation of the final Arg(NO_2) [in position 18] in the synthesis had succeeded with -.21 mMol (∿ 100%) /g of benzhydrylamin polystyrene, whereas the photometric measurement had incorrectly stated, 0.16 mMol Arg(NO_2)-18 (∿ 76%)/g support since just in this last of all close supervisions of the synthesis the uv lamp of the spectrometer was found to be dimming.

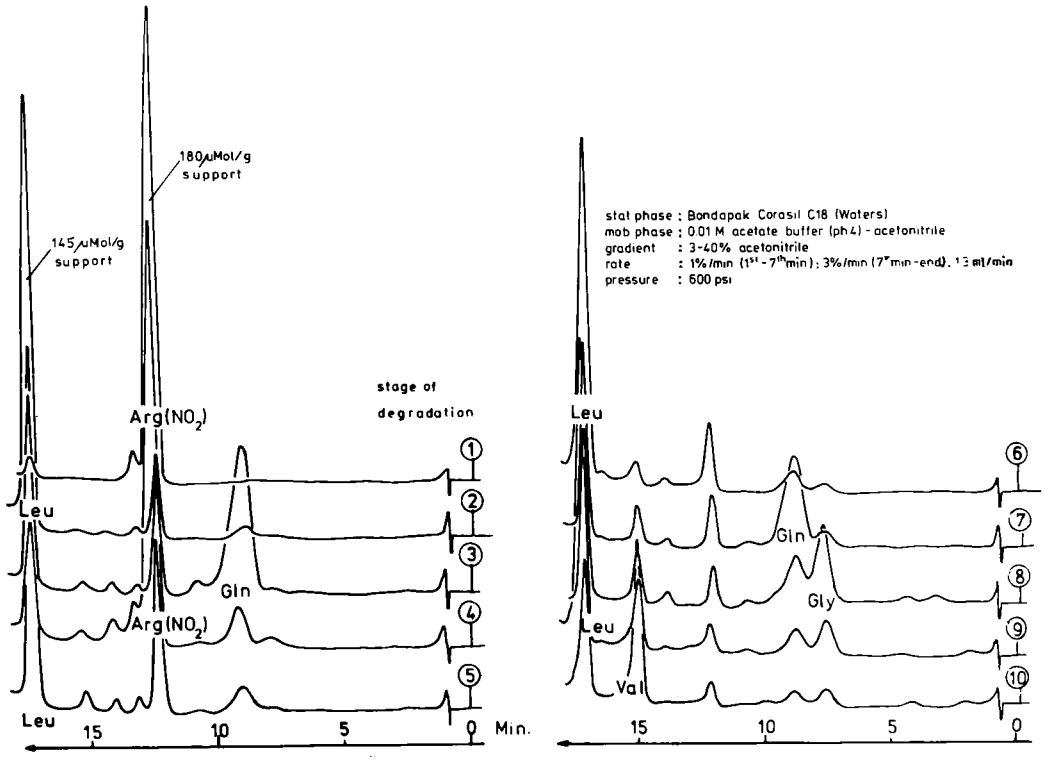

Figure 4: High-pressure liquid chromatograms of the PTH pattern from degradation of the synthetic sequence of 18-27 of secretin, Ddz-Arg(NO_2)-Leu-Gln(Mbh)-Arg(NO_2)-Leu-Leu-Gln(Mbh)-Gly-Leu-Val-NH-benzhydrylpolystyrene.

Ddz-Arg(NO_2)-Leu-Gln(Mbh)-Arg(NO_2)-Leu-Leu-Gln(Mbh)-Gly-Leu-Val-NH-benzhydrylpolystyrene.

stage of degradation		1	2	3	4	5	6	7	8	9	10
target sequence	95.7 %	Arg(NO_2)	Leu	Gln	Arg(NO_2)	Leu	Leu	Gln	Gly	Leu	Val — NHsupport
	0.6	Leu									Val
	0.4	Leu				Gly					Val
	0.9	Leu				Gly	Leu				Val
	0.2	Leu				Gln	Gly	Leu			Val
	0.9	Arg(NO_2)		Gln	Leu	Leu		Gln	Gly	Leu	Val
	0.7	Arg(NO_2)		Gln	Leu	Leu		Gly			Val
	0.6	Arg(NO_2)	Leu	Gln	Gly			Leu			Val
	4.3 %	of total synthetic product									

Figure 5: Quantitative evaluation of the high-pressure liquid chromatograms of the phenylthiohydantoins obtained directly from the automatic solid phase Edman degradation of the secretin sequence 18-27 synthetized on benzhydrylamine-polystyrene by the Redox condensation method.

Acknowledgments

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AN IMPROVED POLYSTYRENE SUPPORT FOR SOLID PHASE PEPTIDE SYNTHESIS

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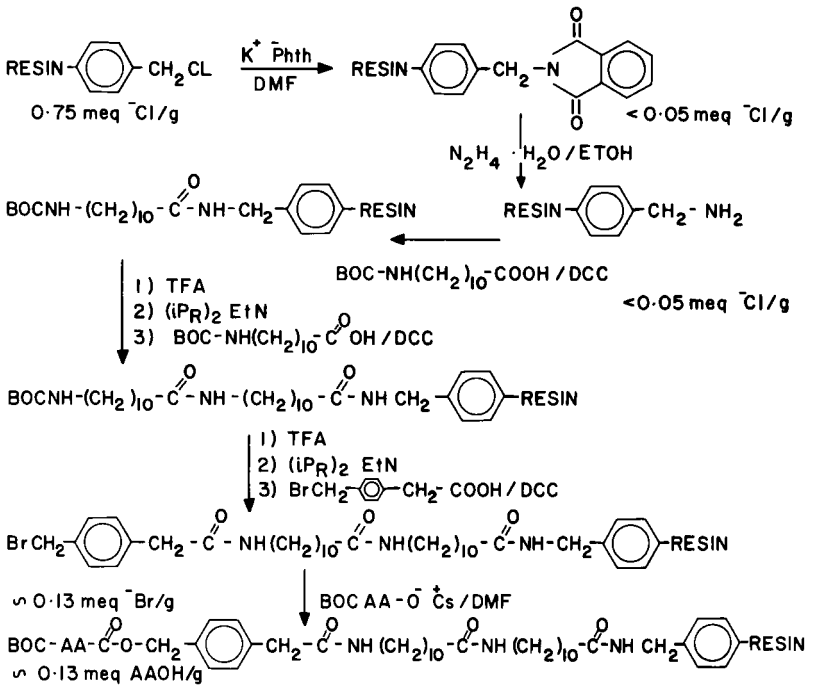
THE SOLID PHASE SYNTHESIS of peptide fragments of the very low density serum apolipoprotein apoC-III has been reported.[1] This synthesis used a 1% divinylbenzene polystyrene support, and was attended by low yields of approximately 5%; all attempts to increase the yields by modifications of the automatic synthesizer procedure were ineffective. Thus, studies were initiated to develop an improved resin support. Bayer *et al.*[2] has described the preparation of a resin with an *n*-hexyl spacer chain. In the present report, we describe the synthesis of a resin support which has a much longer spacer chain attached to the polystyrene matrix to separate the first amino acid from the matrix. Scheme I summarizes the synthetic route for the synthesis of this new support.

Experimental

Chloromethyl polystyrene was treated overnight with a 100% excess of potassium phthalimide at 50° in DMF. After treatment the chloride content of the resin was less than 0.05 meq per gram, and the infrared spectrum of the washed resin exhibited characteristic phthalimide bands at 1710 and 1775 cm^{-1}. After removal of the phthalimide moiety with hydrazine hydrate in refluxing ethanol, the resin lacked these bands. After extensive washing, this resin, as expected, was strongly fluorescent when treated with fluorescamine and viewed under ultraviolet illumination. Upon treatment with Boc-11-amino-undecanoic acid



Scheme I.

(prepared in DMSO from the amino acid and Boc-azide) and dicyclohexylcarbodiimide, a resin was obtained which was not fluorescamine positive. After treatment with 25% trifluoroacetic acid in methylene chloride to remove the protecting group, a second residue of Boc-11-amino-undecanoic acid was added. Finally, after deprotection, dicyclohexylcarbodiimide coupling with p-bromomethyl-phenylacetic acid in 50% DMF/methylene chloride gave a resin which was fluorescamine nagative and contained approximately 0.13 meq of bromide per gram of resin. Treatment of this resin with the cesium salt of the first Boc-amino acid according to the procedure of Gisin[3] gave a resin which contained approximately 0.13 meq of amino acid per gram of resin.

Results

Using a Boc-alanine resin prepared by both this new procedure and our previously reported method,[1] we have synthesized a 19-residue peptide, H-Phe-Ser-Glu-Phe-Trp-Asp-Leu-Asp-Pro-Glu-Val-Arg-Pro-Thr-Ser-Ala-Val-Ala-Ala-OH, sequence 61-79 of apolipoprotein C-III from human serum very low density lipoprotein, and have compared the resulting products. During the peptide synthesis on this new support, we observed increased incorporation of the amino acids residues, as determined by amino acid analysis performed on the peptide resin. Cleavage with hydrogen fluoride and chromatography on BioGel P-10 in 0.1 M ammonium bicarbonate gave a symmetrical peak (Figure 1)

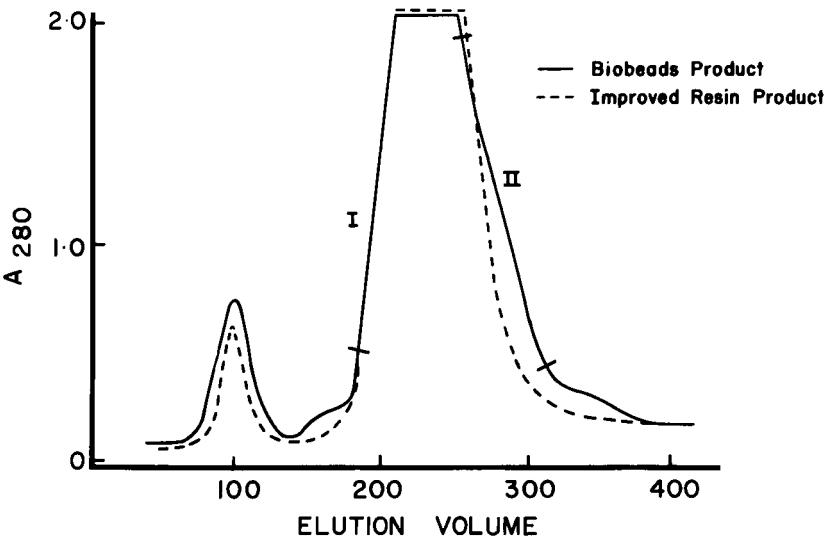


Figure 1: Elution profile of the HF cleaved products on BioGel P-10 in 0.1 M ammonium bicarbonate. Curve A. (——) Biobeads; Curve B. (---) Modified-Resin.

which analyzed correctly for the 19-residue fragment (Table I). The total yield of this peptide from the BioGel column was 38.7%, based on 0.13 mmoles of alanine per gram of resin. Further chromatography on AG 1-X2 permitted 70% recovery of pure peptide for an overall yield of 28.9%. A similar purification of the material (31.7%

Table I

Amino Acid Analysis of Peptide Fractions From BioGel P-10

Amino Acid	*Improved Resin*		*Biobeads*		*Theoretical*
	I	*II*	*I*	*II*	
Aspartic Acid	2.03	0.92	2.14	2.14	2
Threonine	0.96	0.64	0.96	0.85	1
Serine	1.80	1.04	1.83	1.70	2
Glutamic Acid	1.99	1.08	2.11	1.94	2
Proline	1.90	1.29	2.11	2.19	2
Alanine	3.00	3.00	3.00	3.00	3
Valine	2.05	1.51	2.26	2.14	2
Leucine	0.99	0.42	1.01	1.02	1
Phenylalanine	2.06	0.64	2.05	1.61	2
Arginine	1.09	0.72	1.04	1.13	1
Yield of Peptide	57.56 mg 38.7%	7.81 mg	84.43 mg 31.6%	27.08 mg	

yield from P-10, Figure 1, Table I) obtained from the synthesis on the commercially available BioBeads gave only 27.6% recovery of the pure peptide from AG 1-X2 for an overall yield of 8.7%, based on 0.25 mmoles of alanine per gram of resin.

Discussion

From these results and those obtained from the synthesis of similar peptides, we conclude that this new resin offers two important advantages over the BioBeads: (1) higher overall yields of hydrogen fluoride cleavable peptide and (2) peptides of greater homogeneity. It seems plausible that the increased yield of peptide results from a decrease in interaction of the growing peptide chain with the polystyrene matrix and thereby could result in a decrease in premature termination of the growing chains. In Figure 3 we present electron paramagnetic resonance spectra of two peptide resins labelled at the NH_2-terminus with 1-oxido-2,2,6,6-tetramethylpiperidinyloxycarbonyl. The spin label on the unmodified peptide resin (A) is significantly

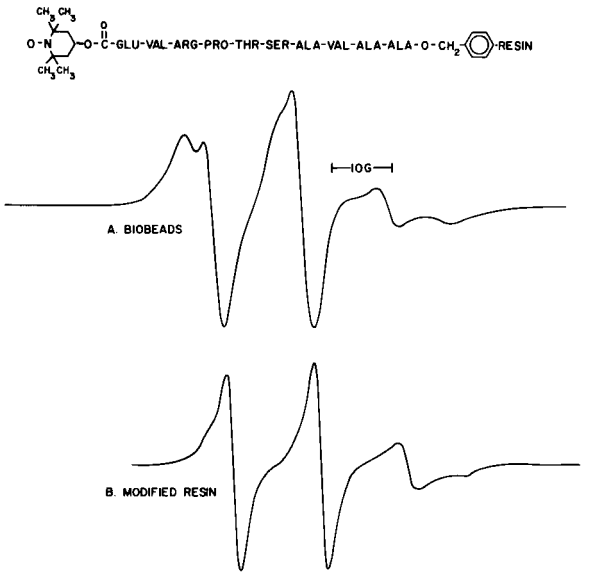


Figure 2: Electron paramagnetic resonance spectrum of 1-oxido-2,2,6,6-tetramethylpiperidinyloxycarbonyl-peptide resins in methylene chloride. Curve A. Peptide-BioBeads; Curve B. Peptide Modified-Resin.

less mobile than the label on the improved peptide resin (B), suggesting increased peptide chain mobility on the improved support. This evidence supports our conclusion that the spacer group serves to decrease peptide resin interactions, which could diminish coupling efficiency.

Acknowledgments

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AUTOMATICALLY PROGRAMMED SYNTHESIZER FOR THE LIQUID PHASE SYNTHESIS

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THE LIQUID PHASE peptide synthesis utilizes a linear, soluble polymer with functional groups which can be coupled to the carboxylic group of the COOH-terminal N-protected amino acid or peptide. The desired peptide is then synthesized by stepwise coupling of amino acids or peptide fragments.[1,2] Bi- or mono-functional polymers such as polyethylene glycol mono ethers or polyethylene glycol have proved to be especially good. Coupling reactions are carried out under homogeneous conditions; and rate constants for the coupling reactions are equal to those in classical peptide synthesis[3,4] and are linear, contrary to the rate constants in solid phase synthesis. The solubilizing effect of polyethylene glycol enables homogeneous reactions also in many cases where the non-polymer bound peptide is no longer soluble.[5] The control of the peptide synthesis can be automatically monitored in homogeneous solution by using ninhydrin or fluram reactions[6] or potentiometric titration.[7] Liquid phase peptide synthesis is done in repetitive cycles like solid phase synthesis and therefore can be automated.

However, a cycle in liquid phase synthesis requires more operations than in the solid phase method, where only mixing during the coupling and deprotection reaction, filtration and washing are necessary. The reaction vessel for automatic liquid phase synthesis has to be more versatile. The following working procedures are necessary during one cycle in the "crystallization" version of the method.

1. Addition of coupling reagent and N-protected amino acid to the solution of the polymer-bound amino acid or peptide.
2. Mixing of reagents and polymer-peptide (or amino acid) and coupling.
3. Separation of excess coupling reagents by adding diethyl ether and "crystallization" of the polymer-peptide and filtration.
4. Dissolving the precipitate, "recrystallization" with diethyl ether, filtration and evaporation.
5. Dissolving the precipitate, addition of reagents for removal of the N-terminal protecting group.

In step 5, aliquots are used for estimating the free amino acid groups available for the next coupling step. In step 2, the coupling yields are monitored. In some coupling steps it may be necessary to evaporate a part of the solvent *in vacuo*.

A scheme of the synthesizer is shown in Figure 1. The reaction vessel 6 is in horizontal position for reaction and dissolving procedures. For filtration the whole assembly is automatically turned by 90°.

Solvents and reagents are fed in by tubes 8 and 7 from both sides of the vessel to achieve good mixing. Washing solvents used for filtration are only fed in through 7. The assembly is constructed similar to a rotary evaporator. The motor 2 rotates reaction vessel 6 for mixing and during occasional evaporation of solvents. Vessel 6 can be cooled or heated. Through 5 both vacuum or pressure (2 atm N_2) can be applied to the reaction vessel. The automatic programming follows that described for solid phase peptide synthesis[8,10] with the necessary changes in the sequence of operations required for liquid phase synthesis.

For a synthesis cycle, the dissolved polymer-amino acid is placed into the reaction vessel 6 (horizontal position) so that the total volume fills approximately one-half of the "bulge." The coupling reagents are added through tubes 7 and 8 with continuous rotation of the reaction vessel. After completion of coupling solvent is added through 7 and 8 in order to precipitate the polymer-peptide under vigorous rotation (400 cpm) and cooling to 0°C.

Then the assembly is turned around 90° and, applying slight nitrogen pressure (2 atm) at 5, the solution is filtered through a sintered disk into the reservoir and washed. The "crystallization" is repeated by returning to

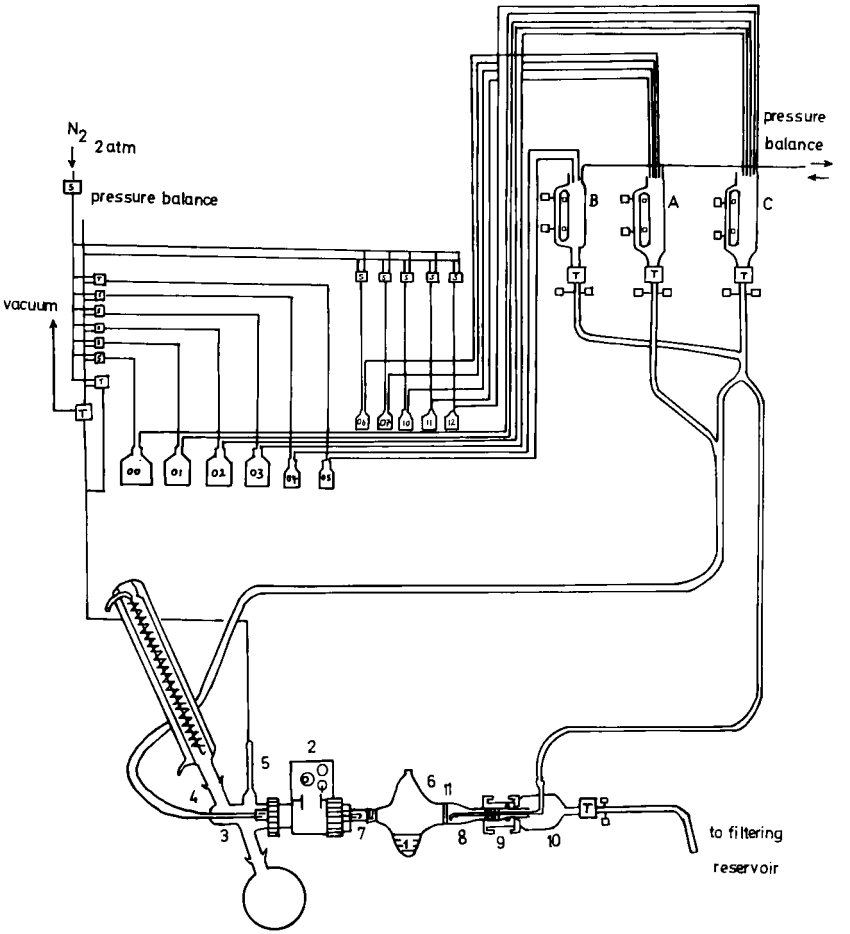

Figure 1: Reaction vessel for the liquid phase peptide synthesis (vertical position).

1 Homogeneous solution in reactor, 2 motor for rotating, 3 tube and 7 tube for inlet of reagents, solvents, washing solvents, 4 inlet of tube, 5 tube for vacuum or pressure (2 atm N_2), 6 reaction vessel, 8 tube for inlet of reagents, solvents, 9 ball bearing, 10 distribution part, 11 sintered glass filter dish.

00-12 solvents and reagents. A. B, C etc. Measuring and mixing vessels for reagents and solvents; S and T three- and two-way solenoid valves.

the horizontal position, applying vacuum at 5, adding solvent through 8 and 7, "crystallization" and filtration from excess reagents as described.

The N-terminal protecting group is split off the polymer-peptide by adding the reagent through 7 and 8. Crystallization and purification of the N-deprotected polymer-peptide is achieved as already described. After redissolving, the next cycle starts. A total programmed cycle for connecting one amino acid to the polymer takes between 1 and 5 hours, depending upon the type of coupling reaction and amino acid. Different peptides have been synthesized with this synthesizer unit and compared with already published synthesis by hand operation with the liquid phase method. The purity and coupling yield of the synthesis were always similar. However, less loss of polymer occurred in the programmed synthesizer, and the time for one synthesis cycle is reduced by a factor of 1.5 to 2. The completeness of the coupling reaction can be monitored continuously by the ninhydrin reaction described earlier for liquid phase peptide synthesis.[6]

Potentiometric titration produced unreliable results with larger than hexapeptides. In all cases polyethylene glycol is exclusively used as basic polymer in the synthesizer. Other basic polymers like polyvinylalcohol are not suitable, since they do not show reproducible precipitation and solubility behavior.

It was not yet possible to construct a synthesizer which could use diafiltration to remove excess instead of crystallization and filtration, since the completion of diafiltration is different for individual polymer-peptides and becomes the slowest step in a cycle.

Time programming on the basis of preselected coupling times is easier to handle than automatic monitoring of coupling and deprotection. In liquid phase peptide synthesis the rate constants are linear and not dependent upon the degree of completion of the reaction. Absolute rate constants have been reported.[3],[4] Assuming linear rates, the measurement of relative reaction rates enables the prediction of the necessary reaction time for individual coupling. A model system for accumulating relative reaction rates has been chosen by competitive coupling, *i.e.*, by reacting a polymer bound N-terminal deprotected amino acid (*e.g.*, Boc-Pro, Boc-Leu, Boc-Ile) and equimolar amounts of two amino acid derivatives to be estimated. After all amino groups of the polymer-amino acids have been reacted, the excess of reagents is removed, the polymer peptide "crystallized," hydrolyzed and the relative amounts of amino

acids bound to the polyethylene glycol estimated. The relative molar amounts of amino acids are proportional to the relative reaction rates. In Tables I-V relative reaction rates for the coupling of different amino acid derivatives to different polymer-amino acids are reported relative to Boc-Pro (=100). The different amino acids vary in their relative rates by a factor of approximately 20, and we can classify three groups (Table I). The relative rates of trifunctional amino acids can very much depend upon the protecting group as Table II shows for Arg and Asn. Table III demonstrates the dependence of the reaction rate on the amine component, which is very often neglected in synthesis. Table III also shows that the molecular weight of the polymer does not influence the relative kinetics of the coupling reaction. This is in agreement with results obtained determining the absolute rate constants.[4]

Table I

Relative Reaction Rates (V_{rel}) of Different BOC-Amino Acids Coupled to H-Gly-OPEG Measured by the Competitive Method Coupling with DCCI in CH_2Cl_2 by Preactivation[19]

Group I	*Group II*	*Group III*
Pro (100)	Cys(S-isoProp) (65)	Ser(O-Bzl) (31)
Gly (93)	Met (60) $^{++}$	Try^{++} (28)
Ala (85)	Glu(OtBu) (54)	Asn (27)
	Tyr(O-Bzl) (53)	Arg (HCl) (23)
	Lys(Z) (53)	His (N_{im}-Bzl)$^{++}$ (22)
	Phe (53)	Val (18)
	Leu (52)	Arg(NO_2) (17)
	Gln (49)	Thr(O-Bzl) (10)
	Arg$(Z)_3$ (49)	Z-Asn(Mbh) (11)
	Asp(OtBu) (44)	Ile (6)

The dependence of the relative reaction rates on the amine component is more pronounced than in the solid phase method, where obviously the polymer skeleton dominates the rates. In Table V different coupling procedures are compared. With the exception Phe no remarkable differences

Table II

Relative Reaction Rates V_{rel} of Different Protected Arg and Asn to H-Gly-OPEG with DCCI in CH_2Cl_2[11]

	BOC-Arg(H^+Cl^-)	*BOC-Arg(NO_2)*	*(Z_3)Arg*	*BOC-Asn*	*Z-Asn(Mbh)*
V_{rel}	23	16	47	27	11

Table III

Relative Reaction Rates of BOC-Amino Acids Coupled to Different Polymer Bound Amino Acids with DCCI in CH_2Cl_2[11]

Aminocomponent	*Pro*	*Gly*	*Phe*	*Leu*	*Ile*
H-Ala-OPEG (m.w. 10,000)	100	86	54	53	6
H-Ala-OPEG (m.w. 4,000)	100	85	53	53	6
H-Val-OPEG (m.w. 10,000)	92	100	46	45	4
H-Pro-OPEG (m.w. 10,000)	6	100	15	10	2

Table IV

Relative Reaction Rates for Different Versions of Coupling with DCCI (Coupling to H-Gly-OPEG)

Coupling Method	*Pro*	*Gly*	*Phe*	*Leu*	*Ile*
DCCI	100	86	54	53	6
Symm.Anh.[14]	100	98	53	55	6
DCCI + HOBt	100	83	93	54	6

Table V

V_{rel} for the Coupling of Different BOC-Amino Acids to H-Ala-OPEG in Different Solvents with DCCI and Separate Preactivations with DCCI (*)

Solvent	*Pro*	*Gly*	*Phe*	*Leu*	*Ile*
CH_2Cl_2	54	100	65	60	16
DMF	58	100	100	70	36
Benzol	59	100	97	73	30
DMG + HOBt	69	79	100	58	33
CH_2Cl_2* (separate)	96	100	18	73	53
DMF* (separate)	71	100	14	82	63

are to be seen. In Table V the relative reaction rates of different amino acids in different solvents are listed. The twofold faster relative rate of Boc-Ile and Boc-Phe in DMF than in CH_2Cl_2 is remarkable. This selection of relative rates shows that no uniform standard coupling time can be used. These investigations are of interest not only for the programming of the liquid phase peptide synthesis. Since the reaction rates in classical and liquid phase peptide synthesis are equal, these values may be also useful for classical peptide synthesis. However, due to the easy isolation procedure of polyethylene glycol-bound peptides, a quantitative estimation by competitive measurements will be much more difficult if not impossible.

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COMBINED USE OF SOLUBLE SUPPORTS AND SOLID POLYMER REAGENTS FOR PEPTIDE SYNTHESIS

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A NEW APPROACH FOR REPETITIVE peptide synthesis is described which combines the advantages of peptide synthesis on soluble supports with those of solid polymer bound reagents. This technique includes two cycles (Figure 1). For cycle A, a solid polymer reagent is used with delivers N-protected amino acids in the activated state and which can be reused after the coupling step. The peptide sequence is built up according to cycle B using polyethyleneglycol (PEG) as solubilizing carboxylic protecting group.[1] Solid

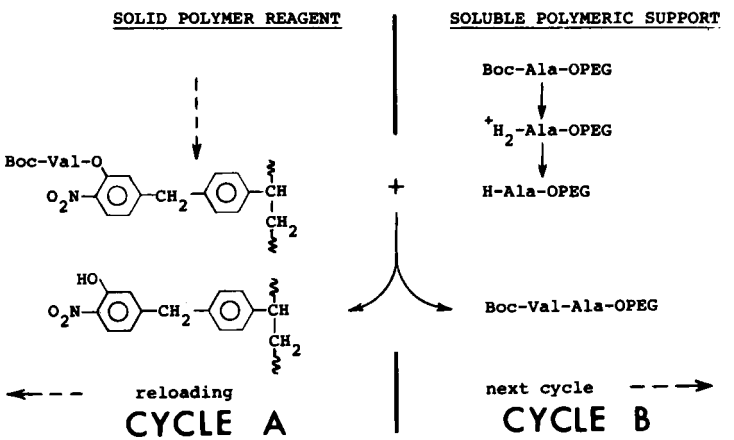

Figure 1: Reaction scheme for peptide synthesis using solid polymer reagents (cycle A) and soluble polymeric support (cycle B).

polymer and PEG-peptides are in contact only during the coupling steps. This approach offers the following advantages: An exact control of each coupling and deprotecting step and the possibility for repetition of the reaction are given. Problems arising from the growing peptide chain are avoided as long as the properties of the PEG-chain are dominating. Therefore, peptides up to about 15 residues can be built up at MW 10,000 of the PEG. On the other hand, the storable, insoluble polymer reagents are readily available sources of activated N-protected amino acids which can be used several times.[2] There is no loss of excess coupling components since the amino terminus of the solubilized peptide chain will pick up only the required amount of activated amino acid.

The repetitive, easily automated technique used here improves both the polymer reagent and the liquid phase strategy: During the coupling step there is no formation of soluble or insoluble side products and no excess of amino acids has to be removed.

In principle this would allow an elimination or at least shortening of the so far rather time-consuming purification steps of the PEG-bound peptides after quantitative coupling. We tested our method successfully in the synthesis of several tripeptides with alternating alanine and ornithine residues (Table I). The first surprising result was the observation that there are no serious complications arising from the inhomogeneous reaction between the two polymers. Quantitative yields were reproducible by repeating the coupling in 5 to 10 molar excess of the polymer reagent. Initially, we used the 2-hydroxynitrophenylesters based on 4-nitro-3-hydroxy-benzoic acid and chloromethylated cross-linked polystyrene.[3] This reagent seems to be of rather limited stability since the reaction mixture was always dark colored by soluble by-products. Much better results are obtained using the stable polymer reagents based on insoluble (4-hydroxy-3-nitro)benzylated polystyrene (PHNB).[2]

A typical coupling procedure is as follows: 10 g H-L-Orn(Z)-PEG (MW 10,000, esterified OH-groups, 56% corresponding to 1.1 mMol) were coupled with 13 g solid polymer bound Boc-L-alanine-*o*-nitrophenylester (5.5 mMol) in 180 ml CH_2Cl_2 for 5 hr. The pH of the mixture was adjusted to pH 7-8 during the coupling. The coupling yield was controlled by titration of the free amino groups, and a yield of 72% was determined. A second coupling using the same excess and reaction time yielded 90% and the third coupling gave a quantitative result. The Boc group is split off by 1.2 N HCl/CH_3COOH. For neutralization we used N-methylmorpholine. In all other coupling steps we used analogous conditions.

Table I

Yields of Tripeptides Synthesized on Solubilizing PEG Supports
Using Solid Polymer Reagents

Peptides	*MW*	*PEG-Peptide*		*Yield of the Crude Products After Deprotection*		*Yield of the Pure Peptides*	
		Weight (g)	*mMol*	*mg*	*%*	*mg*	*%*
H-L-Ala-Orn-Ala-OH	274	1.1	0.28	36	47	15	20
H-L-Orn-Orn-Ala-OH	317	1.6	0.41	73	56	30	23
H-L-Orn-Ala-Orn-OH	317	4.0	0.45	83	58	48	34
H-L-Ala-Ala-Orn-OH	274	3.5	0.39	68	63	37	34
H-L-Orn-Ala-Ala-OH	274	2.3	0.59	62	38	23	14
H-L-Ala-Orn-Orn-OH	317	4.5	0.50	104	66	62	39
H-L-Orn-Orn-Orn-OH	360	3.4	0.38	101	73	64	46

Soluble Supports: Polyethyleneglycols (PEG)

H-L-Ala-PEG: MW 6,000, 78% loaded (1 g = 0.26 mMol)

H-L-Orn-PEG: MW 10,000, 56% loaded (1 g = 0.11 mMol)

Saponification and Deprotection

3.5 g Boc-L-Ala-Ala-Orn(Z)-PEG (0.39 mMol) were stirred in 1/10 N NaOH for two hours, neutralized and lyophilized. The white residue was taken up in warm ethanol. The PEG crystallized in the cold and was filtered off. The solvent of the filtrate was evaporated and the dried residue was reacted with HBr/CH_3COOH for 40 min. The free peptide was precipitated by ether and dried. Remaining traces of PEG were removed by an extraction with CH_2Cl_2. The residual peptide was taken up in water and chromatographed after neutralization on Sephadex G-15. Six peaks were eluted with water. The first (exclusion) peak (I) represented residual traces of PEG which was cleanly separated from the peptide, H-Ala-Ala-Orn-OH, in the major peak (IV). Four additional minor peaks were due to alanine (II,III), ornithine (V,VI) and several unidentified impurities which were all completely separated from the peptide (IV). Peptides (Table I) are generally obtained in pure form by such criteria as TLC, thin-layer electrophoresis (Figure 2, showing H-Ala-Ala-Orn-OH, IV, as an example) and by amino acid analysis data in full agreement with theoretical values.

Some of the peptides containing two or three ornithine residues (Table I) were water-insoluble in their N-protected form and could be isolated as such by centrifugation of the acidified mixture after saponification of the PEG-peptides.

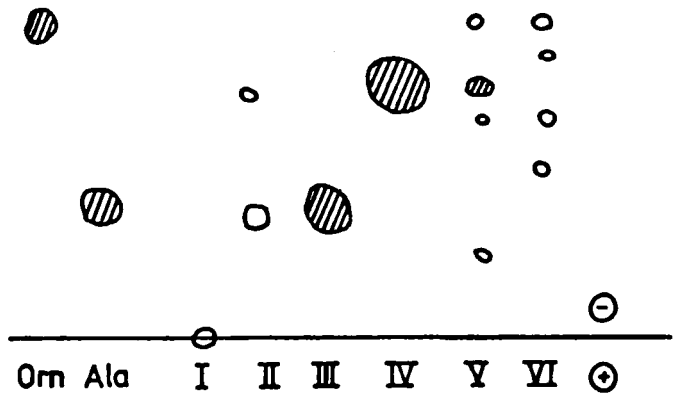

Figure 2: Thin-layer electrophoresis of H-Ala-Ala-Orn-OH (IV); (1% HCOOH, AcOH, pyridine in H_2O, pH 4.2, 300 V, 35 mA, 1 hr). I-VI, Fractions from Sephadex G-15 chromatography.

Using this technique peptide synthesis is highly simplified if we consider the chain building alone. However, there are some serious problems which are not yet solved for the polymer reagent and the liquid phase approach. First, the preparation of activated polymer bound amino acid esters requires large excesses of N-protected amino acids and, despite extensive washings, we still do find some impurities after the coupling steps. In the saponification step for the removal of the peptide from the PEG rather drastic conditions are needed and relatively low yields are obtained. Therefore, anchor groups based on benzyl residues are needed which can be removed by acidic treatments or catalytic hydrogenation.

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INVESTIGATION OF SOLID SUPPORTS AND CARBOXYL-PROTECTING GROUPS IN ALTERNATING LIQUID-SOLID PHASE PEPTIDE SYNTHESIS

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POLYMERS HAVE BEEN USED to simplify peptide synthesis since their introduction by Merrifield[1] in basically two ways: (1) the growing peptide chain is *always* fixed to the polymeric support, acting as the carrier of a protecting group; and (2) the polymer carries the coupling reagent; the growing peptide chain is never fixed to the polymeric support.[2]

In the first category three different approaches have been investigated: (a) the Merrifield procedure using insoluble polymers as carrier for a carboxyl-protecting group; (b) the liquid phase method using a soluble polymer instead;[3] and (c) the Letsinger procedure wherein an insoluble polymer serves as the carrier of an amino-protecting group.[4]

All three methods have been designed to avoid time-consuming clean-up procedures, but they revealed a congenital defect: every side reaction and incomplete reaction of the support-attached partner leads to an accumulation of undesirable side products.[5] They cannot be separated until the peptide is split off after the final coupling step. In the second category a number of polymer-bound coupling reagents have been described. This procedure has not been extensively applied to peptide synthesis at this time.

In order to circumvent the necessity for practically 100% yield in every reaction step of the Merrifield procedure and its variations we have recently introduced a new

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synthesis concept: the alternating liquid-solid phase peptide synthesis,[6] by which the separation of reacted and unreacted compound (*i.e.*, peptide chain) is effected by simple filtration. At every reaction step the desired product is either bound to or released from a solid support selectively and simultaneously during the reaction.

In Figure 1 the general procedure is shown. The rectangle represents a solid support, bearing amino acid residues represented by small circles. During our first studies we attached these amino acids to the resin with their amino function, so the solid support carries the amino-protecting group of the amino acid represented by X. The polymer-bound amino acid is then coupled with the growing peptide represented by a row of small circles and a larger circle, the carboxyl-protecting group. Uncoupled peptide remains in solution and is removed by filtration.

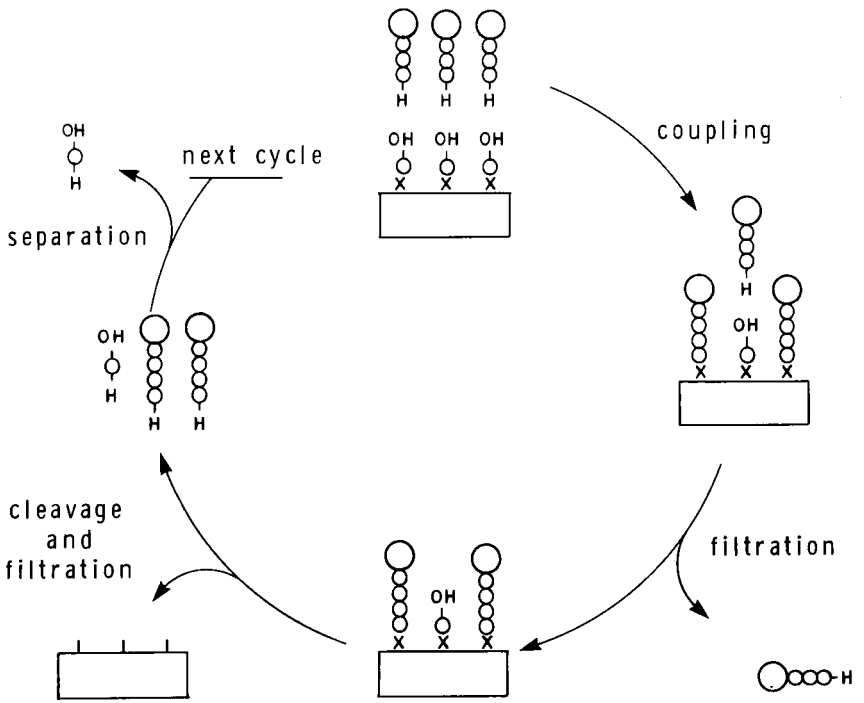
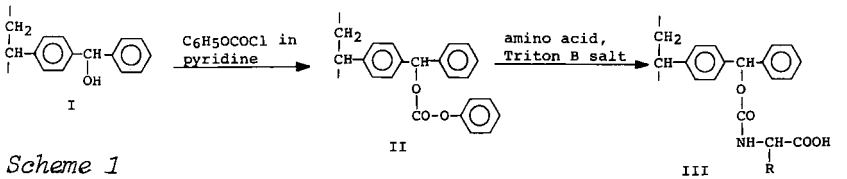


Figure 1: - General scheme of the alternating liquid-solid phase peptide synthesis.

After cleavage of the amino-protecting group, which is also the linkage to the solid support, unreacted amino acid and the peptide elongated by one residue are separated by gel permeation chromatography or precipitation methods. The next amino acid can be coupled in the same way or by any other method. With this procedure incomplete coupling and deprotection reactions as well as an irreversible blocking of the amino-function do not contribute to impurities. Every uncoupled and unelongated peptide chain is removed by filtration. Every chain not deblocked at the amino-terminus stays attached to the solid support and is removed by filtration. Every chain blocked thereafter cannot be reacted and attached to the polymeric support at the next coupling step and is again removed by filtration.

The selectively cleavable polymer-bound amino-protecting group is an analog of the diphenylmethyloxycarbonyl-group, where one phenyl ring is contributed by polystyrene. The first two steps of the synthesis providing compound I (Scheme 1) have been described in the literature.[7] The remaining two steps in the synthesis of α-polystyryl-benzyloxycarbonyl-(PBOC)-amino acids (III) are brought to completion by a fourfold excess of the corresponding reagents. In Table I the solid supports used are listed.



Scheme 1

Table I

Functionalized Solid Supports

Solid Support	*Protecting Group (analog)*
Copolymer Polystyrene - 2% Divinylbenzene	
Copolymer Polystyrene - 1% Divinylbenzene	Diphenylmethyloxycarbonyl-
Controlled Pore Glass, Pore Size 3000 A	
Copolymer Acrylic acid methylester-p-Vinylphenyldimethylcarbinol	Phenylisopropyloxycarbonyl-

The PBOC-amino acids can be stored in the dry state at 0°C and are totally stable under coupling conditions. On the other hand the anchor group is readily cleaved by 10% trifluoroacetic acid within 5 minutes.

During our first synthesis of the model peptide glycyl-valyl-glycyl-alanyl-proline,[8] we realized that we were faced with two problems which had not been investigated previously: (1) the reaction rate of coupling is dependent on the size of the soluble macromolecule and the structure of the insoluble polymer, and (2) how can the separation of an amino acid trifluoroacetate or hydrochloride from a peptide be facilitated on a gram scale? Both problems are partially governed by the features of the employed carboxyl-protecting group.

In our first synthesis we used the picolyl ester.[9] Though we ended up with a chromatographically pure product, the separations of peptide and amino acid caused a considerable loss of product after each cycle. We investigated then the usefulness of polyoxyethylenes of different molecular weight for the same synthesis, *i.e.*, polyoxyethylene monostearylether (mol wt 700) or monoethylether (mol wt 1500) and polyoxyethylene monostearates of mol wt 4000 or 6000. They are soluble in all of the solvents used and their high molecular weight makes the separation of oligopeptides and amino acids possible either by gel permeation chromatography or, with polyoxyethylenes above a molecular weight of 4000, by precipitation with ether or ethanol. The reaction rate is markedly dependent on the molecular weight of the employed polyoxyethylene (Table II).

Table II

Coupling Reaction of Monoprolylpolyoxyethylene Derivatives and PBOC-Alanine

Carboxyl-Protecting Group Mol. Weight	*Excess PBOC-Alanine*	*Yield After 1 hour (%)*
700	1.5	98
1500	4	86
4000	4	71
4000	8	85
6000	4	4
6000	8	15
6000	4*	75

*Controlled pore glass, pore size 3000 Å.

The optimal molecular weight is about 4000 in combination with 1% cross-linked polystyrene as the insoluble partner; the coupling rate is considerably improved by using functionalized glass beads, but so far from a practical point of view the amount of functional groups per gram is too low and the glass beads are too expensive.

To prove our presumption of a clean end-product, we synthesized the model peptide again, starting with monoprolylpolyoxyethylene monostearate, mol wt 4000, by the previously described liquid phase method and the new alternating liquid-solid phase method. The crude products at the tri-, tetra-, and pentapeptide stages were analyzed by ion exchange chromatography[10] (Figure 2). The pentapeptide polymer of the liquid phase method (LPPS) was separated from low molecular weight peptides and amino acids by filtration through an ion exchanger prior to cleavage of the peptide from the soluble support. Though the amount of impurities is small, they are still detectable (Figure 2f). From the alternating liquid-solid phase synthesis the crude product appeared to be homogeneous (Figure 2e).

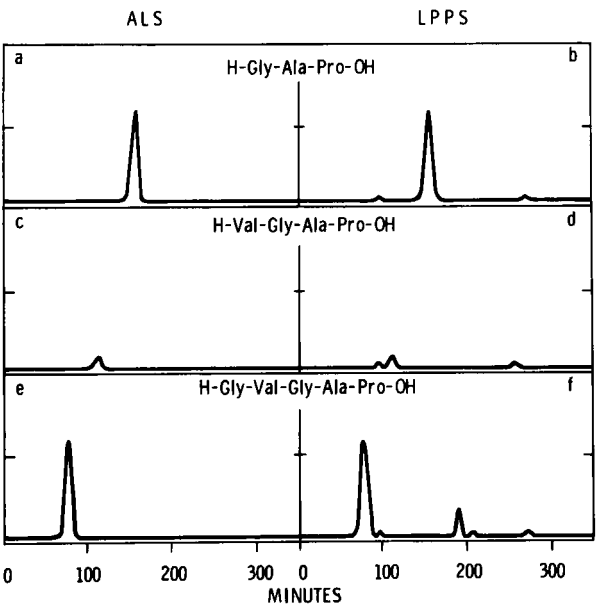


Figure 2: Ion exchange chromatography of oligopeptides synthesized by alternating liquid-solid phase method (ALS), a, c, e; and by liquid phase method (LPPS) b, d, and f.

In Table III some features of solid phase peptide synthesis (SPPS), liquid phase peptide synthesis (LPPS), and alternating liquid-solid phase method (ALS) are compared. Certainly the method in its present form is improvable. Especially the employment of less cross-linked polystyrenes[11] as well as chemically different supports and anchor groups will be investigated. However, in its present stage of development, the procedure can advantageously be used in a single-step application, when in a conventional solution synthesis a sequence-dependent low coupling yield leads to difficulties in separation of the resulting two peptides.

Table III

Comparison of Solid Phase (SPPS), Liquid Phase (LPPS), and Alternating Liquid-Solid (ALS) Peptide Synthesis

	SSPS	*LPPS*	*ALS*
Determination of yield of deblocking amino groups	low accuracy -	low accuracy -	not necessary ++
Deprotonation of amino groups	prior to coupling −	during coupling +	during coupling +
Determination of coupling yields	low accuracy -	high accuracy +	high accuracy +
Prerequisite on coupling yield	must be 100% -	must be 100% -	should be 98% +
Removal of excess amino acid	very easy ++	easy +	less easy -
Clean-up of crude peptide	very necessary -	necessary±	not necessary ++

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β-PHENACYL ESTERS OF ASPARTYL PEPTIDES TO MINIMIZE SUCCINIMIDE FORMATION DURING HF TREATMENT

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IN THE COURSE OF THE solid phase synthesis[1] of an active fragment, H-Arg-Glu-Leu-Glu-Asp-Gly-Thr-Pro-Arg-OH, of bovine growth hormone (125-133), a side reaction in the Asp-Gly sequence was encountered. This side reaction was shown to be the formation of a succinimido derivative during the HF cleavage step. Imide formation in Asp(OBzl)-Gly peptides has been reported to occur in HBr-TFA[2] and in HF[3] and these strong anhydrous acids also promote cyclization in other aspartyl sequences.[3,4] It was known,[2,4] however, that Asp-Gly or Asp-Ser sequences containing a free β-carboxyl showed little tendency to undergo cyclization under these same conditions. Consequently a synthetic strategy to circumvent this rearrangement was designed in which the protecting group on the β-carboxyl of aspartic acid was removed *prior* to the acid cleavage step. For that purpose aspartic acid was incorporated as its β-phenacyl ester (OPac) and was deprotected by sodium thiophenoxide before cleaving the peptide from the resin support by HF.

The sequence dependence of succinimide formation was examined for a series of small β-benzyl aspartyl peptides and then the effect of replacing the benzyl ester by a β-phenacyl ester was determined. To that end several model di-, tri-, and tetrapeptides related to the parent growth hormone sequence were synthesized by standard solid phase procedures, using the benzyl group for side chain protection of Asp, Glu, Ser, and Thr. The peptides were cleaved and deprotected by HF containing 10% anisole at 0° for 1 hr. The di- and tripeptides were separated from the corresponding imides by preparative paper electrophoresis at pH 6.5 in 0.1 *M* pyridine-acetic acid buffer (Figure 1). The

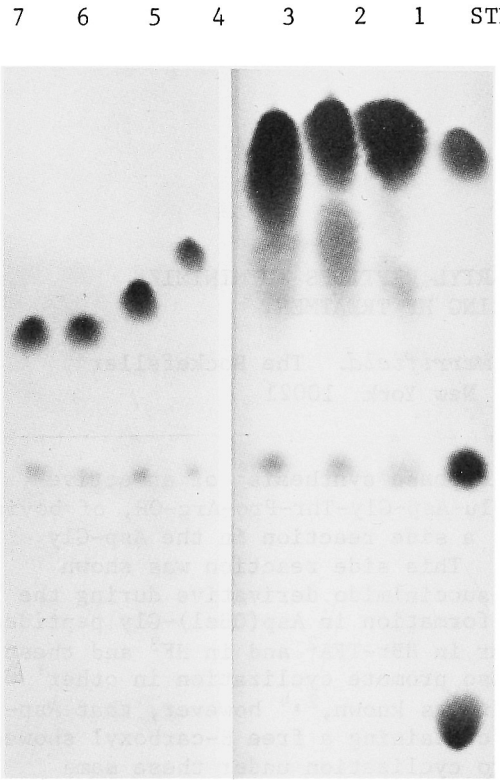


Figure 1: Preparative paper electrophoresis of HF-treated di- and tripeptides. (pH = 6.5, pyridine-acetic acid 0.1 *M*).

relative proportions of α-peptides and imides were estimated by amino acid analysis of acid hydrolyzates of eluted peptides (Table I). Most of these peptides showed little tendency to undergo the acid catalyzed succinimide formation, ranging from 1.4 to 11.9%. The α-aspartyl tetrapeptides H-Glu-Asp-Gly-Thr-OH, H-Glu-Asp-Ala-Leu-OH and H-Glu-Asp-Ser-Leu-OH synthesized in the same way were separated from the aspartimide peptides and quantitated on a Dowex 50 column (55x1 cm, at 55°) using pH 3.25, 0.2 *N* sodium citrate buffer for 100 min followed by pH 4.25 (0.2 *N* sodium citrate buffer) (Table II). The peptides were isolated on a similar column by elution with 0.1 *M* pyridine-acetate buffer. The imide-containing peptides were then hydrolyzed in 1% aqueous Et_3N for 24 hr to give a mixture of α- and β-peptides.

Table I

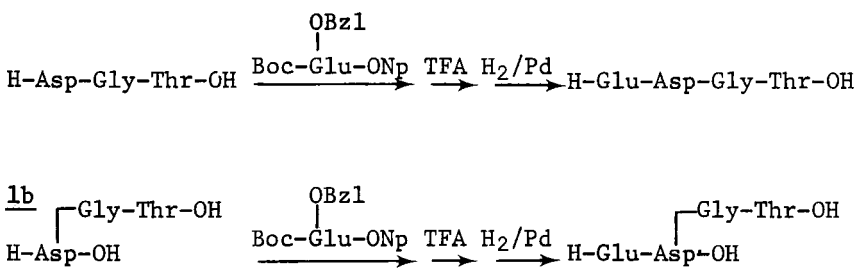
Relative Amounts of Imide and Desired Peptide

Peptide-Resin[a]	*Desired Peptide*[b]	*Imide Peptide*[b]
H-Asp(OBzl)-Gly-R	98.6	1.4
H-Glu(OBzl)-Asp(OBzl)-Gly-R	96.6	3.4
H-Asp(OBzl)-Gly-Thr(Bzl)-R	98.4	1.6
H-Glu(OBzl)-Asp(OBzl)-Ala-R	92.2	7.8
H-Glu(OBzl)-Asp(OBzl)-Ser(Bzl)-R	88.1	11.9
H-Asp(OBzl)-Ala-Leu-R	95.9	4.1
H-Asp(OBzl)-Ser(Bzl)-Leu-R	96.7	3.3

[a] For the relative size of spots, see Figure 1. Due to the presence of free C-terminal amino acids, neutral spots were much more ninhydrin-positive than their actual peptide content.

[b] Based on amino acid content of the individual spot. The N-terminal residue was used to calculate percentages.

These isomers were quantitated and isolated on the same Dowex 50 columns (Table III). They were characterized by amino acid analyses, digestion with leucine aminopeptidase, and aminopeptidase *M*, and by comparison with standard tetrapeptides that were synthesized according to Scheme 1.



Scheme 1.

Table II

Distribution of Tetrapeptides[a]

Peptide	Peptides after HF Cleavage (%)						Peptides in Et_3N Hydrolyzate of Imide %		
	β-Benzyl-Asp Peptide			β-Phenacyl-Asp Peptide[b]					
	α	β	Imide	α	β	Imide	α	β	Imide
Glu-Asp-Gly-Thr	1	0	99	97.6	0	2.4	29	71	0
Glu-Asp-Ala-Leu	75	0	25	95.8	0	4.2	26	74	0
Glu-Asp-Ser-Leu	75	0	25	90.5	0	9.5	20	80	0

[a]Using the ion exchange column described in Table II.

[b]The phenacyl ester groups were removed in sodium thiophenoxide prior to HF cleavage.

Table III

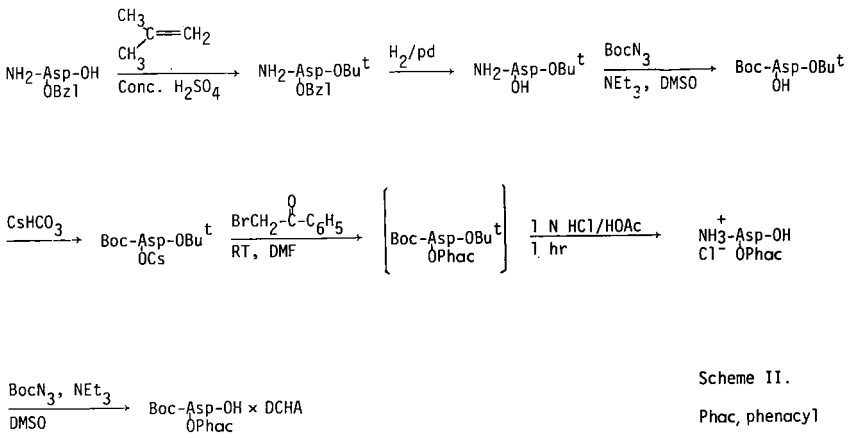
Elution Times of the Model Tetrapeptides

Peptide	*Elution Time (min)*[a]		
	β-*Peptide*	α-*Peptide*	*Imide*
Glu-Asp-Gly-Thr	54	70	116
Glu-Asp-Ala-Leu	102	130	139
Glu-Asp-Ser-Leu	97	131	157

[a]Separations were made on a Beckman Automatic Amino Acid Analyzer (Model 121, long column, 55 cm). Starting buffer was Durrum Pico A for 100 min (pH = 3.25, 0.2 N Na^+) followed by Beckman Buffer (pH = 4.25, 0.2 *N* sodium citrate). Flow rate for both buffers was 66 ml/hr. Column was maintained at 55°.

These tetrapeptides, in which the aspartyl residue was protected by a β-benzyl ester, were very extensively cyclized under the HF cleavage conditions, ranging from 99% for the peptide containing the Asp-Gly sequence to 25% for those containing either Asp-Ala or Asp-Ser. The rearrangement was, therefore, dependent on the steric and electronic effects as determined both by sequence and position of the Asp-X bond within the peptide. When both termini were in peptide bonds the cyclization was much more extensive than when one or both ends were not in amide linkage. Thus H-Asp(OBzl)-Gly-Thr(Bzl)-R gave only 1.6% while H-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-R gave 99% imide. Analog studies have eliminated the requirement for Glu, Ser or Thr residues in this side reaction and hence the involvement of lactone formation or N to O shifts to account for the altered mobility of products. In all three cases examined, the tetrapeptide imides opened in base to give 70 to 80% of the β-aspartyl peptide and 30 to 20% of the α-isomer.

To test the new synthetic strategy, N^{α}-Boc-β-phenacyl aspartic acid [Boc-Asp(OPac)-OH] was prepared according to *Scheme 2*. It was used to synthesize the tetrapeptide resins H-Glu(OBzl)-Asp(OPac)-Gl-Thr(Bzl)-R, H-Glu(OBzl)-Asp(OPac)-Ala-Leu-R, and H-Glu(OBzl)-Asp(Opac)-Ser(Bzl)-Leu-R. The phenacyl group was removed by treatment with 1 *M* sodium



Scheme II.

Phac, phenacyl

Scheme 2: Phac, phenacyl.

thiophenoxide in DMF at 25° for 8 hr.[5-8] Then the peptide-resins, containing a free β-carboxyl group, were cleaved and deprotected with HF-anisole at 0° for 30 min. The proportion of imide in the cleavage mixture was again determined by ion exchange chromatography and found (Table II) to be 2.4, 4.2 and 9.5%, respectively. It is unlikely that a significant fraction of the imide was formed during the removal of the phenacyl ester by thiophenoxide since the isolated H-Glu-Asp-Ala-Leu-OH tetrapeptide also yielded 4% of imide following treatment with HF-anisole at 0° for 30 min. The cyclization of the carboxylic acid may depend on an acylium ion intermediate, whereas imide formation from the ester probably does not.

For the Asp-Ala and Asp-Ser peptides this approach resulted in an appreciable decrease in the side reaction and in the case of the Asp-Gly peptide it led to a very significant 40-fold reduction. Although it did not completely eliminate the problem, the selectively removable β-phenacyl ester for the protection of aspartic acid represents an improvement which can be very helpful in the synthesis of certain sequences.

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SIDE-REACTIONS OF GLUTAMYL PEPTIDES IN LIQUID HYDROGEN FLUORIDE/ANISOLE MIXTURES

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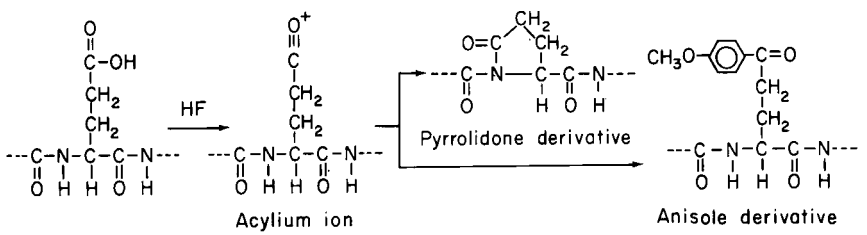
IN RECENT YEARS, PEPTIDES containing multiple glutamic acid residues were found difficult to obtain by solid phase synthesis.[1-3] On several occasions in our hands, glutamyl peptides whose assembly had proceeded smoothly nevertheless gave rise to by-products lacking glutamic acid. The side reactions producing this phenomenon were traceable not, as might be expected, to the coupling steps of the synthesis, but occurred during cleavage of the peptide from the resin in liquid hydrogen fluoride containing anisole as a carbonium ion scavenger.[4] Reaction of anisole with glutamic acid had been postulated for synthetic peptides[5] and native ribonuclease[6] treated with such mixtures and is, in fact, one of two major side-reactions which glutamyl peptides can undergo during cleavage in HF/anisole. Under catalysis by liquid hydrogen fluoride, the γ-carboxyl group of glutamic acid gives an acylium ion[7] which was found to undergo Friedel-Crafts acylation of anisole or to react with the α-amido nitrogen to yield a pyrrolidone derivative.



Conditions of By-Product Formation

The catalysis is highly temperature-dependent, so that by-product formation can be minimized or enhanced by altering the temperature and length of exposure of the peptide to the cleavage medium.

Thus, when the model hexapeptide-resin Bpoc-Leu-Lys(Z)-Glu(OBzl)-Ala-Val-Gly-resin, synthesized by standard solid phase techniques,[8] was treated for 0.5 hr at 0° in a 10% anisole/HF mixture, the resulting hexapeptide Leu-Lys-Glu-Ala-Val-Gly (1A) (see Figure 1) contained a full equivalent of glutamic acid by amino acid analysis, and showed only one component by electrophoresis at pH 6.5. The electrophoretic mobility of the peptide (R_{Lys} 0.17) indicated that the peptide was neutral, and its homogeneity showed that the synthesis itself had proceeded without detectable side-reactions.

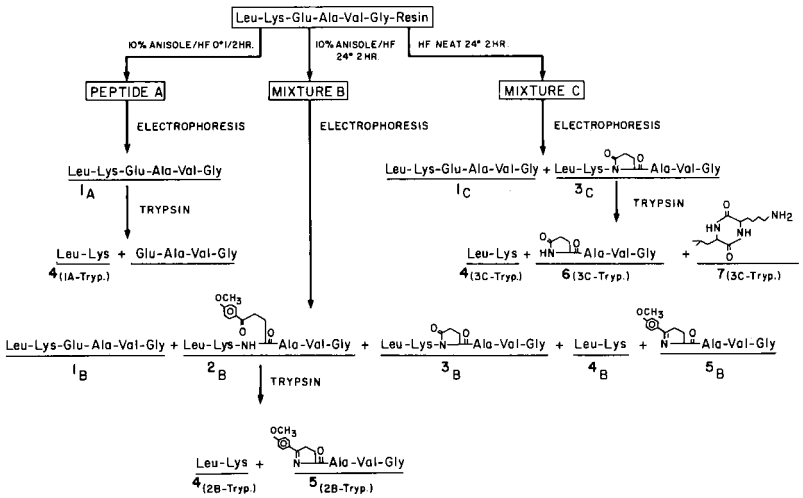


Figure 1: Modifications of a glutamic acid-containing hexapeptide under various conditions.

When the cleavage temperature was raised to 24° and the time extended to 2 hr, the result was a peptide mixture B which was electrophoretically resolved into five distinct peptides. The expected hexapeptide 1B was accompanied by two major by-products: the anisole acylation hexapeptide 2B and the pyrrolidone hexapeptide 3B. The two minor com-

ponents 4B and 5B proved to be breakdown fragments from 2B. When the cleavage was performed at 24° for 2 hr with anisole entirely omitted, a mixture C containing only the expected hexapeptide 1C and the pyrrolidone by-product 3C was produced.

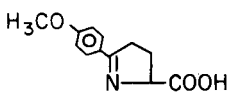
Structure of the Pyrrolidone By-Product

The pyrrolidone hexapeptide by-product 3C, isolated by elution from the electropherogram, had a mobility (R_{Lys} 0.52) indicative of the loss of a carboxyl group. Yet, it gave a full equivalent of glutamic acid when hydrolyzed in 6 *N* HCl and analyzed. When cleaved in weak base containing trypsin, the pyrrolidone 3C gave the expected dipeptide leucyllysine 4(3C-Tryp) and the diketopiperazine 7(3C-Tryp) arising from internal aminolysis by the leucine α-amino group. The tetrapeptide fragment pyroglutamyl-alanyl-valyl-glycine 6(3C-Tryp) had a net negative charge (R_{Lys} -0.48) and was unreactive with ninhydrin, indicating that the amino terminus had indeed reacted with the γ-carboxyl group. The structures of the fragments were further verified by comparison with synthetic standards prepared by independent routes.

Structure of the Anisole By-Product

Like the pyrrolidone, the anisylated hexapeptide by-product 2B had an electrophoretic mobility (R_{Lys} 0.45) indicative of a net positive charge, consistent with a modified carboxyl group. Amino acid analysis of hydrolyzates of 2B gave full equivalents of five residues, but unlike the pyrrolidone, the glutamic acid was not regenerated. By-product 2B exhibited an ultraviolet absorption in water (276 nm, $\varepsilon = 1.72 \times 10^4$) comparable to *p*-methoxyacetophenone in ethanol (276 nm, $\varepsilon = 1.6 \times 10^4$),[9] and the molecule formed an orange derivative with dinitrophenylhydrazine under conditions in which the intact hexapeptide 1, lacking a keto group, did not react.

Cyclization of Anisole By-Products



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The acid hydrolyzate of hexapeptide 2 contained no ninhydrin-positive chemical modification of the original glutamic acid residue. However, the ninhydrin-negative pyrroline carboxylic acid 8 was shown

to be present by chlorine/tolidine spray[10] on a thin-layer chromatogram of the hydrolyzate. This molecule, the cyclic Schiff base form of the anisylated glutamic acid, had an ultraviolet absorption maximum at 262 nm in base that shifted to 310 nm ($\varepsilon = 2.75 \times 10^4$) after acidification, and co-chromatographed with authentic 2-*p*-methoxyphenyl-1-pyrroline-5-carboxylic acid prepared from α-methyl glutamate. This sample was identified by UV, IR, NMR and elemental analysis.

The existence of the pyrroline 8 after 6 *N* acid hydrolysis indicates how strongly the cyclization to the Schiff base is favored when the glutamate amino terminus is free. Thus, when the anisylated hexapeptide 2B was subjected to tryptic digestion, the resulting tetrapeptide 5(2B-Tryp) also terminated in the cyclic form of the derivative. It was unreactive with ninhydrin and was nearly neutral at pH 6.5 (R_{Lys} -0.10). This same pyrroline-terminal tetrapeptide was produced when Boc-Glu(OBzl)-Ala-Val-Gly-resin was cleaved at room temperature in 10% anisole/HF, and its structure was confirmed by coupling pyrroline 8 with alanyl-valyl-glycine.

Quantitation of By-Product Formation

This was accomplished by ion-exchange chromatography of the peptides and, for the anisylated derivatives, by measurement of the ultraviolet absorption of their hydrolyzates. Table I shows that both hexapeptide and tetrapeptide resins gave nearly 80% of their respective anisylated by-products when cleaved for 2 hr at 24° in 10% anisole/HF, with a further 11% being converted to the pyrrolidone by-product in the hexapeptide. Significantly, pure hexapeptide 1A re-treated with HF/anisole at 24° for 2 hr produced nearly the same ratio of by-products as protected peptide-resins originally treated at 24°, indicating that formation of by-products is not dependent on the presence of either resin or protecting groups, but proceeds through an acylium ion intermediate generated from the γ-carboxyl group of glutamic acid. The formation of all by-products was reduced to 1% or less by cleaving at 0° for 0.5 hr, a level of side-reaction which should allow isolation of most peptides containing glutamic acid.

Acknowledgments

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Table I

Effect of Cleavage Conditions on By-Product Formation in HF-Treated Glutamyl Peptides

Cleavage Conditions[a]		Cleavage Yield %	Determined by Column Chromatography Peptide[b] (Mol %)				
			Intact Hexapep. 1	Anisylated Hexapep. 2	Pyrrolidone Hexapep. 3	Leu-Lys 4	Total Anisyl-Glu 2 + 4
Z OBzl Boc-Leu-Lys-Glu-Ala-Val-Gly-Resin							
10% Anisole/HF	0° ½ hr	77	98.8	1.08	0.04	0.0	1.12[c]
10% Anisole/HF	0° 1 hr	37	94.5	4.7	0.4	0.0	5.12[c]
10% Anisole/HF	0° 2 hr	17	91.3	8.3	0.4	0.0	8.3
10% Anisole/HF	24° 1 hr	64	50.6	39.2	6.9	3.2	42.4
10% Anisole/HF	24° 2 hr	73	10.2	66.5	11.1	12.2	78.7
HF (neat)	24° 2 hr	48	31.1	-	66.1	0.0	-
10% Anisole/HF re-treated for	0° ½ hr 24° 2 hr	77	17.0	63.4	10.2	9.3	72.7
Boc-Glu(OBzl)-Ala-Val-Gly-Resin							
10% Anisole/HF	0° ½ hr	67					0.4[c]
10% Anisole/HF	24° 2 hr	57					79.4[c]

a. 100-200 mg of peptide-resin substituted 0.12-0.18 mmoles/g in 10 ml HF mixture.
b. Structures of peptides are shown in Fig. 1.
c. Determined by ultraviolet absorption of peptide hydrolyzate.

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DISCUSSION OF THE PAPER BY R. S. FEINBERG AND R. B. MERRIFIELD

R. Hirschmann

I should like to elaborate briefly on the work by Dr. E. Harris and Miss S. Chodroff to which Dr. Feinberg referred in his lecture (ref. 6, above), which I had reported at the International Peptide Symposium in Madison, Wisconsin two years ago. Harris and Chodroff had clarified the chemistry of one of the side reactions which can occur when peptides containing glutamic acid residues are treated with anhydrous HF in the presence of anisole (10% v/v). That an unwanted reaction can occur had first been reported by S. Sano and S. Kawanishi (ref. 5, above). We observed

the side reaction initially during the HF-catalyzed conversion of poly-γ-benzyl-L-glutamic acid to polyglutamic acid. Harris and Chodroff had shown the side reaction to involve Friedel-Crafts acylation of anisole by UV and NMR spectroscopy. In the case of polyglutamic acid, the extent of ketone formation was determined by comparison of the molecular extinction at 275 nm with that of *p*-methoxypropiophenone prepared from anisole and propionic acid in HF. After 1 hr at 0°, 3.4% of the γ-carboxy residues of polyglutamic acid had undergone Friedel-Crafts acylation, while at 19°, 16.1% had reacted. The extent of acylation by the three glutamic acid residues of the amino-terminal decapeptide of the β-chain of porcine hemoglobin was determined using ^{14}C-labeled anisole. After 1 hr, Harris and Chodroff had found 2.3% and 18.4% Friedel-Crafts acylation at 0° and 19°, respectively. Similarly, after 1 hr at 19°, 0.59 mole of ^{14}C-labeled anisole had been incorporated per mole of ribonuclease A corresponding to a 5.9% reaction of the ten glutamic acid residues of the enzyme. Neither L-alanine or D,L-acetylalanine afforded detectable amounts of ketone after treatment with HF/anisole for 1 hr at 19°. Our results and those reported by Dr. Feinberg this morning are thus in excellent agreement qualitatively and quantitatively for this Friedel-Crafts acylation.

LARGE SCALE SOLID PHASE SYNTHESIS OF ACTH

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THE ADRENOCORTICOTROPIC HORMONE - ACTH - was first obtained from pituitary tissue in 1930.[1] Partial purification was accomplished in 1943.[2,3] Further purification of porcine ACTH was accomplished in 1950 and this material was first offered for clinical use in 1950.[4] In the years following, ACTH from various species were isolated, purified and structures determined. The structure of human ACTH was elucidated in 1961 by the Lee and Lerner group,[5] and revised in 1972 by Riniker *et al.*[6] by changing positions 25-27 and 30 (previously[5]: -Asp-Ala-Gly- and -Gln-) as follows:

H-Ser-	Tyr-	Ser-	Met-	Glu-	His-	Phe-	Arg-	Trp-	Gly-	Lys-	Pro-	Val-	Gly-	Lys-
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Lys-	Arg-	Arg-	Pro-	Val-	Lys-	Val-	Tyr-	Pro-	Asn-	Gly-	Ala-	Glu-	Asp-	Glu-
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Ser-	Ala-	Glu-	Ala-	Phe-	Pro-	Leu-	Glu-	Phe-OH
31	32	33	34	35	36	37	38	39

The solid phase procedure reported here can be used on either of these sequences with slight modifications of protecting groups and purification techniques.

After the introduction of natural porcine ACTH as a drug substance in 1950, the use rose steadily so that in 1954, 200 million international units were used; in 1960, 400 million units were used; and the rate continues to

increase steadily. This continued increase has caused some problems in finding adequate supplies of pituitary glands and it became increasingly desirable to produce a synthetic ACTH for commercial use. With the introduction of solid phase peptide synthesis by Merrifield in 1964,[7] a practical method for the commercial synthesis of the total ACTH molecule became a possibility and our group embarked on this endeavor.

After many years of failures and successes, a manufacturing procedure was arrived at for the large scale synthesis described in this typical run.

Three hundred-forty (340) grams of Boc-L-phenylalanine resin with a titer of 0.35 meq/gm was loaded into a five-liter reaction vessel of a large scale peptide synthesizer. The modified large volume solvent delivery systems are programmed using commercially available peptide synthesizers.

The general method of attachment of each amino acid residue comprises deprotection by trifluoroacetic acid, washing to remove excess acid, neutralization with organic base, washing to remove formed salt, coupling of the next amino acid using dicyclohexylcarbodiimide or an active ester coupling in the case of asparagine or glutamine residues, and finally washing to remove excess reagents and by-products. The synthesis is carefully monitored at each coupling by use of the ninhydrin test previously described.[8]

The yield of resin peptide after final deprotection, washing and drying was 890 grams (64.9% based on first amino acid titration value). The finished peptide is removed from the resin by treatment with anhydrous hydrogen fluoride using 1 liter Kel-F reaction vessels.

The crude peptide (391 g, 87% based on peptide resin) was purified on columns containing microcrystalline carboxymethyl cellulose. ACTH was absorbed on the CMC column and fast running impurities eluted with 30 to 40 column volumes of 4 millimhos ammonium acetate buffer at 6.7 pH. The active ACTH peak was then eluted with 3 millimhos ammonium acetate buffer at 7.5 pH. A typical elution pattern is shown in Figure 1. The ACTH is under the shaded portion.

After a concentration step, the material was finally subjected to gel filtration on a column of G-25 Sephadex, affording purified ACTH (71.2 g, 18.2% based on crude product). Table I shows the amino acid analysis of five large scale batches of synthetic ACTH. Vertical polyacrylamide slab gel electrophoresis showed essentially single bands for each preparation. Analytical Sephadex G-25 (superfine) chromatography (1 mg sample, 0.9 x 100 cm column, OD 280) showed typically one peak with very minor shoulders

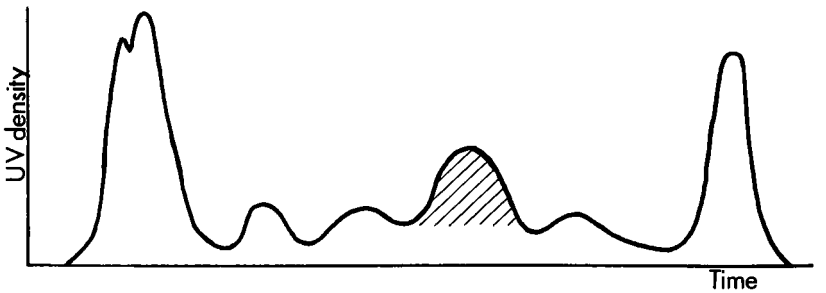

Figure 1: Typical elution pattern of column chromatography of crude synthetic ACTH on carboxymethyl cellulose using 4 m*M* and 3 m*M* ammonium acetate, pH 6.7 and 7.5, respectively. ACTH elutes in shaded area.

Table I

Amino Acid Analysis[a] of Batches of Synthetic ACTH, Prepared by Large Scale Solid Phase Synthesis

		Found				
	Moles Theory	*744005 N10*	*658236 R9*	*658247 W9*	*691074 P8*	*691075 T10*
Aspartic Acid	2	2.11	2.04	1.99	2.02	2.00
Serine	3	2.79	2.82	2.85	2.83	2.81
Glutamic Acid	5	5.09	5.00	5.01	4.98	4.93
Proline	4	4.08	4.03	4.11	3.99	4.02
Glycine	3	3.03	2.98	2.94	3.01	2.94
Alanine	3	3.03	3.05	2.98	3.02	3.02
Valine	3	2.97	3.04	3.00	2.94	2.98
Methionine	1	.98	.94	.93	.94	.91
Leucine	1	1.04	1.03	1.02	1.03	.98
Tyrosine	2	1.88	1.91	1.90	1.93	1.91
Phenylalanine	3	3.05	2.98	3.03	3.02	2.96
Histidine	1	1.05	1.03	.97	.97	1.04
Lysine	4	3.97	4.04	4.04	4.06	3.95
Arginine	3	3.06	3.01	2.96	2.96	3.06

[a] 6 N HCl - 20 hours hydrolysis Durrum Amino Acid Analyzer.

on each side. Analytical gradient carboxymethylcellulose chromatography (Figure 2) showed one peak.

The biological activity by U.S.P. assay of two samples of highly purified natural human ACTH was 104 $\pm$ 10 units

per milligram and 112 ± 9 units per milligram. The bioassays of various lots of purified synthetic ACTH are shown in Table II. Pharmacological and clinical studies have shown these synthetic products to be equal in steroidogenesis with natural porcine ACTH.

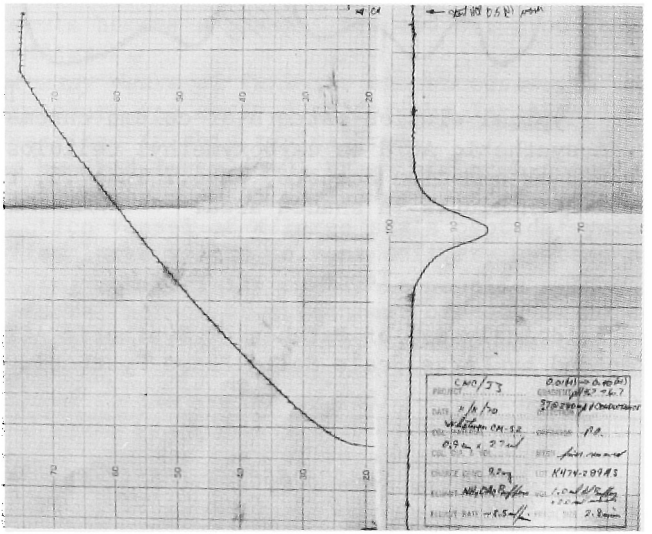

Figure 2: Analytical gradient carboxymethyl cellulose chromatography of synthetic ACTH. Column 0.9 x 10 cm; Whatman CM-52; buffers: 0.01 molar ammonium acetate - pH 4.7 and 0.40 molar ammonium acetate - pH 6.7; gradient: linear with respect to molarity and metered at 4.5 bed volumes per hour; detection: 280 nm recording spectrophotometer.

Table II

Biological Activity (Ascorbic Acid Depletion Assay) of Different Lots of Purified Synthetic ACTH

Lot Number	*U.S.P. ACTH Activity Units Per Mg*
528267 - C9	125 ± 18
691029 - R9	103 ± 15
688028 - W9	123 ± 11
691042 - 110	106 ± 11
658260 - N10	114 ± 13
691058 - R10	129 ± 16
691118 - E11	119 ± 13
691126 - F11	99 ± 12
691141 - O11	125 ± 15

Weighted mean assay of at least two samples.

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LARGE SCALE SOLID PHASE PEPTIDE SYNTHESIS OF THYROTROPIN-RELEASING HORMONE AND LUTEINIZING HORMONE-RELEASING HORMONE

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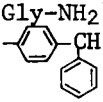
THYROTROPIN-RELEASING HORMONE (TRH) and luteinizing hormone-releasing hormone (LH-RH) have been synthesized on a large scale (50 grams of resin) with reported yields after purification, TRH 60% and LH-RH 40% based upon the amount of C-terminal amino acid substituted on the resin. The synthetically produced TRH and LH-RH have full biological activities, as measured on *in vitro* bioassays, and are active at dosage levels as low as 0.3 ng. The large scale solid phase syntheses of TRH (<Glu-His-Pro-NH_2) and LH-RH (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) were accomplished using benzhydrylamine resin, P-  -CH NH_2 (BHA) (0.5 meq-NH_2/g) as the solid support. The C-terminal Boc-amino acid was coupled onto the resin by the Merrifield procedure. The peptides were synthesized using the appropriate protected amino acids. Aoc-L-Arg(Tos) and Z-<Glu were the only amino acids used that were not Boc-protected. The resulting protected peptide resins, Z-<Glu-His(Tos)-Pro-BHA and Z-<Glu-His(Tos)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-BHA, were treated with anhydrous HF in the presence of pure anisole and the crude peptides purified with suitable column chromatography, to afford pure TRH and LH-RH.

TRH and LH-RH have been synthesized by the Merrifield procedure, by classical peptide chemistry, and by a combina-

tion of classical and solid phase techniques. The references list 44 publications describing the various syntheses of <Glu-His-Pro-NH_2[1] and <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2[2], 22 each.

We have synthesized TRH and LH-RH in high yield, using the benzhydrylamine resin[1,u] (BHA) prepared in-house as the solid support, with the routine Merrifield procedure on a large scale peptide synthesizer.

In the synthesis of TRH, Boc-Pro (2.5 molar excess) was attached to a benzhydrylamine resin (50 g, 0.5 meq/g) using DCC as the coupling reagent. The success of the coupling reactions was monitored by the semi-quantitative ninhydrin or the fluorescamine test.[3] The following steps were used to couple Boc-His(Tos) and Z-<Glu to the Boc-Pro-BHA resin:

1) Washing with $CHCl_3$ (3 x 400 ml)
2) Prewashing with 50% TFA in $CHCl_3$ (1 x 400 ml)
3) Deprotection with 50% TFA in $CHCl_3$ (1 x 400 ml, 20 min)
4) Washing with $CHCl_3$ (3 x 400 ml)
5) Washing with 33% dioxane in CH_2Cl_2 (3 x 400 ml)
6) Washing with CH_2Cl_2 (3 x 400 ml)
7) Prewashing with 5% diisopropylethylamine in CH_2Cl_2 (1 x 400 ml)
8) Neutralization with 5% diisopropylethylamine in CH_2Cl_2 (1 x 400 ml, 10 min)
9) Washing with CH_2Cl_2 (5 x 400 ml)
10) Protected Boc-amino acid (2.5 molar excess) in DMF (50 ml) and CH_2Cl_2 (250 ml) was added
11) DCC in CH_2Cl_2 (0.5 M, 125 ml) was added and the reaction time was up to 320 min
12) Washing with CH_2Cl_2 (3 x 400 ml).

The resulting Z-<Glu-His(Tos)-Pro-BHA resin was washed well with 50% TFA in $CHCl_3$ (1 x 400 ml), $CHCl_3$ (3 x 400 ml) and then absolute EtOH (1 x 400 ml). After drying *in vacuo* overnight, Z-<Glu-His(Tos)-Pro-BHA resin (59.7 g) was collected. A part of this protected tripeptide BHA resin (4.7 g) was cleaved by HF (40 ml) in the presence of anisole (20 ml) for one hour at room temperature. After evaporation *in vacuo* to dryness, the reaction mixture was

washed well with anhydrous ether (3 x 50 ml) and the desired product dissolved in MeOH (200 ml), the resin filtered off, and the organic solvent evaporated to afford the crude peptide (560 mg). The peptide was purified on a silica gel column with elution by MeOH-$CHCl_3$ (3:7 v/v), giving the pure TRH (430 mg, 60% yield based on a capacity of 0.5 meq/g of BHA resin), with $[\alpha]_D^{20}$ - 42.4° (c 1.0, MeOH). TLC (silica gel showed one single spot, positive to Pauly and I_2 reagent. The R_f was identical with authentic peptide[1j] in three different solvent systems: R_f^1 0.23 (n-BuOH:HOAc:EtOAc:H_2O 1:1:1:1); R_f^1 0.64 ($CHCl_3$:MeOH:NH_4OH 60:45:20), and R_f^3 0.41 (EtOH:H_2O 7:3). Amino acid analysis showed: Glu 0.99, His 0.99, Pro 1.02, and NH_3 1.00.

In the synthesis of LH-RH, Boc-Gly (2.5 molar excess) was attached to benzhydrylamine resin (44.8 g, 0.5 meq/g), using DCC as the coupling reagent. To this Boc-Gly-BHA resin the following protected amino acids were successively added: Boc-Pro, Aoc-Arg(Tos), Boc-Leu, Boc-Gly, Boc-Tyr(Bzl)-Boc-Ser(Bzl), Boc-Trp, Boc-His(Tos) and Z-<Glu. A 2.5 molar excess of each protected amino acid was used. The conditions for introducing the protected amino acids were the same as those described above. After coupling of the Trp moiety, the removal of the Boc-group using 50% TFA in $CHCl_3$, was carried out in the presence of 1% indole and 1% mercaptoethanol. The desired protected decapeptide BHA resin, Z-<Glu-His(Tos)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(Tos)-Pro-Gly-BHA, was dried *in vacuo* (78.2 g). A part of this resin (2 g) was cleaved by HF (20 ml) with anisole (4 ml) and mercaptoethanol (0.5 ml) at room temperature for one hour, and the decapeptide (681 mg) was collected. This was purified twice on a CM-cellulose column with elution by NH_4OAc solution (0.025M to 0.2M) to give pure LH-RH (273 mg, yield 40% based on the capacity of BHA resin). TLC (silica gel) shows one single spot which is identical with the authentic sample:[2h] R_f^1 0.49 (EtOAc:Pyridine:HOAc:H_2O 5:5:1:3) and R_f^2 0.64 (n-BuOH:HOAc:H_2O:EtOAc 1:1:1:1), positive spot with Pauly and I_2 reagents. $[\alpha]_D$ -45.1° (c 0.61, 1% HOAc), amino acid analysis (6N HCl, 110°C during 16 hr, 72.3% recovery of amino acids): Ser 0.92, Glu 1.05, Pro 1.08, Gly 2.04, Leu 1.06, Tyr 0.82, His 0.97 and Arg 1.03.

The synthetic TRH and LH-RH from the large scale peptide syntheses were quantitatively examined by Dr. C. Y. Bowers for the characteristic hormonal activities and potencies in comparison with the Tulane standard samples. Data on the *in vitro* bioassays[4,5] of releasing TSH, LH and FSH from the synthetic TRH and LH-RH were obtained. The synthetically produced TRH and LH-RH have full biological activities and are active at dosage levels as low as 0.3 ng.

Acknowledgments

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SECTION IV

BIOLOGICALLY ACTIVE PEPTIDES

A NEW ROUTE TOWARD THE SYNTHESIS OF HUMAN INSULIN

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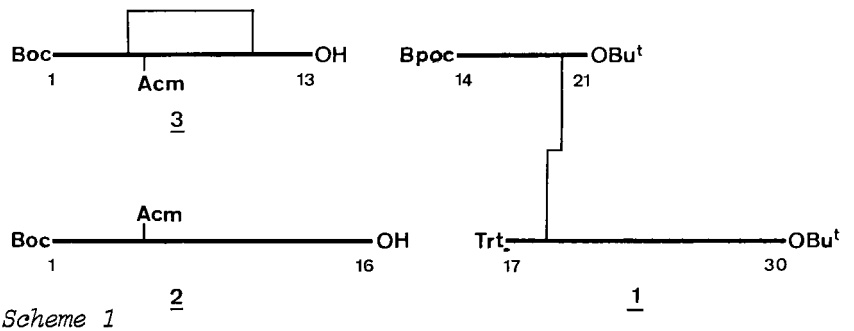
THE CHARACTERISTIC FEATURE OF THE new route we have elaborated for the synthesis of human insulin is the stepwise manner of forming the three disulfide bridges. This was accomplished by way of a fragment-condensation approach, Scheme 1. The open-chain, asymmetrical cystine peptide A(14-21)-B(17-30) (1), already containing the disulfide



Scheme 1

bridge between A20 and B19, constituted the amino component in two fragment condensations, first with B(1-16) (2) and secondly with A(1-13) (3). In the latter fragment, the "loop" from A6 to A11 was already present. Finally, the third disulfide bond between A7 and B7 was formed.

A number of requirements had to be fulfilled before this method of synthesis could be successfully put into practice:

1. First, the formation of the disulfide bonds in 1 and 3 had to take place under conditions in which the other protecting groups present would not be attacked.
2. Second, the cystine peptide 1 had to have protecting groups at its amino ends that could be cleaved off selectively, permitting the two chains to be elongated step by step.
3. Third, the synthesis of the A(1-13) fragment (3) depended on there being two selectively removable sulfur protecting groups because a precursor of this compound contained three cysteine residues (A6, A7 and A11), of which two (A6 and A11) had to be selectively converted to a cystine residue.
4. Finally, the formation of the disulfide bridge A7-B7 had to take place in the presence of two already existing disulfide bonds.

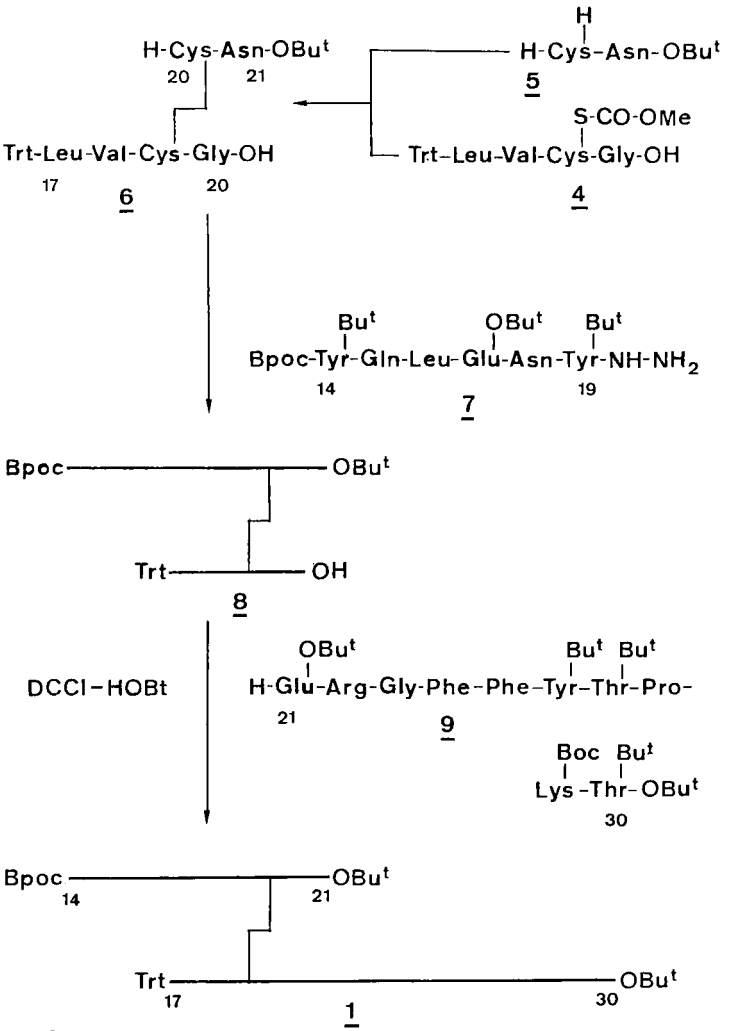
As it so happened, it proved possible to satisfy all these requirements employing only protecting groups already long in use in peptide chemistry, *i.e.*, *t*-butylester, N-Boc, and *t*-butylether for "permanent" protection (His and Arg side chains being unprotected), and methyl ester, N-Z, N-Trt, N-Bpoc,[1] S-Trt,[2,3] and S-Acm[4] for temporary protection. It was necessary, however, to develop new techniques of cleavage. In particular, conditions had to be found under which N-trityl could be removed in the presence of Bpoc, and Bpoc in the presence of "permanent" protecting groups of the *t*-butyl type. We also developed a new technique for the transformation of two S-trityl cysteine residues into cystine without attacking the S-Acm cysteine residue.

Synthesis of A(14-21)-B(17-30), Intermediate 1
Scheme 2

The asymmetrical cystine peptide A(20-21)-B(17-20), compound 6, was obtained by way of an already published reaction sequence[5,6] featuring the conversion of a sulphenylthiocarbonate (4) with the thiol derivative 5. Condensation with the fragments A(14-19), compound 7, and B(21-30), compound 9, yielded intermediate 1. 1 and 8 were purified by countercurrent distribution.

Selective Cleavage of Nα-trityl in the Presence of Bpoc in Trifluoroethanol[7]

Kinetic data relating to the acidolytic cleavage of Nα-trityl and Bpoc in 90 percent trifluoroethanol



Scheme 2

at "pH" 4* in some model peptides are given in Table I. It is evident that the rate of cleavage of the trityl group varies considerably according to the amino acid involved,

*pH denotes the value determined with a combined glass electrode. This value is about 2.5 units lower than that obtained with the same concentration of acid in water. During the reaction, the pH was kept constant with HCl in 90% trifluoroethanol by means of a pH-stat.

Table I

Selective Acidolytic Cleavage of Trityl, Bpoc, and Boc Amine Protecting Groups in Trifluoroethanol

Reagent / *Model Peptide*	90% TFE at pH 4*			90% TFE at pH 1*		
	$K_1(h^{-1})$	K_{rel}	t/2 (min)	$K_1(h^{-1})$	K_{rel}	t/2 (min)
Trt-Val-Val-Ome	138	900	0.3			
Trt-Leu-Val-OMe	52	350	0.8			
Trt-Gly-Val-OMe	7.4	50	5.6			
Bpoc-Val-Val-OMe	0.15			32	ca.	1.3
Bpoc-Leu-Val-OMe'	0.15	1		38	2500	1.1
Bpoc-Gly-Val-OMe	0.20			31		1.3
Boc-Leu-Val-OMe				0.013	1	
Z-Lys(Boc)-OMe				0.02	1.5	

*Determined with combined glass electrode.

whereas that of Bpoc is more or less constant. Except when trityl is bound to glycine, its cleavage takes place about 350 times more rapidly than that of Bpoc. (In 80% acetic acid the ratio is 7:1 only.[8]) This difference in reactivity affords a satisfactory degree of selectivity as regards cleavage for preparative purposes.

Selective removal of the Bpoc group in the presence of "permanent" protecting groups of the *t*-butyl type is attained in trifluoroethanol at "pH" 1 (Table I). The established factor of approximately 2,500:1.5 at pH 1 guarantees that the reaction takes place smoothly. The conditions employed in the synthesis of insulin for the selective release of the amino groups at Leu B17 and Tyr A14 are indicated in Scheme 5.

Synthesis of B(1-16), Boc-Phe-Val-Asn-Gln-His-Leu-Cys(Acm)-Gly-Ser(But)-His-Leu-Val-Glu(OBut)-Ala-Leu-Tyr(But)-OH, Intermediate 2

This sequence was obtained by condensation of fragments B(1-8) and B(9-16) (DCCI-HOBt) and subsequent hydrolysis of the methyl ester. Throughout the synthesis, difficulties arose owing to the poor solubility of the intermediate products, which were particularly serious in preparing fragment B(1-8).

Selective Oxidation of S-trityl in the Presence of S-Acm

The direct conversion of S-tritylcysteine peptides and S-Acm cysteine peptides to cystine peptides with the aid of iodine was described some years ago.[9,10] The influence exerted by various solvents was studied, and it turned out that trifluoroethanol was an ideal medium for the discrimination of these two sulfur protecting groups.

Scheme 3 depicts an experiment in which the different behavior of the two protecting groups was demonstrated. An equimolar mixture of the S-trityl derivative a and the S-Acm derivative b was subjected to oxidation. In methanol, in which the two groups reacted at approximately the same rate, a statistical mixture of the three cystine peptides c, d and e was obtained. When trifluoroethanol was used as solvent, on the other hand, 95% of a was converted to its symmetrical oxidation product c and only 5% to the asymmetrical derivative d. Compound e was not detected, that is to say, 95% of b was recovered unchanged.*

Ac-Gly-Cys(Trt)-Gly-OMe

a

Z-Phe-Cys(Acm)-Gly-OMe

b

Ac-Gly-Cys-Gly-OMe
|
Ac-Gly-Cys-Gly-OMe — c

Ac-Gly-Cys-Gly-OMe
|
Z-Phe-Cys-Gly-OMe — d

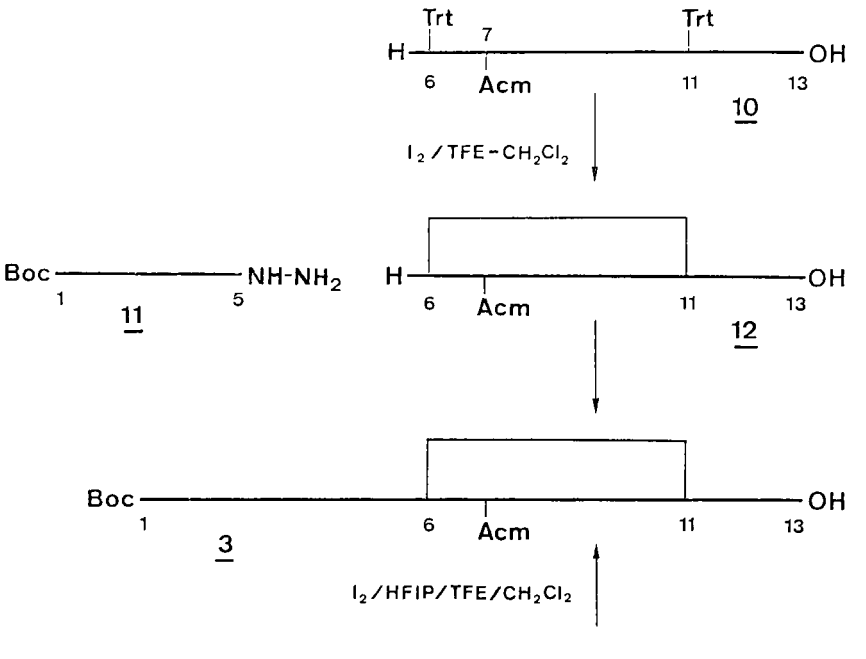
Z-Phe-Cys-Gly-OMe
|
Z-Phe-Cys-Gly-OMe — e

Scheme 3

Synthesis of A(1-13), Intermediate 3

The application of the foregoing reaction in the synthesis of 3 is illustrated in Scheme 4. Closure of the cysteine ring was effected at the stage of both sequence A(6-13),

*Since under these conditions, b alone likewise yields no e, it is obvious that S-Acm is resistant to iodine, but is attacked to a slight extent by an intermediate product of the conversion of a to c, most probably its sulphenyliodide derivative.



Scheme 4

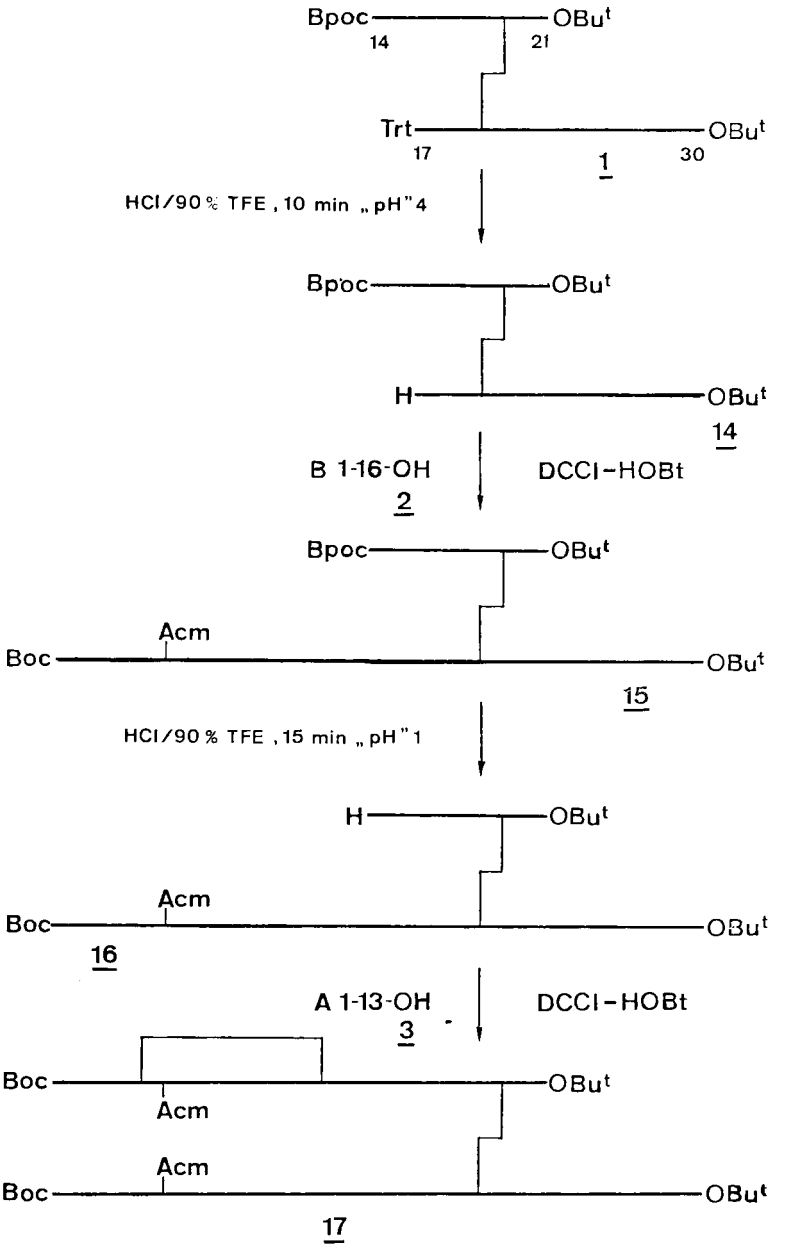
compound 10, and sequence A(1-13), compound 13. Since the latter compound was insufficiently soluble, even in trifluoroethanol, in this case the peptide was added to the iodine solution in hexafluoroisopropanol (HFIP).* In the iodine oxidation reaction HFIP behaves similarly to trifluoroethanol towards S-trityl and S-Acm. The crystalline product 3 was obtained with a yield of 65% in relation to fragments 10 and 11.

Condensation of Fragments 1, 2 and 3 and Final Steps in the Synthesis

The liberation of the amino groups from Leu-B17 and Tyr-A14 and the linkage of fragments 1, 2 and 3 are illustrated in Scheme 5. Both 15 and 17 were purified by countercurrent distribution.

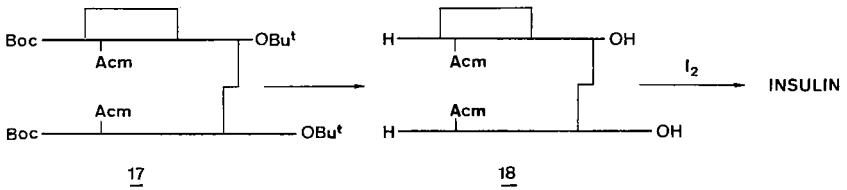
The conversion of 17 to insulin was now feasible in either of two ways. The formation of the cystine bridge

*HFIP vapors are very toxic, and great caution is indicated when using HFIP.


Bpoc
OBu^t
14
21
Trt
OBu^t
17
30
1
HCl/90 % TFE , 10 min „pH" 4
Bpoc
OBu^t
H
OBu^t
14
B 1-16-OH
2
DCCI-HOBt
Bpoc
OBu^t
Acm
Boc
OBu^t
15
HCl/90 % TFE , 15 min „pH" 1
H
OBu^t
Acm
Boc
OBu^t
16
A 1-13-OH
3
DCCI-HOBt
Boc
OBu^t
Acm
Acm
Boc
OBu^t
17

Scheme 5

from A7 to B7 could be effected either before or after the removal of the "permanent" protecting groups. Tentative experiments proved that both these procedures were practicable. Since the "free" peptide 18 (Scheme 6) permitted a further purification step by countercurrent distribution, we chose this way in the first instance for preparative purposes. Sixty percent acetic acid proved to be a suitable solvent for the iodine oxidation reaction. At this stage it was important to protonate 18 with a mineral acid, as otherwise a slight degree of iodination would take place. Pure synthetic insulin could now be isolated from the crude oxidation product by way of two countercurrent distributions in two different systems. It could then be readily crystallized in the form of a zinc complex by a procedure described in the literature.[11]



17 18

Scheme 6

Characterization of Synthetic Human Insulin and Biological Activity

Amino acid analysis of the synthetic peptide and natural extractive human insulin revealed no differences. The identity of the two substances was further confirmed by thin-layer chromatography and electrophoresis in various systems. The synthetic and the natural hormone also showed an identical pattern of enzymatic breakdown by trypsin, chymotrypsin, subtilisin and thermolysin, as determined by thin-layer chromatography.

Synthetic and natural human insulin possessed identical biological activity in the rat in two *in vivo* systems, the first measuring the hypoglycemic effect and the second the increase in diaphragm glycogen.

In this synthesis of human insulin, the disulfide bonds have been formed for the first time in separate and distinct chemical reaction steps. The procedure we employed also differs fundamentally from the approach involving cross-linked chains, recently elaborated in various laboratories[12-15] in which the correct pairing of the sulfur-sulfur bonds of the six cysteine residues is ensured by the "natural" folding of the reduced peptide chains. A conformational

aid of this sort is not necessary in the method described, very probably not even in the formation of the disulfide bridge between A7 and B7. This may be of some significance in the synthesis of insulin analogues ("unnatural" insulins).

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STUDIES ON THE SYNTHESIS OF OVINE INSULIN

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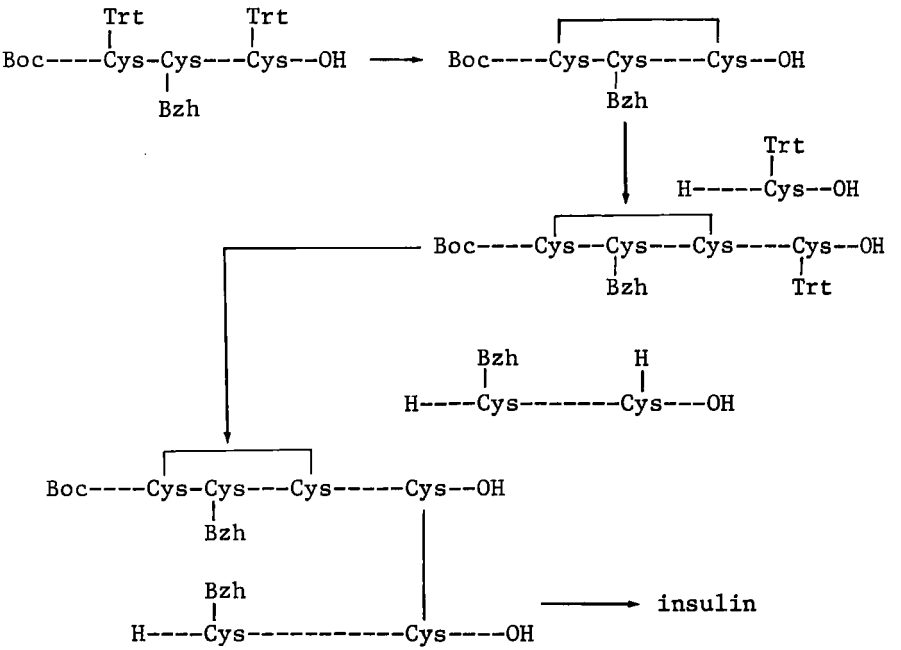
THE DEVELOPMENT OF METHODS FOR THE direct conversion of appropriately protected cysteine side chains to correctly paired cystine residues in a polypeptide chain has opened routes to the total synthesis of natural polypeptides containing two or more sulfur-sulfur bonds.[1] The schemes developed for this conversion have utilized the reactivity of certain thioethers, hemi- or dithioacetals, and thiazolidine derivatives of peptide-found cysteine residues toward such reagents as thiocyanogen, alkyl sulfenylthiocyanates, iodine, or carbomethoxysulfenyl chloride.[1,2]

The relative rates of reaction between, for example, *S*-benzhydryl [Bzh] and *S*-tritylthioethers [Trt] of cysteine and thiocyanogen are sufficiently different to allow the selective oxidative removal of the latter in the presence of the former (a similar difference in reactivity also exists between the *S*-acetamidomethyl [Acm] and *S*-trityl derivatives). This difference in reactivity has been exploited in the synthesis of a model *tris*-cystine peptide;[3] the scheme was tested as a possible route for the synthesis of insulin or various analogs and forms the basis for the strategy of the present study.

The key features of the synthetic scheme involved the preparation of an A chain containing an A6-11 cystine loop and *S*-protective groups of differing lability at A7 and A20. The preparation of this material in the model scheme utilized the difference in reactivity of *S*-trityl and *S*-benzhydryl thioethers of cysteine toward thiocyanogen; cyclization of the counterpart of the A6-11 loop in the model system proceeded smoothly and in good yield.[2,3] Extension

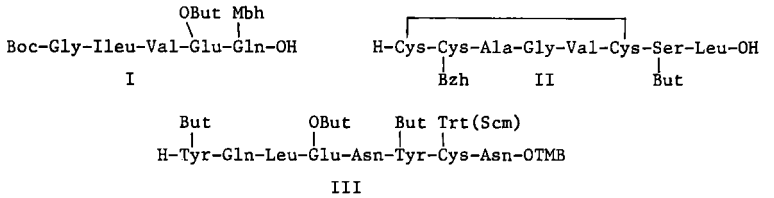
of the A-chain by coupling of the loop fragment to the COOH-terminal portion of the A-chain provided the polypeptide containing the desired features (Scheme 1). The second stage of the synthetic scheme required the preparation of a suitably protected B-chain containing differential protection at positions in the model corresponding to the Cys-B7 and Cys-B19 residues in insulin. Combination of the A- and B-chains by the Cys-A20 to Cys-B19 disulfide bond again utilized the difference in reactivity of the *S*-trityl and *S*-benzhydryl thioethers toward thiocyanogen. The final stage, the formation of the Cys-A7 to Cys-B7 sulfur-sulfur bond involved the action of thiocyanogen on the *S*-benzhydryl thioethers under more stringent conditions.



Scheme 1

The preparation of the three peptide fragments required for the application of this scheme to the actual synthesis of the insulin A-chain were subsequently reported.[2,4] These included the NH_2-terminal pentapeptide derivative I; the A6-13 fragment containing the cystine loop and a protected Cys-A7 residue, II; and the COOH-terminal portion of the A-chain, the octapeptide derivative, III, containing either

the *S*-trityl protective group or the *S*-carbomethoxysulfenyl [Scm] group at Cys-A20.



The present report describes the synthesis of the fully protected A-chain from these fragments and the preparation of a B-chain derivative from natural insulin suitable for the projected synthetic route.

Fragment coupling of I (as the *N*-hydroxysuccinimide ester) with II provided the A1-13 peptide fragment in 54-74% yield. The tridecapeptide derivative (V) was generally insoluble and could not be characterized by TLC or electrophoretic techniques; however, amino acid analyses were in good agreement with the proposed structure (Table I).

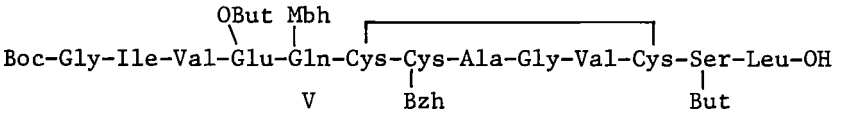


Table I

Analytical Data on A_{1-13} Fragment Amino Acid Analysis[a]

	106 hr	*36 hr*	*36[d] hr*	*Theory*
$CySO_3H$	b	c	2.30,2.37	3
Ser	0.54,0.57	0.87,0.84	0.88,0.82	1
Glu	2.00,2.12	2.18,2.12	2.10,2.15	2
Gly	2.00,2.01	2.06,2.00	1.95,2.00	2
Ala	0.94,1.00	1.00,0.97	1.00,1.02	1
Val	1.96,2.09	1.61,1.56	1.56,1.60	2
Ile	0.95,1.01	0.64,0.64	0.61,0.62	1
Leu	0.92,0.98	1.00,0.97	0.93,0.95	1

a Anisole added to the peptide before hydrolysis.
b 1.2,1.3 residues cystine/2 detected.
c 1.93,1.87 residues cystine/2 detected.
d Peptide oxidized with performic acid prior to hydrolysis.

The preparation of the fully protected A-chain of ovine insulin was achieved by fragment condensation of V and III using the dicyclohexylcarbodiimide *N*-hydroxybenzotriazole or *N*,*N*-carbonyldiimadazole methods. The A-chain obtained by either route was soluble in chloroform-methanol and could be chromatographed on Sephadex LH-20 and studied in various TLC systems. The analytical data obtained by amino acid analysis of the A-chain derivative is reported in Table II; the ratios of Asp/Ala and Tyr/Gly as well as the homogeneity of various preparations of the synthetic protected A-chain derivative in several TLC systems support the purity of the polypeptide derivative.

Table II

Analytical Data on A_{1-21} Amino Acid Analysis[a]

	36[b] hr	*36[c] hr*	*Theory*
Asp	2.00,2.00	2.00,2.00	2
Ser	0.87,0.84	0.81,0.86	1
Glu	4.13,4.01	4.24,4.19	4
Gly	2.01,2.00	1.93,1.97	2
Ala	1.03,1.05	1.00,1.00	1
Cys[d]	-- --	-- --	4
Val	1.68,1.64	1.63,1.55	2
Ile	0.65,0.67	0.55,0.55	1
Leu	2.04,2.03	1.96,1.95	2
Tyr	2.10,2.08	1.87,1.90	2

a Conducted in the presence of phenol.
b Via DCC-HOBt
c Via CDI
d Performic acid oxidation followed by hydrolysis indicated 3.25 residues of $CysO_3H$.

The preparation of a B-chain derivative of natural insulin which could be combined with the synthetic A-chain to generate first the A20 to B19 and in a later step the A7 to B19 sulfur-sulfur bonds (Scheme I) was achieved in the following manner. The reduced dithiol form of the B-chain (obtained from insulin by oxidative sulfitolysis and reduction) was allowed to react with 1.1 equiv. of acetamidomethylcarbinol in TFA at 0°. The reaction mixture was then treated with sodium sulfite/sodium tetrathionate and the mixed *S*-acetamidomethyl/*S*-sulfonate B-chain was purified

by chromatography on a DEAE Sephadex A-25 column equilibrated with 0.1 *M* Tris buffer (pH 8.0) in 7 *M* urea. The mixture could be resolved into several components (Figure 1); peak D-1 eluted at 70-120 ml and was shown by attempted *S*-carboxymethylation and amino acid analysis to be the dialkylated B-chain derivative, B-$(Acm)_2$. Fraction D-2 eluted at 220-380 ml and consisted of monoalkylation products, B-(7-Acm, 19-SSO_3) and B-(7-SSO_3, 19-Acm); fraction D-3 consisted of unreacted B-chain as shown by the fact that two *S*-carboxymethylcysteine residues could be detected by amino acid analysis after treatment of this fraction with β-mercaptoethanol followed by iodoacetic acid.

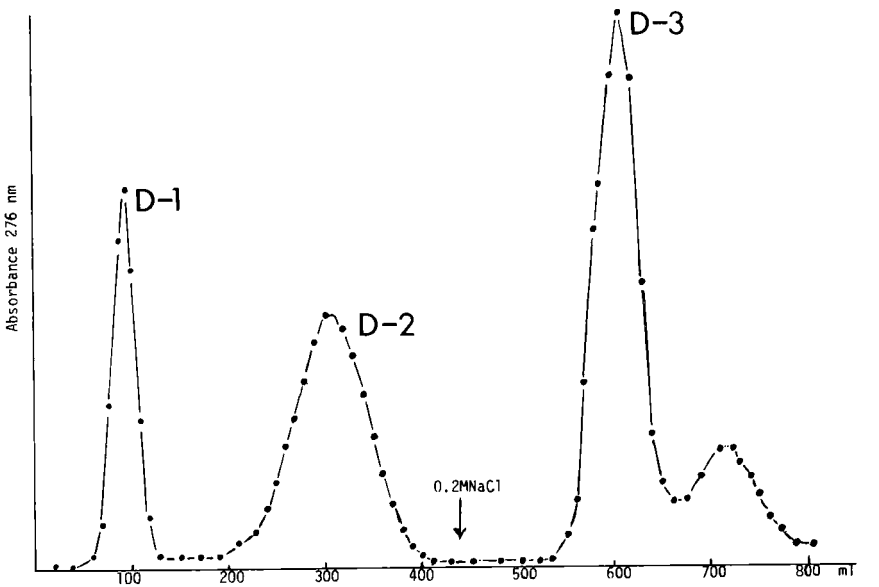


Figure 1: Chromatography of 7,19-bis-*S*-acetamidomethyl B-chain [B$(Acm)_2$] of bovine insulin on a column (1.6 x 95 cm) of DEAE-Sephadex A-25. Eluant: 0.1 *M* Tris-HCl, 7 *M* urea, pH 8.0; 0.2 *M* NaCl after fraction 44.

Resolution of the D-2 fraction into the Cys(Acm)-B7 and Cys(Acm)-B19 derivatives was accomplished by chromatography on a phosphocellulose column (Figure 2). The D-2 fraction (as the *S*-sulfonate derivative) was dissolved in 0.05 *M* Na citrate buffer (pH 3.7) containing 7 *M* urea; elution from the column with a 0-0.15 *M* sodium chloride gradient provided two fractions, P-1 and P-2, which were pooled, collected, and desalted using a Sephadex G-25 column with 5% acetic acid as the eluant. P-1 was shown to be B-(19-Acm, 7-SSO_3); P-2 was identified as B-(7-Acm, 19-SSO_3). The ratio of P-1/P-2 was 1.71 indicating some regiospecificity in the alkylation reaction.

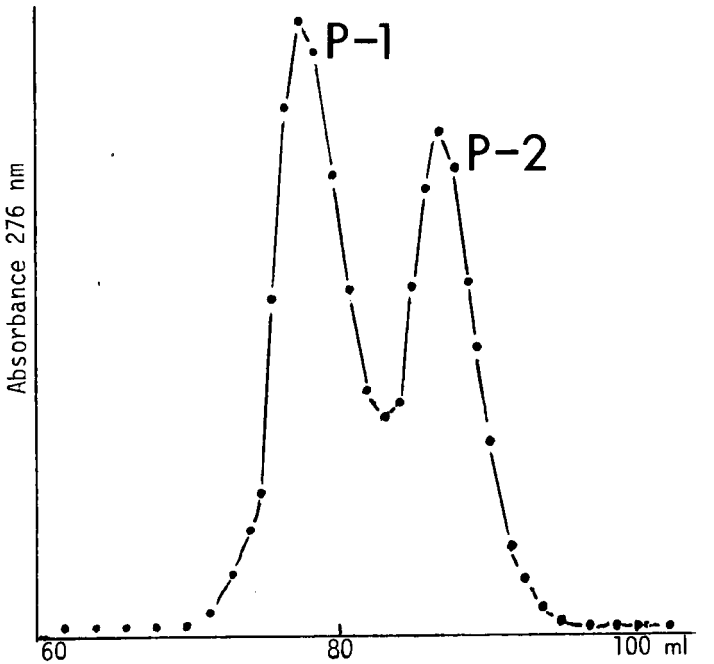


Figure 2: Chromatography of fraction D-2 (Figure 1), mixed *S*-acetamidomethyl/*S*-sulfonate B-chain, on Phosphocellulose (BioRad). Column size: 1.5 x 90 cm. Eluant: 0.05 *M* Na citrate, 7 *M* urea, pH 3.7 with a gradient of 0-0.15 *M* NaCl.

The structure of P-1 and P-2 were established by fingerprinting studies on the tryptic digests of the *S*-aminoethylated [SAE] derivatives of the two fractions, (P-1-SAE, P-2-SAE). Each fraction was dissolved separately in Tris

buffer (pH 8.7) containing 7 *M* urea and treated with β-mercaptoethanol to liberate the thiol from the polypeptide. *S*-Aminoethylation was achieved by the addition of excess ethyleneimine to each solution; after 30 min the reaction was quenched with acetic acid and desalted on a Sephadex G-25 column. The amino acid analysis of various SAE-B-chain derivatives is given in Table III.

Table III

Amino Acid Analysis of *S*-Aminoethylated B-Chain Derivatives

	$B(SAE)_2$	D_2SAE	P_1SAE B(19-Acm,7-SAE)	$P_2(SAE)$ B(7-Acm,19-SAE)
Asp	1.00	0.99	0.98	1.07
Thr	0.89	0.95	0.97	0.92
Ser	0.83	0.82	0.98	1.10
Glu	3.13	3.02	3.10	3.06
Pro	0.97	1.26	1.25	0.99
Gly	3.00	3.00	3.03	3.06
Ala	2.01	2.00	1.99	1.96
Cys	--	0.25	0.24	0.66
Val	2.96	2.98	3.02	2.84
Leu	3.91	3.94	4.04	3.86
Tyr	1.94	0.77	1.95	1.87
Phe	2.86	2.72	2.88	2.70
Lys	1.01	1.00	1.05	1.03
His	1.91	2.03	2.08	2.15
Arg	1.00	1.00	1.00	1.00
Cys-SAE	1.71	0.86	0.85	0.89

Treatment of each SAE-B-chain derivative (4 mg) in 0.1 *M* ammonium bicarbonate buffer, pH 8.3, with trypsin (enzyme: substrate ratio, 1:40) was carried out for 5 hr at 25°. The solutions were lyophilized and applied to Whatman No. 3 paper for descending chromatography using *n*-butanol-water-acetic acid (4:5:1) as the developing solvent. After 16 hr the papers were dried and used for electrophoresis in the second dimension. Electrophoresis was conducted in pyridine-acetic acid buffer (pH 3.7) using 3700 V for 1 hr. The paper was dried, dipped in cadmium acetate-ninhydrin reagent and dried overnight. The developed peptide spots were cut from the paper sheet and the peptide eluted from the paper with 1 *N* hydrochloric acid solution. The solutions were lyophilized and the residues hydrolyzed with 6 *N* hydrochloric acid

prior to amino acid analysis. The results of these studies are given in Table IV. The fact that P-1-SAE represented the B-chain derivative containing the acetamidomethyl group at Cys-B19 with Cys-B7 as the thiol (prior to conversion to the *S*-aminoethyl derivative) was clearly evident since the tripeptide B-20-22, present in $B(SAE)_2$ and P-2-SAE, was not observed in the map of P-1-SAE. However, the heptapeptide B1-7 produced by tryptic hydrolysis of either $B(SAE)_2$ or P-1-SAE could be isolated. The identity of P-2-SAE (and hence P-2) followed from the absence of B1-7 peptide (indicating alkylation by acetamidomethyl cation at Cys-B7) and the appearance of B20-22 (indicating the presence of the *S*-aminoethyl group at Cys-B19). Seely *et al.*[6] noted that alkylation of the insulin B-chain with excess acetamidomethylcarbinol in TFA provided significant amounts of the *C*-alkyl derivative of tyrosine. The present results (Tables III, IV) indicate that this problem can be circumvented using lowered quantities of the alkylating agent.

These studies have provided sufficient quantities of the synthetic A chain and a suitable B chain derivative for further studies on the combination of the A- and B-chains by formation of the Cys-A20 to Cys-B19 disulfide bond. This should afford an H-peptide derivative of insulin similar to that prepared by the CIBA-GEIGY group. The conversion of the H-peptide to ovine insulin can be accomplished by the iodine oxidation method employed in their recent elegant total synthesis of human insulin.[7]

Acknowledgment

This investigation has been supported by grant AM-03416 from the National Institute of Arthritis, Metabolism and Digestive Diseases, U.S. Public Health Service.

Table IV

Amino Acid Compositions of Peptides Isolated from Tryptic Hydrolysis of SAE-B-Chain Derivatives

	Color with Ninhydrin	*$B(SAE)_2$ 7,19-SAE*	*$B(Acm)_2$ 7,19-Acm*	*19-Acm,7-SAE*	*$P_2(SAE)$ 7-Acm 19-SAE*
1-7	red		a		a
Phe		0.35[c]		0.28[c]	
Val		0.52		1.00	
Asn		1.00		1.00	
Gln		1.07		1.03	
His		0.93		0.83	
Leu		0.97		0.93	
SAE-Cys		0.67		0.63	
23-29	yellow				
Gly		0.45[c]	0.55[c]	0.55[c]	0.24[c]
Phe		2.00	2.00	2.00	2.00
Tyr		0.93	0.96	0.93	0.97
Thr		1.01	1.04	1.11	1.04
Pro		1.05	1.17	1.25	1.05
Lys		0.97	0.92	1.06	0.93
20-22	yellow		a	a	
Gly		0.41[c]			0.27[c]
Glu		1.00			1.00
Arg		0.93			0.51
30	red				
Ala		b	b	b	b

a Peptide was not present in the two-dimensional map of the tryptic digest.

b Present in two-dimensional map, but not eluted and quantitated.

c Amino acid partially destroyed by the ninhydrin reagent used to locate the peptide.

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A1-B1-CROSS-LINKED INSULINS FOR STRUCTURE-FUNCTION AND REDUCTION-REOXIDATION STUDIES*

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IN THE INSULIN MOLECULE THE α-amino group of A1-Gly is very close to the ε-amino group of B29-Lys, whereas from the α-amino group of B1-Phe it is separated by 20 Å.[1] A1-B29-cross-linked insulin derivatives are structurally very similar to insulin and comparable to proinsulin with respect to reduction/reoxidation.[2-6]**

The objective of the work presented here was to cover the comparatively large distance between the *N*-termini with bridges of varying length and to study the effect of these modifications on the properties of the hormone and the ability of the derivatives to readopt their structure upon disruption and reformation of the disulfide bonds.

Insulin Derivatives

$(Boc)_2$-Dsu-$(ONp)_2$, Ad(Lys(Boc)-ONp)(Lys(Boc)-ONp), and Z-Lys-ONp, Ad, Z-Lys-ONp

---

*Abbreviations: Dsu = L,L-2,7-diaminosuberoyl, Ad = adipoyl.

**And references therein.

were prepared for use as bifunctional reagents. The first should be cleavable by Edman degradation, the second by trypsin and the third by deprotection[7] before reoxidation followed by Edman degradation thereafter. The protected derivatives I,[8] III and IV (see Table I) were prepared[2] by reacting beef insulin with 1.2 equivalents of the activated esters in DMSO in the presence of N-methylmorpholine. They were isolated in homogeneous form by gel filtration on Sephadex G-50 fine in 10% acetic acid and subsequent chromatography of the monomeric material (yields about 50%) on SP-Sephadex in 1.5 *M* HOAc/7 *M* urea by gradient elution (0.05 to 0.2 *M* NaCl). II and IV were obtained by deblocking I and III with TFA.

Table I

Properties of A1-B1 Cross-Linked Insulins

	Derivative	*Bridge*		*Yield*	*% Biological Activity*
		Atoms	*Maximal Length(Å)*	*%*	*(Fat Cells[9])*
I	$(Boc)_2$-Dsu	8	11	8	0.3
II	Dsu	8	11		1.5-2.6
III	Ad[Lys(Boc)]$_2$	12	14.5	5	0.4
IV	Ad$(Lys)_2$	12	14.5		1.5
V	$(Z\text{-}Lys)_2$(Ad)	20	25	4	0.4

Independent of the length of the bridge, the *in vitro* potencies (stimulation of lipogenesis[9]) of the derivatives (Table I) are extremely low. A slight increase is observed after deprotection. The CD spectra (Figure 1) are rather similar and differ from that of insulin with respect to the 195, 222 and 274 nm ellipticities which are considerably reduced. Only V in the far UV appears to be more insulin-like, the superelevated 208 nm band being in part due possibly to the blue shift of the lowest wavelength band. It appears that the long bridge is accommodated more easily. It cannot be specified from the present data to what proportion the observed spectral differences are resulting from differences in the tertiary structure or in the association behavior as a consequence. Addition of Zn^{++} produced spectral changes in all derivatives similar to those observed with unmodified insulin. Thus, the ability to form hexamers seems to be preserved although one of the sites the bridge is attached to is part of the dimer/dimer interface.[1]

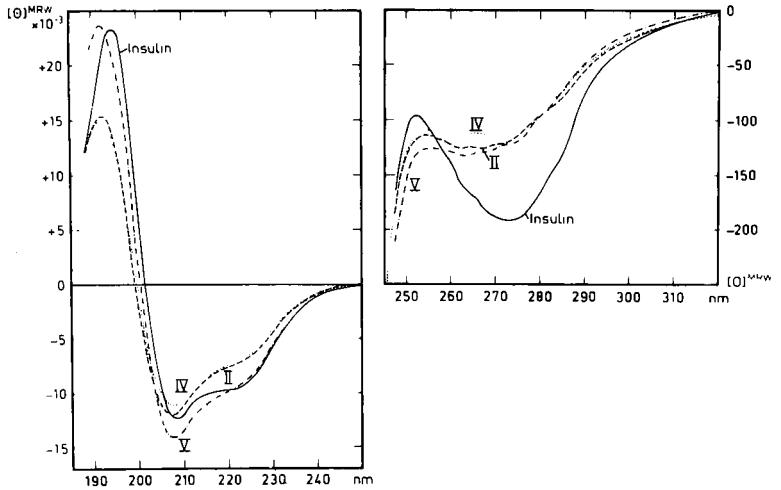

Figure 1: CD spectra of A1-B1 cross-linked insulin derivatives and unmodified insulin. Dsu-insulin (II) ————, Ad(Lys)$_2$-insulin (IV) · · · ·, (Z-Lys)$_2$(Ad)-insulin (V) — · — · —, insulin ———. C ≃ 3·10^{-5}*M*, 0.025 *M* Tris, HCl (pH 7.8), zinc-free solution.

Reductions/Reoxidations

Compounds II, IV and V were reduced and isolated as described for A1-B29 cross-linked insulins.[3] During a reoxidation experiment at an analytical scale[6,10] the CD spectrum (Figure 2) developed toward that of II although identity was not achieved, indicating that correct folding should have occurred to a substantial extent. Preparative air oxidations (0.05 *M* NH_4HCO_3/1·10^{-6} *M* Cu^{++}) with 5-25 mg of derivative gave products the fractionations of which are shown in Figure 3a. Formation of polymers and oligomers could be reduced by lowering the concentration. At 2 nanomoles per ml the amount of monomers isolated (II', IV', V') decreased with increasing length of the bridge. Reoxidations were also carried out with the glutathione redox system[11] (70-80 equivalents GSSG/350-400 equivalents GSH per molecule; 0.08 *M* Tris/HCl ph 8; 24°C; 4 hr). For II and IV the elution patterns were similar to those obtained after air oxidation at three times lower concentrations (see Figure 3b). The products of V were insoluble.

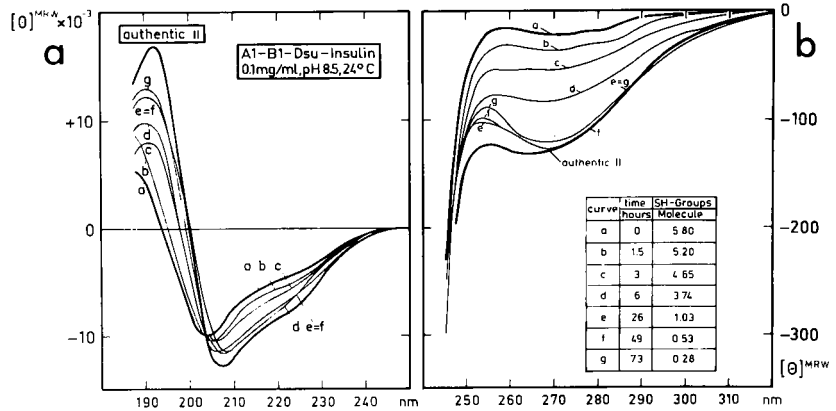

Figure 2: (a) Far UV CD spectrum and (b) near UV CD spectrum and average number of free SH-groups (insertion) in the course of the air reoxidation of reduced Dsu-insulin.

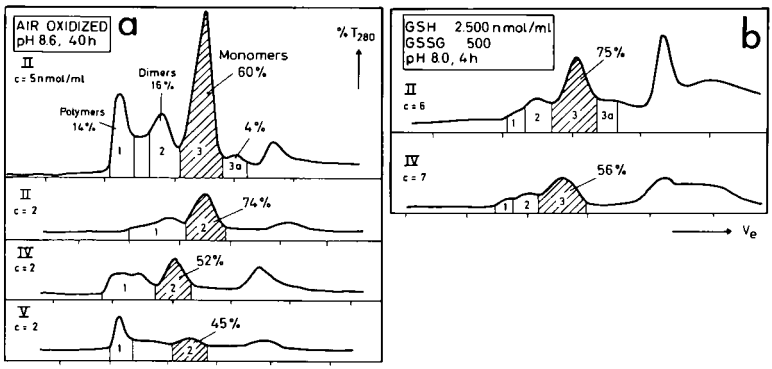

Figure 3: Gel filtration of the reoxidation products obtained from the reduced derivatives II, IV and V: (a) oxidation by air; (b) oxidation by glutathione redox system. Sephadex G-50 fine, 10% acetic acid, column 1.5 x 150 cm. Values given are percentage of total protein eluted.

A comparison of Figures 1 and 4 shows that the CD spectra of the air reoxidized monomers II', IV' and V' are only partially corresponding to those of the authentic derivatives. Not the longest bridge (in V) but on the contrary the shortest one (in II) seems to be more favorable for refolding. The spectra of the monomeric fractions isolated from the thiol-disulfide interchange system were comparable (not depicted).

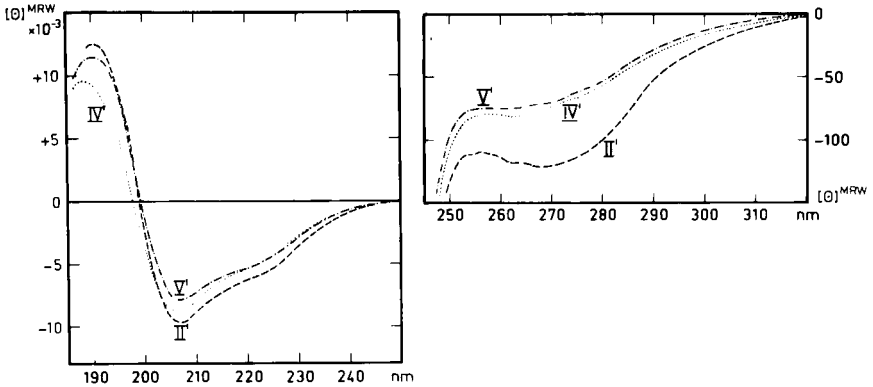

Figure 4: CD spectra of the monomeric fractions of the air reoxidation products of A1-B1 cross-linked insulins. Dsu-insulin (II') – – – –, Ad(Lys)$_2$-insulin (IV') · · · ·, (Z-Lys)$_2$(Ad)-insulin (V') – · – ·. c ≈ 3 · 10^{-5} *M*, 0.025 *M* Tris, HCl (pH 7.8), zinc-free solution.

To remove the Dsu-cross-link, II was reacted with 50 equivalents of phenylisothiocyanate in 90% pyridine for 4 hr at 25°C. Fully active crystalline insulin containing 10-20% of the B29-phenylthiocarbamoyl derivative was obtained after TFA cleavage of the crude reaction product. Edman degradation of II' (60% yield) gave a partially crystalline product with about 30% insulin activity.

Discussion

Going from A1-monosubstituted to A1-B29-cross-linked and further to A1-B1-cross-linked insulin derivatives, an increasingly pronounced loss of biological activity is observed which for the latter is almost complete. Since A1 is not likely to be centrally involved in receptor binding the reduction of activity should be due to conformational consequences of the cross-link. The strain on the molecule should increase in the order V < IV < II if it were merely dependent on the length of the bridges. This is reflected in the CD spectra to some extent (Figure 1). However, additional effects must be considered, as for instance indicated by the increase of activity upon deprotection.

With respect to the reoxidation behavior, it seemed plausible to expect that the greater the strain on the original conformation the more a derivative would tend to avoid it in refolding. Contrary to this expectation the elution pattern and the spectral quality of the monomeric products

were best for II with the shortest cross-link. Active insulin crystals were obtained from II' demonstrating that correct recombination of A1-B1 cross-linked chains is feasible. Though less efficient than A1-B29 cross-linking, these findings represent a basis for an alternative approach to the strategy of insulin synthesis.

Acknowledgment

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SYNTHESIS OF THE DOCOSAPEPTIDE CORRESPONDING TO THE ENTIRE AMINO ACID SEQUENCE OF PORCINE MOTILIN

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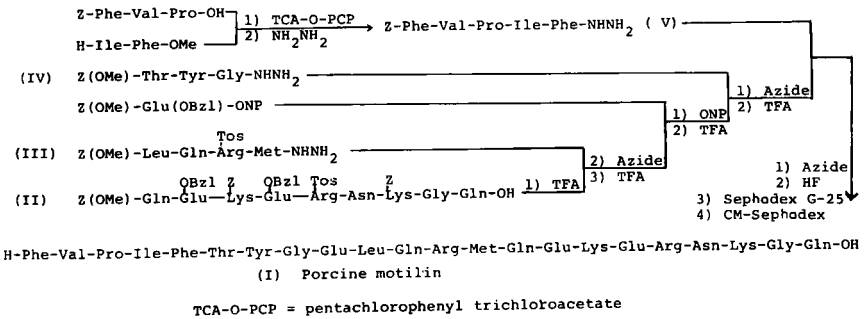
THE ENTIRE AMINO ACID SEQUENCE OF porcine motilin, a gastric motor activity stimulating polypeptide (I), was determined by Brown *et al.*[1] in 1974, after a minor correction of their 1973 formula.[2] The Gln residue, instead of Glu, was placed at position 14 in their new formula.

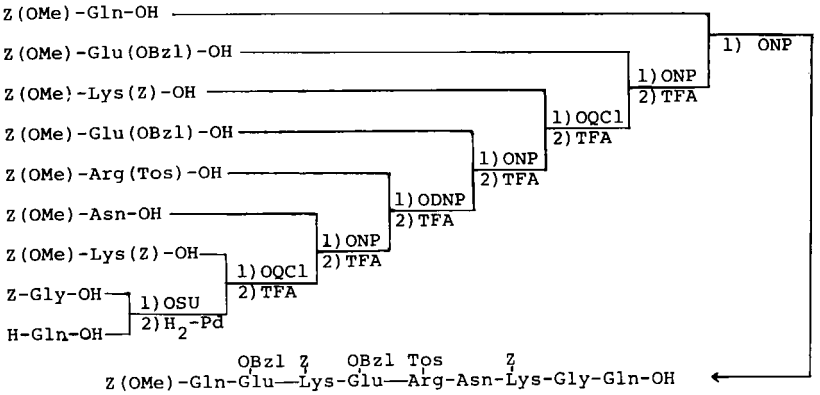
We completed the synthesis of the docosapeptide corresponding to the newly revised sequence of motilin by the method (Scheme I) different from that employed by Wünsch *et al.*[3] who synthesized [13-Nle, 14-Glu]-motilin in 1973.

The COOH-terminal protected nonapeptide, Z(OMe)-Gln-Glu-(OBzl)-Lys(Z)-Glu(OBzl)-Arg(Tos)-Asn-Lys(Z)-Gly-Gln-OH (II), was synthesized in a stepwise manner by the active ester procedure, starting from H-Gly-Gln-OH.[4] The *p*-methyloxybenzyloxycarbonyl Z(OMe) group removable by trifluoroacetic acid (TFA)[5] served as the temporary protection of respective



Scheme 1: Synthesis of motilin.

amino acids incorporated. Z(OMe)-Asn-OH, Z(OMe)-Gln-OH and Z(OMe)-Glu(OBzl)-OH were introduced to the peptide chain by the *p*-nitrophenyl ester procedure,[6] Z(OMe)-Lys(Z)-OH by the 5-chloro-8-quinolyl ester procedure[7] and Z(OMe)-Arg(Tos)-OH by the 2,4-dinitrophenyl ester procedure[8,9] as shown in Scheme II.

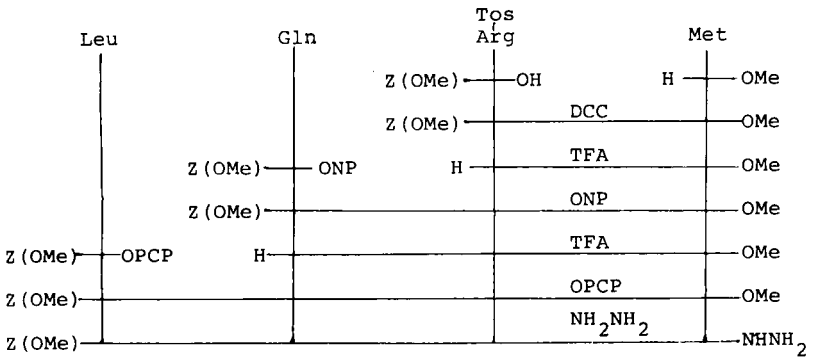


Scheme II: Synthesis of protected nonapeptide, Z(OMe)-(motilin 14-22)-OH.

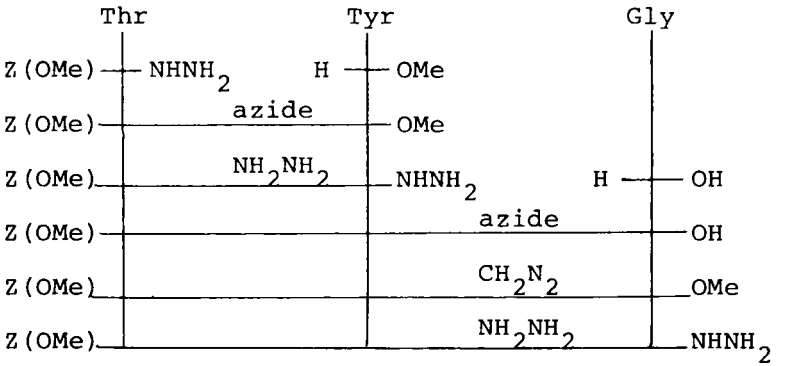
For the synthesis of the protected tetrapeptide hydrazide, Z(OMe)-Leu-Gln-Arg(Tos)-Met-$NHNH_2$ (III), the stepwise elongation method was similarly employed starting with Z(OMe)-Arg(Tos)-Met-OMe, which was obtained by the dicyclohexylcarbodiimide (DCC) condensation procedure.[10] The *p*-nitrophenyl ester and pentachlorophenyl ester procedure[11] were employed for introduction of Z(OMe)-Gln-OH and Z(OMe)-Leu-OH, respectively. The resulting Z(OMe)-Leu-Gln-Arg(Tos)-Met-OMe was treated with hydrazine to give (III) as shown in Scheme III.

In order to prepare Z(OMe)-Thr-Tyr-Gly-$NHNH_2$ (IV), Z(OMe)-Thr-Tyr-$NHNH_2$ was condensed with the triethylammonium salt of Gly and the resulting protected tripeptide was converted to (IV) through the corresponding methyl ester as shown in Scheme IV.

In order to prepare the N-terminal pentapeptide hydrazide, Z-Phe-Val-Pro-Ile-Phe-$NHNH_2$ (IV), Z-Phe-Val-Pro-OH was treated with pentachlorophenyl trichloroacetate[12] to give the corresponding pentachlorophenyl ester, which was allowed to react with H-Ile-Phe-OMe and the resulting pro-



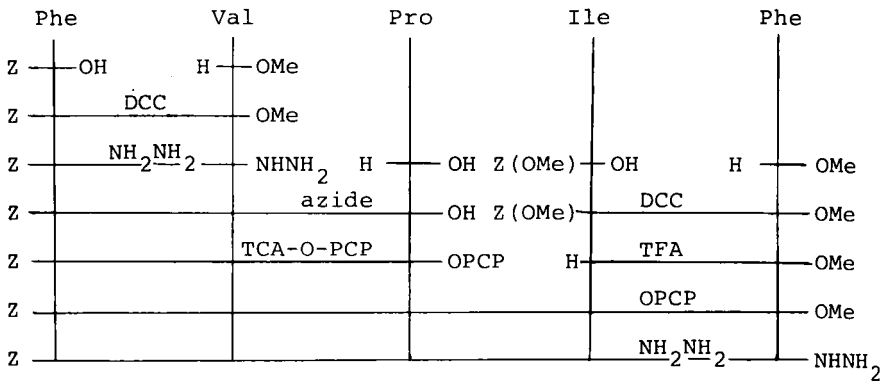
Scheme III: Synthesis of protected tetrapeptide hydrazide, Z(OMe)-(motilin 10-13)-$NHNH_2$ (III).



Scheme IV: Synthesis of protected tripeptide hydrazide, Z(OMe)-(motilin 6-8)-$NHNH_2$ (IV).

tected pentapeptide ester was converted to V in the usual manner as shown in Scheme V.

Construction of the entire amino acid sequence of motilin was carried out as illustrated in Scheme I. Except for the introduction of the Glu(OBzl) residue, each fragment condensation was performed by the modified azide procedure[13] to avoid the risk of racemization. Every intermediate and the fully protected docosapeptide, Z-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu(OBzl)-Leu-Gln-Arg(Tos)-Met-Glu(OBzl)-Lys(Z)-Glu(OBzl)-Arg(Tos)-Asn-Lys(Z)-Gly-Gln-OH, were purified by column chromatography on silica using the solvent system of chloroform-methanol-water (8:3:1, v/v), and characterized by elemental analysis and acid hydrolysis.



Scheme V: Synthesis of protected pentapeptide hydrazide, Z(OMe)-(motilin 1-5)-$NHNH_5$ (V).

Removal of all protecting groups from the above protected docosapeptide was performed by hydrogen fluoride[14] at 0° for 60 minutes. Anisole and Met were used as scavengers. The deblocked product was treated with Amberlite IR-4B (acetate form) and then purified by column chromatography on Sephadex G-25 followed by CM-Sephadex. Elution of the desired peptide was established by 1% acetic acid in the former step and by gradient elution with ammonium acetate buffer (0.2 *M*, pH 6.9) in the latter step. Ammonium acetate in the desired fractions was removed by lyophilization. The synthetic docosapeptide thus purified exhibited a single spot on thin layer chromatography and its homogeneity was assessed by amino acid analysis in an acid and an AP-M hydrolysis.

Upon intravenous infusion to dogs, our synthetic peptide exhibited characteristic contractile activity patterns of the stomach and the duodenum, which were quite similar to those of the burst activity of those organs.

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PROGRESS IN THE SYNTHESIS OF THE PORCINE PANCREATIC SECRETORY TRYPSIN INHIBITOR II (KAZAL)

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THE SYNTHESIS OF SIX SUITABLE protected fragments (A to F) (Figure 1) spanning the entire amino acid sequence of the porcine pancreatic secretory trypsin inhibitor II (PSTI II)[1-3] and a scheme for the construction of the entire peptide chain of this inhibitor have been already presented.[4]

A) Z-Arg(NO_2)-Glu-Ala-Thr-Cys(Acm)-Thr-Ser-Glu(OBu^t)-Val-Ser-N_2H_2-Boc
1 10

B) Trt-Gly-Cys(Acm)-Pro-Lys(Tfa)-N_2H_2-Boc
11 14

C) Nps-Ile-Tyr(Bu^t)-Asn-Pro-Val-Cys(Acm)-Gly-Thr-Asp(OBu^t)-Gly-N_2H_2-Boc
15 24

D) Boc-Ile-Thr-Tyr-Ser-Asn-Glu(OBu^t)-Cys(Acm)-Val-Leu-Cys(Acm)-Ser-N_2H_3
25 35

E) Boc-Glu(OBu^t)-Asn-Lys(Tfa)-Lys(Tfa)-Arg-Gln(Mbh)-Thr-Pro-OH
36 43

F) Boc-Val-Leu-Ile-Gln-Lys(Tfa)-Ser-Gly-Pro-Cys(Acm)-OH
44 52

Figure 1: Synthetic fragments spanning the entire sequence of porcine PSTI II.

In this paper we outline briefly the results we got in the assessment of the stereochemical homogeneity of the synthetized peptides and present an alternative synthetic scheme designed to investigate the possibility of preparing an active modified inhibitor lacking the Lys 14-Ile 15 peptide bond. Moreover, an account of the synthesis of fragments AB

and EF is given. The stereochemical homogeneity of the synthetic fragments A to F has been checked, after partial deprotection with aqueous 90% TFA, or liquid hydrogen fluoride (fragment A), by enzymic digestion followed by quantitative amino acid analysis. The amino acid composition of the AP-M standard digest[5] of fragment A afforded values that agreed, within experimental error, with those expected by theory, but in all other cases a digestion with papain and AP-M[6] was needed to get the correct amino acid ratios in the enzymic hydrolysates. Twice the standard AP-M concentration and a prolonged incubation time (40-70 h) were requested for a complete digestion.[7] As an example the liberation of amino acids in papain + AP-M digests of fragment C, as a function of the time of incubation with AP-M, is shown in Table I.

Table I*

Liberation of Amino Acids in Papain + AP-M Digests of Isoleucyltyrosylasparaginylprolylvalyl-S-acetamidomethylcysteinylglycylthreonylaspartylglycine Hydrazide bis(trifluoroacetate) as a Function of Incubation Time with AP-M

Amino Acid	*Time of Incubation with AP-M, h*			*Theory*
	3	*40*	*72*	
Asp	0.50	0.76	1.05	1
Cys(Acm)	0.30	0.77	0.80	1
Thr	0.80	0.98	1.00	1
Asn	0.30	0.70	0.92	1
Pro	0.45	0.92	0.95	1
Gly	1.30	1.47	1.83	2
Val	1.01	1.05	1.03	1
Ile	0.99	1.00	1.00	1
Tyr	0.99	0.97	0.99	1

*The values in the table, which denote the number of residues per molecule, are corrected for the amino acids liberated by autodigestion of the enzymes which have been determined by carrying out a separate experiment in absence of the substrate.

Recent studies on the mechanism of temporary inhibition have shown that the inactivation of the porcine PSTI II proceeds *via* formation of several distinct components which are generated by tryptic cleavage of the entire polypeptide chain.[8] The early modification reaction, leading to a still active

modified inhibitor, is the hydrolysis of the peptide bond between Lys 14 and Ile 15 which has been identified as the reactive site of the inhibitor. Analogously to observations with a number of trypsin inhibitors,[9-12] resynthesis of the enzyme-susceptible peptide bond can be achieved in the presence of trypsin.[8] Moreover, it is known that reduction of the three disulfide bonds of porcine PSTI II is accompanied by complete loss of inhibitory activity which can be restored completely by regenerating the native three-dimensional structure upon air oxidation.[13] In order to determine if two structurally disorganized fragments, spanning the entire amino acid sequence of PSTI II, are able of interacting with one another to regenerate an active recombinant by correct pairing of the thiol groups, new synthetic routes have been used for the preparation of fragments B and C. The tetrapeptide corresponding to the sequence 11-14 was synthesized in the form of Boc-Gly-Cys(Acm)-Pro-Lys(Tfa)-OH. Selective removal of the Boc protecting group afforded a partially protected tetrapeptide which was used as the amino component in the preparation of fragment AB. Fragment C (sequence 15-24) was prepared in the form of Z-decapeptide *t*-butyloxycarbonylhydrazide. Removal of the Boc protecting group afforded a Z-decapeptide hydrazide which may be readily converted into the corresponding azide and will be used as carboxyl component for coupling to fragment DEF. The fully deprotected fragments AB and CDEF will be recombined and the mixture oxidized by air. Regeneration of the inhibitory capacity might accompany this process if, in spite of the unusual motility due to the absence of the reactive site peptide bond, the amino acid sequence would be able to determine, through a complex matrix of interactions, the correct folding of the polypeptide chains into the specific geometry of the inhibitor molecule. If this would happen, trypsin should then be able to transform the so obtained modified inhibitor into the native one.[8] The task of joining up the fragments of PSTI II so far prepared has progressed to the point shown in Figure 2. Fragment A was partially deprotected by treatment with aqueous 90% TFA and used to acylate, through the azide procedure,[14] the selectively deprotected fragment B in the form of free acid as well as protected hydrazide. The resulting crude tetradecapeptide derivatives were dissolved in a mixture of 1-butanol/DMF/MeOH/water (1:1:1:1 by vol.) and the solutions were added to a column of Biorad AG-1x2 which was eluted with the same mixture and then with 1-butanol/DMF/MeOH/aqueous AcOH, of increasing acid strength (1:1:1:1 by vol.). In the case of the doubly protected tetradecapeptide hydrazide the free

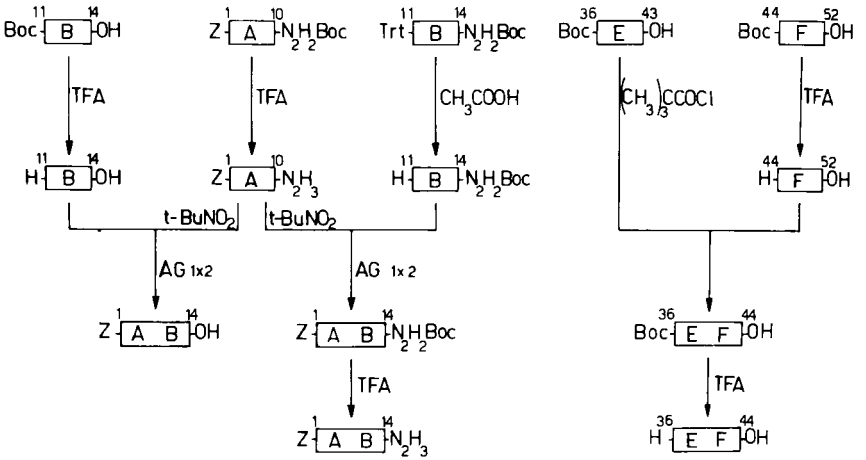

Figure 2: Fragments condensation steps toward the synthesis of porcine PSTI II.

γ-carboxyl group of glutamic acid residues in positions 2 and 8 provided the negative charge necessary for attachment to the resin. Final purification of the products was achieved by precipitation with ether and recrystallization from DMF with ether. Thin layer chromatography did not reveal the presence of contaminants and the amino acid composition of the acid hydrolysates was in agreement with theory. For the synthesis of the heptadecapeptide EF, fragment F was selectively deprotected with aqueous 90% TFA and the ensuing nonapeptide free base was coupled with fragment E by the Anderson modification of the mixed anhydride procedure.[15] Ethanol was added to the reaction mixture and the resulting precipitate was collected by centrifugation. The crude product, which is sparingly soluble in DMF, N,N-dimethylacetamide, hexamethylphosphoramide, DMSO and glacial AcOH proved to be inhomogeneous by TLC. For purification the product was finely ground by prolonged stirring in glacial acetic acid, the resulting suspension was diluted with water and the precipitate was collected by filtration, thoroughly washed with water and ethanol, dissolved in DMF at 60° and reprecipitated with EtOH. The homogeneous peptide produced single spots on TLC and the amino acid composition of an acid hydrolysate agreed with theory. Investigations toward the total synthesis of porcine PSTI II are continued.

Acknowledgment

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PARTIAL SYNTHESIS OF PROTEINS--EXPERIMENTS USING PANCREATIC TRYPSIN INHIBITOR

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PERHAPS THE MAJOR UNSOLVED problem in protein chemistry is the manner in which the polypeptide chain folds itself into a unique three-dimensional structure determined solely by its amino acid sequence. This folding process involves more than a random groping for a minimum energy conformation. A plausible hypothesis is that folding begins in one or more regions which have an intrinsic tendency to form ordered secondary structures (nucleation centers, *e.g.*, α-helices, β-sheets, β-bends), and that these either singly or in combination direct further folding along specific low energy pathways. This hypothesis might be tested if it were possible to prepare sequence variants of globular proteins in which possible nucleation centers were systematically modified. Since the sequence variations desired in any one analog would span at most only a few residues, it seems much more efficient to achieve this by way of partial rather than total synthesis. This paper presents a brief progress report on our attempts to achieve partial synthesis of analogs of the specific globular protein, pancreatic trypsin inhibitor (I).

We focused our attention on the COOH-terminal region of the inhibitor which contains the only extensive region of α-helix in the molecule. In the three-dimensional structure (Figure 1), this helix is located in close proximity to a second possible nucleating center, an extended region of antiparallel pleated sheet spanning residues 16-36, and in fact is covalently bonded to it by the disulfide bridge

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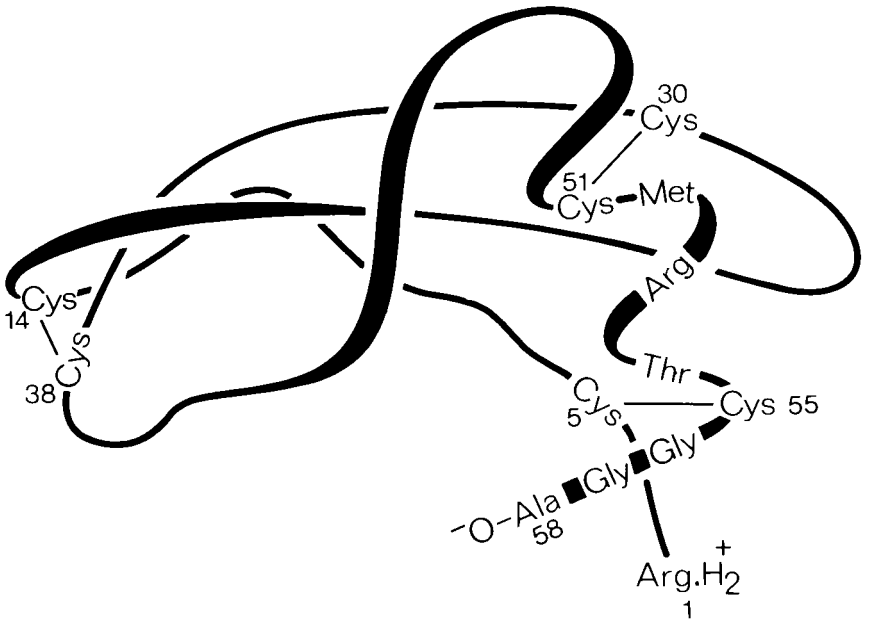
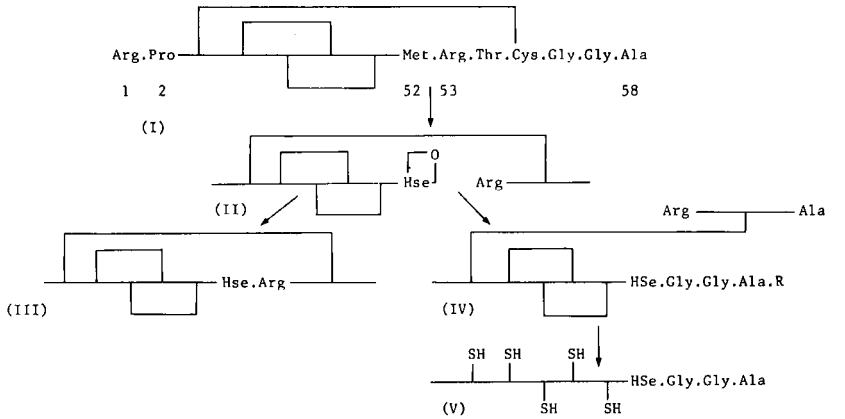


Figure 1: Sketch of the three-dimensional folding of the polypeptide chain in pancreatic trypsin inhibitor. The antiparallel pleated sheet lies immediately behind the C-terminal helix, and is linked to it by the Cys-30, Cys-51 disulfide bridge.

linking cysteine residues 30 and 51. The helix is further linked to the rest of the molecule by a second disulfide bridge between cysteines 5 and 55. In the first instance we planned to remove part of this helical segment and to replace it by synthetic sequences of increased or diminished helicogenicity, and to study the folding properties of these analogs.

The amino acid composition of pancreatic trypsin inhibitor is well suited to this type of investigation. Although there are six half cysteine residues, there is no histidine or tryptophan and only a single methionine. This last is located at position 52, in the center of the COOH-terminal helix. On the other hand, the molecule contains in all 5 amino and 5 carboxyl groups pointing to the need for great selectivity in peptide bond resynthesis if the use of side chain protecting groups is to be avoided.

Chain cleavage of the inhibitor (I) at the single methionine residue was achieved by treatment with a 100-fold excess of cyanogen bromide in the conventional manner.[1]



The seco-lactone (II) was isolated in up to 75% yield by ion-exchange chromatography on CM-cellulose; its characterization by radiolabeling, enzymic digestion, and fingerprinting has been described elsewhere.[1] This method of chain cleavage seemed to us particularly useful because it imparted to the newly liberated carboxyl group a degree of activation through lactone formation which distinguished it chemically from all the other carboxyl groups in the molecule. The homoserine lactone ring of the cleaved inhibitor (II) possessed sufficient reactivity to combine with peptide amino groups (not requiring hydrazinolysis and azide activation, as used by Offord[2] for partial synthesis). After standing in aqueous solution at pH 6.0-6.5 for 4-6 days, (II) was converted spontaneously to at least 85% to a new species (III) in which resynthesis of the full polypeptide chain had taken place. Apparently, hydrolysis of the lactone ring did not compete seriously with peptide bond formation. The structure of (III) as the 52-homoserine (Hse) analogue of the natural inhibitor was firmly established by enzymic digestion as in the case of (II)[1]; it was biologically fully active and very similar in properties to pancreatic trypsin inhibitor.

The smoothness and selectivity of this reaction which has $t_{1/2}$ about 30 hr at pH 7 is remarkable, and is undoubtedly due to substantial retention of the native tertiary structure in the biologically active seco-lactone (II). This places the two reacting groups closely adjacent and perhaps even reduces the rate of competitive hydrolysis by partial exclusion of water. The retention of tertiary structure is itself a consequence of the two disulfide bonds between cysteine residues 5 and 55, and 30 and 51, which

tie the two halves of the cleaved helix firmly to the rest of the molecule. Some attempts were made to liberate the COOH-terminal part of the helix by selective reduction of the 5-55 disulfide. This would have enabled attachment of synthetic cysteine-containing peptides initially by disulfide bond formation with the free sulfydryl of cysteine-5. The spontaneous peptide bond resynthesis reaction would then yield the desired inhibitor analog directly. Not surprisingly, however, this selective reduction could not be achieved, for in the native inhibitor at least, the 14-38 disulfide bond is by far the most reactive. We have therefore had to resort to direct bimolecular attack on the lactone function by added peptides.

In our first experiments, intramolecular resynthesis (II → III) was inhibited by aminopeptidase removal of arginine-53. The α-amino group (of threonine-54) was now held away from the lactone carbonyl by the tertiary structure, and correspondingly, the des-arginine-53 derivative of (II) showed no detectable resynthesis reaction. However, it was difficult to carry out the enzymic removal of arginine-53 cleanly and on a preparative scale, and it proved more efficient simply to slow the intramolecular reaction by dissolution of (II) in a denaturing medium, as 6 *M* guanidine hydrochloride or aqueous or anhydrous dimethylsulphoxide. Under these conditions, resynthesis was sufficiently slow for a high concentration of added synthetic peptide to compete efficiently for the lactone function. Presumably, accessibility to the lactone function by external nucleophiles is also increased in the denaturing medium. The intermolecular aminolysis reaction (II → IV, R=OBu^t) has been studied in some detail using the synthetically readily available COOH-terminal tripeptide (tritiated [1 mCi/mmol] in the terminal glycine) of trypsin inhibitor as model nucleophile. Very large excesses and high concentrations were used in order to compete efficiently with the intramolecular aminolysis.

In a typical experiment, 2 mmoles of H.Gly.Gly.Ala.OBu^t were combined with 10 μmoles of seco-lactone (II) in 2 ml of anhydrous dimethyl sulphoxide at 37°C. Samples were removed at various time intervals and the peptide and protein components separated by gel filtration on Sephadex G-25. A typical elution pattern monitored by absorption at 280 nm and by radioactivity is shown in Figure 2a. The incorporation of radioactivity into the protein component (A) was 76% at 21 hr and 80% at 42 hr. The unchanged peptide emerged as the large radioactive peak (B). The protein was further fractionated on CM-cellulose (Figure 2b) into a

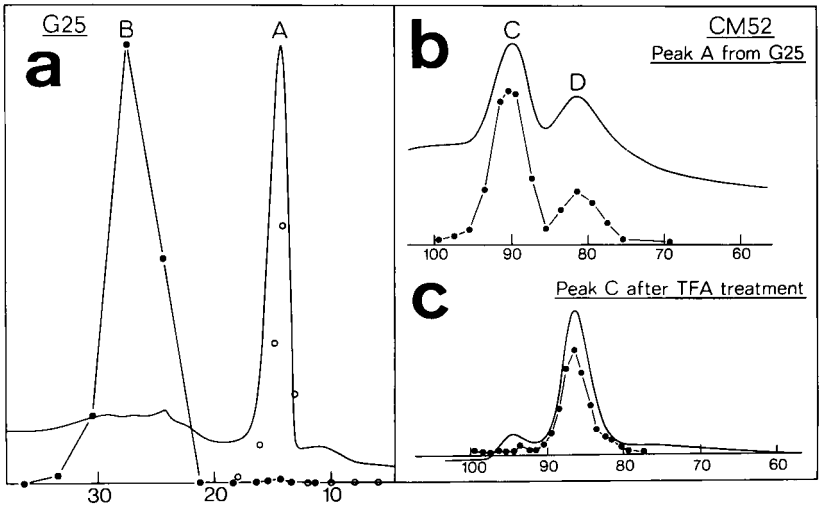


Figure 2: (a) Gel filtration on Sephadex G-25 of the total product from reaction between seco-lactone (II) and H.Gly.Gly.Ala.OBu^t. *Solid line* denotes absorbance at 280 nm; *closed circles*, radioactivity; *open circles*, radioactivity x 100. (b) Chromatography of the protein fraction (A) on carboxymethyl cellulose CM52. (c) Rechromatography of (C) after cleavage of the terminal *t*-butyl ester by treatment with trifluoroacetic acid.

major radioactive peak (C), having the charge difference of +1 from native trypsin inhibitor expected for IV with R = OBu^t, and a second smaller peak (D) which was also appreciably radioactive and had the same charge as native inhibitor. The material in the major peak (C) was collected, treated with trifluoracetic acid, and rechromatographed on the same column. Protein molecules which had incorporated the tripeptide should undergo a charge change of -1 on this treatment due to cleavage of the *t*-butyl ester to give IV with R = OH. The chromatogram (Figure 2c) showed that virtually all of the radioactive materal had moved from the '+1' to the '0' elution positions.

In later experiments, the partially synthetic protein (IV, R = OH) was fully reduced with dithiothreitol. This cleaved the three disulfide bonds and separated the original C-terminal hexapeptide from the partially synthetic protein (V). The protein component was isolated and characterized by carboxymethylation with ^{14}C-labeled iodoacetate followed by trypsin digestion. The autoradiogram (Figure 3) showed

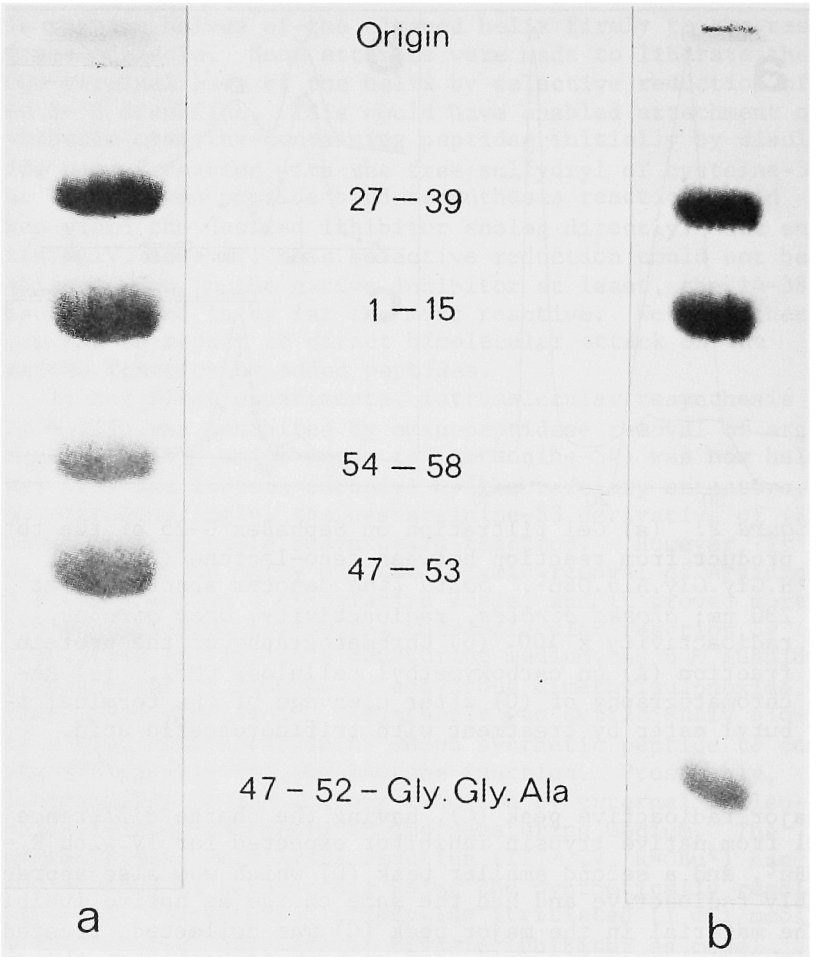


Figure 3: Autoradiograms of electrophoresis maps at pH 6.5 of tryptic digests of ^{14}C-carboxymethylated samples of (a) reduced pancreatic trypsin inhibitor; and (b) the 55-residue analogue (V). Reaction conditions as in reference 1.

the expected pattern of radioactive peptides. In particular, the fastest running, most acidic peptide which arises because of the deletion of arginine-53 from the new sequence had the amino acid analysis $Asp_{1.0}$,$Hse_{0.9}$,$Ser_{1.1}$,$Glu_{1.0}$,$Gly_{2.1}$, $Ala_{2.0}$,$Cmc_{0.2}$, clearly identifying it as the expected C-terminal tryptic peptide from (IV, R = OH). The ^{14}C:^{3}H ratio in this peptide was 1.0

These experiments clearly demonstrate the feasibility of using cyanogen bromide fragments of proteins for the partial synthesis of the analogs. It is noteworthy that no side chain protecting groups were needed at any stage in the transformations described above.

On the other hand, some serious problems remain before the method can be used reliably for the preparation of trypsin inhibitor analogs. Large losses occurred on all occasions when the partially synthetic protein (IV, R = OBu^t) was chromatographed on columns of carboxymethylcellulose, and despite the high level of peptide incorporation, the isolated yield was sometimes as low as 10%. Insofar as the conversion of (II) into (IV) results in displacement of the original C-terminal hexapeptide from its normal position in the globular structure, the large losses on chromatography (which are not found for the proteins I, II or III) may be associated with the inability of (IV) to fold into a compact molecule, leading to aggregation etc. The situation was much worse when incorporation experiments were carried out with a range of synthetic hexapeptide *t*-butyl esters. These problems may be peculiar to the special disulfide linked system in trypsin inhibitor and we are continuing to examine them and develop alternative schemes.

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CONFORMATIONAL ANALYSIS OF GROWTH HORMONES AND SYNTHESIS OF A FRAGMENT OF HUMAN GROWTH HORMONE*

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GROWTH AND LACTOGENIC HORMONES are a group of proteins closely related by their similar molecular weights, primary structures and biological functions.[1-3] The homology is high between h,b,o, and eGH and hPL: about 90% of the positions in the polypeptide chains are occupied by identical amino acids or highly acceptable replacements.[3-7] There is less homology in the sequence of oP, but the alignment of its amino acids with those of hGH and hPL shows regions of close similarity, supporting the view that all three may have evolved from a common ancestral molecule.[7-11]

There are many experimental observations that indicate separate localization of the various activities displayed by GH.[10,12-14] Sequence comparisons intended to correlate segments of the polypeptide chain with specific activities have been unsuccessful[7] or have shown only evolutionary relationships.[9] On the other hand, knowledge of the three-dimensional structure of these proteins may help to localize active sites. Unfortunately, this information is not available and the only possible approach is to attempt the prediction of conformation from the primary sequence.

We have applied the method of Chou and Fasman[15] to predict the secondary structures of hGH, bGH, oGH, hPL and oP. Using the latest revised sequences, we have estimated regions

*Abbreviations used: h, human; b, bovine; o, ovine; e, equine; GH, growth hormone; P, prolactin; PL, placental lactogen; Xan, xanthydryl; DCM, dichloromethane.

of α-helix, β-sheet, and β-turn in each of these proteins. Residues not included in one of the above conformations are considered to be random coil. The comparison of the predicted conformations of these proteins shows homologous regions, and one of these has been correlated with the growth promoting site.

Figure 1 shows the results of the comparisons made, arranged by pairs of hormones. Hatched areas between lines indicate zones with identical or closely homologous secondary structures.

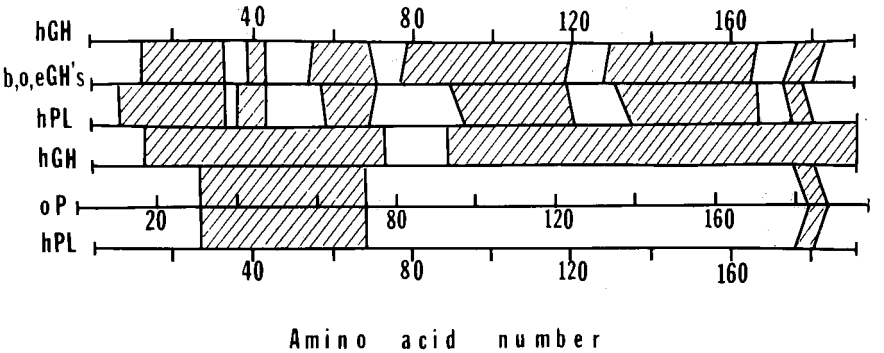

Figure 1: Zones of conformational homology or identity (hatched areas) between hGH, bGH, oGH, hPL and oP.

Examination of Figure 1 reveals three major areas of homology among the molecules having somatotropic activity, but only one among all those with lactogenic activity. This latter region, roughly residues 30-70, should most likely represent the active site for lactogenic activity. The somatotropic site, then, should be one of the other two, corresponding to residues 77-120 or 130-170 of hGH. The latter region is ruled out on chemical grounds, since several investigators have shown that the full biological activity resides in the hGH 1-134 fragment.[16] The conformational homology of the 77-120 region seen in hGH and bGH (Figure 2) is not shared fully by hPL; this is consistent with the lower somatotropic activity of hPL. The bGH tryptic fragment 95-132 isolated by Sonenberg[12] has low GH activity and contains part of the predicted active site. Among synthetic peptides, hGH 88-124[10] and hGH 95-136[17]

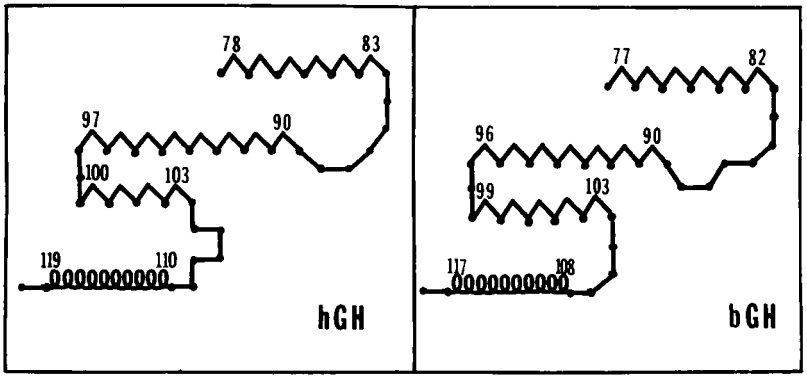

Figure 2: Schematic diagram of the predicted secondary structure for the growth region in hGH and bGH.

contain parts of the active site, and have been reported to have low but definite activity in the tibia test.

We are attempting to confirm the conformational prediction of the active site by synthesis. We report here the synthesis of hGH 75-120-NH_2 by the solid phase method, using Beckman 1% cross-linked benzhydrylamine (BHA) resin as carrier and the Beckman 990 synthesizer. The BHA resin contained 0.28 mmole/g of Boc-Gly. Boc amino acids, obtained from Beckman or Bachem or synthesized in this laboratory, were used throughout. Side chain protecting groups used were: Ser(Bzl), Glu(OBzl), Asp(OBzl), Arg(Tos), Tyr(2-BrZ), Lys(2-ClZ), Asn(Xan), and Gln(Xan). Tryptophan was used with and without the formyl blocking group, with comparable results. Deprotection (prewash + 30 min) was done with 100% TFA containing 1 mg/ml indole; the reagent was stored at least 24 hr before use. Neutralization was done with 10% triethyl amine in DCM for 5 min following a prewash. Coupling reactions in DCM were carried out with 2.5-fold amounts of Boc amino acids and DCC for 2 hr; insoluble derivatives were used in DMF-DCM mixtures. All coupling reactions were monitored with the Kaiser ninhydrin test. Those still positive after 2 hr were recoupled under the same conditions; the recoupling protocol included a repetition of the neutralization step. If the ninhydrin test was still positve after recoupling, the peptide was acetylated with a 5-fold amount of acetyl imidazole in DCM. The points where recoupling and acetylation were needed are indicated in Figure 3. The peptide was cleaved from the resin with anhydrous HF (anisole) at 0° for 60 min. At several points

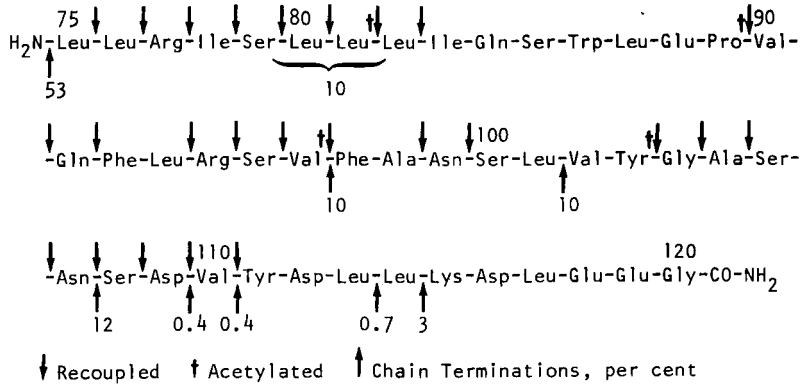

Figure 3: Synthesis of human growth hormone 75-120.

during the synthesis aliquots of resin were removed and cleaved with HF, and aliquots of the peptides were analyzed for amino acid content after enzymatic and acid hydrolysis.

Two types of extraction of the peptide from the resin were used: (a) extraction of all peptides with 50% HOAc, lyophilization and extraction of the dry peptide with pyridine-HOAc buffer, pH 6.5 (2.3 *M* in pyridine), or (b) extraction of the resin with pyridine-HOAc buffer followed by extraction of the resin with 50% HOAc. The peptide containing the entire 46 amino acid sequence was insoluble in buffer, and either type of extraction served to separate it from soluble shorter peptides.

Chromatography of the buffer-insoluble peptide on Bio-Gel P-30 in 50% HOAc gave a major peak preceded by some material with higher molecular weight; material from both peaks had, after hydrolysis, the same amino acid composition which was that expected for the desired peptide: Lys, 1.05; Trp, 1.02; Arg, 2.00;, Asp, 5.11; Ser, 5.68; Glu, 5.20; Pro, 1.08; Gly, 2.20; Ala, 2.16; Val, 4.13; Ile, 1.86; Leu, 10.55; Tyr, 1.95; Phe, 1.89. Partition chromatography of the material from the major peak on silica gel in 1-BuOH:HOAc:H_2O:EtOAc (1:1:1:1) gave a single symmetrical peak. SDS acrylamide gel electrophoresis (0.1 *M* phosphate, pH 7.2) gave one band with an apparent molecular weight of about 6000. TLC on silica gel gave one spot in two different systems, and dansylation gave only dansyl leucine. Trypsin digestion and fingerprint analysis gave the expected four peptides, each of which was eluted and hydrolyzed and found to have the correct amino acid composition. The purified peptide had activity in the tibia test, although assay results were variable.

Data from gel chromatography of the buffer-soluble part of the product combined with information from the resin aliquots taken at different places during synthesis allowed us to determine that measurable chain termination occurred at eight places during the synthesis although the ninhydrin test was negative at every step. The places and amounts of the terminations are indicated in Figure 3; 53% of the chain remained viable at the end of the synthesis. Deliberate termination by acetylation was not the major source of truncated sequences. The most likely cause of the terminations is incomplete deprotection. In a subsequent synthesis of hGH 73-128, similar terminations were also observed in the early part of the synthesis, but after 29 amino acids had been assembled a double deprotection program was adopted (30 min in 100% TFA followed by 30 min in 25% TFA in DCM) and most termination was eliminated. At the end of this synthesis 73% of the chain was still viable. Recoupling was used more extensively in this synthesis. A repetition of this latter synthesis using double deprotection from the outset is now under way, and no significant termination has been seen during the first thirty residues.

This work provides abundant evidence for incomplete deprotection as a cause of truncated sequences in solid phase peptide synthesis, confirming prior evidence of several investigators. The very stable peptide-resin bond in the BHA resin allowed us to use sufficiently vigorous deprotection conditions to remove essentially all Boc groups. Use of such deprotection conditions on the standard Merrifield resin for synthesis of peptide acids would cause unacceptable loss of peptide from the resin. The logical general solution of this problem is the use of much more labile α-blocking groups.

Acknowledgments

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SYNTHESIS OF LITORIN

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LITORIN IS A NEW NATURAL NONAPEPTIDE isolated from methanol extracts of the skin of the Australian leptodactylid frog *Litoria (Hyla) aurea.*[1] It belongs to a recently discovered peptide family of amphibian origin, which already includes bombesin,[2] alytesin[2] and ranatensin[3] (Figure 1). The prototype may be considered bombesin, on account of the extensive pharmacological studies carried out with this molecule.[4-9]

Bombesin	<Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Alytesin	<Glu-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Ranatensin	<Glu----------Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
Litorin	<Glu----------Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2

Figure 1: The bombesin family.

Other natural analogs of these peptides are known to be present in amphibian skins, namely litorin II,[10] ranatensin C[11] and ranatensin R,[11] and the complete determination of their sequences is in progress.

All the members of this family are characterized by a striking structural similarity. As far as litorin is concerned, its COOH-terminal octapeptide is the same as that of ranatensin, and differs from that of bombesin and alytesin only by substitution of Phe for Leu at the penultimate position. In addition, all four peptides possess an NH_2-terminal pyroglutamic acid residue.

The synthesis[12] of litorin was performed in order to provide independent confirmation of the structure deduced by degradative experiments,[1] and to supply substantial amounts

of peptide for more extensive studies in comparison with bombesin. Our approach (Figure 2) involved the preparation of two fragments (VIII and IV) by stepwise elongation, mainly through the mixed anhydride method.

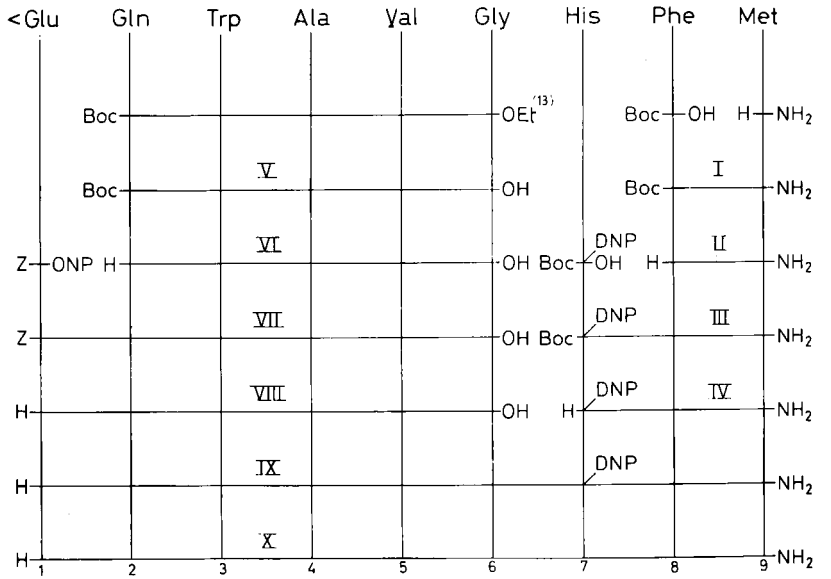

Figure 2: Synthesis of litorin.

The glycine residue at position 6 was an obvious point of division, as the 6-7 bond could be formed without any risk of racemization. The scheme of synthesis was the same for the COOH-terminal nonapeptide of bombesin[13] and bombesin analogues,[14] the only difference being the protection of the imidazole function of histidine by the 2,4-dinitrophenyl (DNP) group. The coupling of the two fragments was carried out via DCCI + HOSu. The protected nonapeptide IX was easily obtained in 66% yield. Deprotection was performed by thiolysis,[15] using a very large excess of 2-mercaptoethanol in a mixture of hexamethylphosphoric acid triamide-DMF-H_2O (4:1:1) maintained at pH 8 with Na_2CO_3.

Litorin (X) was finally secured in 60% yield as the hydrochloride by treatment with HCl/AcOH and desalting through a column of amberlite XAD-2 (eluant: H_2O and then H_2O containing increasing amounts of MeOH up to 50%).

The synthetic product was found to be homogeneous and showed the same electrophoretic and chromatographic mobilities,

the same degradative pattern and the same biological properties as natural litorin.

Synthetic litorin was then compared with synthetic bombesin in a number of *in vitro* and *in vivo* tests.[16,17] A close similarity in the pharmacological effects of the two peptides resulted, however there were some noteworthy differences.

The most striking difference lies in the fact that the actions of litorin are more prompt in onset and in disappearance than those of bombesin, both *in vitro* and *in vivo*. In this respect litorin occupies an intermediate position between bombesin octapeptide and bombesin nonapeptide.

Very recently an immunoreactive bombesin-like peptide was detected in tissue extracts of mammals and birds,[18] and now it has also been shown in human serum after the ingestion of food.[19] Antisera were raised in rabbits against the COOH-terminal bombesin nonapeptide conjugated with BSA. The labeled antigen was obtained by iodination (^{125}I) of the same nonapeptide containing an additional tyrosine residue at its NH_2-terminus.

Attempts to isolate the new peptide hormone are being followed both radioimmunologically and biologically, and the bioassays seem to indicate a greater similarity to litorin than to bombesin.

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MOLECULAR PHARMACOLOGY OF ANGIOTENSIN II RECEPTORS

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SPECIFIC RECEPTOR SITES FOR angiotensin II (AII) and related peptides have been demonstrated in homogenates and subcellular fractions prepared from adrenal cortex, kidney and aortic smooth muscle.[1-3] The binding sites present in adrenal and kidney homogenates were identified with ^{125}I-labeled AII,[1,2] and those in aortic smooth muscle homogenates were demonstrated with tritiated AII (^{3}H-AII).[3-5] Both radioiodinated and tritiated AII have been more recently employed for characterization of angiotensin binding sites in the adrenal cortex[6-9] and the renal glomerulus.[10] The particulate binding sites have not been subjected to highly selective purification procedures for most of the reported binding studies, and were usually recovered as a 20,000 g fraction from homogenates of the target tissue. Where localization of the peptide binding sites has been analyzed during cell fractionation, the AII receptors have been found to be predominantly located in the plasma membrane.[6,11,12]

An important aspect of the validity of AII binding by tissue fractions is the need to demonstrate a close correlation between binding specificity, usually evaluated by binding-inhibition studies, and the biological potencies of angiotensin analogues and fragments. Such a correlation has been consistently observed during binding studies on particulate adrenal receptors with ^{125}I-A II and ^{3}H-A II.[6] However, in binding studies with ^{3}H-A II and microsomal membranes[4] or solubilized material[5] from rabbit aorta, the biologically inactive 2-8 hexapeptide fragment exhibited

the same binding-inhibition activity as the intact A II molecule. In the solubilized aortic material, the biologically inactive 1-7 heptapeptide fragment was almost half as effective as A II in terms of binding-inhibition.[5] These observations suggest that the binding which is measured with tritiated A II in such aortic preparations may not be representative of the true receptor sites for A II in smooth muscle.

Angiotensin II Tracers

Tritiated A II offers the advantage of high biological activity in the tracer employed for binding studies,[13] but its relatively low specific activity (30-50 Ci/mmole) is not satisfactory for the demonstration of high affinity binding sites. For this purpose, ^{125}I-labeled A II of specific activity approximately 1000 Ci/mmole has proved to be more satisfactory, and has several advantages as tracer for binding studies in adrenal cortex preparations. Radioiodinated A II tracer for binding studies is relatively easily prepared by iodination of pure synthetic A II by the chloramine-T method. The residual biological activity of monoiodo-A II has been variously estimated to be from 25 to 80%, while that of diiodo-A II is considerably lower (2 to 24%).[14] For this reason, it is essential to prepare the monoiodinated peptide for receptor binding studies. This is most readily achieved by labeling A II to relatively low specific activity (*e.g.*, 1 mCi of ^{125}I to 50 µg of A II) followed by isolation of the monoiodo-peptide by ion exchange chromatography,[15] or polyacrylamide gel electrophoresis.[16] The presence of the monoiodo-peptide can be confirmed by pronase digestion and chromatography of the labeled amino acid residues. The specific activity of ^{125}I-A II prepared by this method is usually in the region of 600-1000 µCi/µg. Monoiodinated A II is extremely stable during storage, and retains receptor binding activity and high specific activity for prolonged periods after labeling. These properties are attributable to the process of "decay catastrophe," the destruction of radioiodinated peptides by the intense ionization which accompanies the radioactive decay of ^{125}I. The resulting simultaneous disappearance of both radionuclide *and* peptide causes relatively prolonged retention of high specific activity.[16] After preparation, the labeled peptide should be stored as frozen aliquots at -60°C, and used only once for binding studies after thawing. When quantitative binding studies are to be performed, with derivation of association constants and binding site concentrations, it is

essential to characterize the labeled peptide in terms of its biological activity and its ability to bind to excess receptor sites. Suitable corrections for these factors should then be employed during the computation of binding site concentration and affinity.[11]

Preparation of Binding Fractions

Although vascular and uterine smooth muscle have been shown to bind A II, the most abundant binding sites are located within the adrenal cortex. The marked uptake of tritiated A II by rat adrenals was noted during an early *in vivo* study on the metabolism of labeled angiotensin II.[19]

For preparation of bovine adrenal binding fractions, the adrenals are obtained immediately after slaughter and kept in ice-cold buffered saline until processed. The cortical layers are rapidly dissected out and homogenized in 20 mM sodium bicarbonate in a Dounce homogenizer, then filtered through fine nylon mesh. More than 90% of the A II binding sites in the filtered homogenate could be recovered in the 1500 g sediment, and in the 20,000 g sediment of the 1500 g supernatant solution. The concentration of receptor sites per milligram of protein is about twice as high in the 20,000 g fraction as in the 1500 g sediment.[6] When more concentrated preparations of A II binding sites are required, the initial dissection of the bovine adrenal cortex can be performed to exclude the majority of the cortical layer, with retention only of the capsule and glomerulosa layer. For most binding-inhibition studies of A II agonists and antagonists this additional step is not necessary, since adequate binding activity is obtained by homogenization of the whole adrenal cortex.

A similar procedure for preparation of particulate binding fractions has been employed for studies on the A II receptors of the rat and dog adrenal cortex. The binding activity of rat adrenals is somewhat more stable to storage than that of the bovine adrenal, and glands frozen immediately can be stored at -70°C with retention of binding activity. Again, higher concentrations of A II binding sites per milligram of protein can be obtained by selective homogenization of the capsular layer, which contains the majority of the zona glomerulosa.

Binding Studies

Uptake of tritiated and radioiodinated A II by particulate adrenal cortex fractions is characterized by relatively rapid kinetics, with attainment of a steady-state after

incubation for 30-45 minutes with concentrations of A II in the range 10^{-9} to 10^{-11} *M.*[6] Degradation of both A II receptors and the labeled peptides can occur relatively rapidly at high temperatures, and for this reason most binding studies have been performed at room temperature most commonly at 20°C. At lower temperatures, the degradation of receptor sites and tracer is relatively slow, but the rate of binding of A II is considerably reduced, and much longer times are required for the establishment of an equilibrium or steady state. When binding is performed for more prolonged periods at 20°C, a decline from the steady-state value is commonly seen, probably reflecting the marked degradation of free hormone in the incubation medium and the consequent dissociation of bound hormone from receptor sites. This process is also partly attributable to a degree of degradation of the peptide receptor sites. A suitable compromise for quantitative binding studies is to perform incubation at 20°C for relatively short time periods, from 30-60 minutes. For derivation of receptor binding constants, it is important to ensure that the chosen time interval is adequate to permit all concentrations of peptide hormone employed during binding studies to reach a steady state.

Characteristics of Adrenal Cortex Receptor Sites

1. *Stability.* The binding activity of adrenal receptors is highest when fresh tissue is employed for preparation of particulate binding fractions. However, satisfactory binding assays can be performed with homogenates of adrenal tissue which have been collected into liquid nitrogen and stored at -60°C. After homogenization, the binding properties of bovine adrenal cortex fractions are maintained for up to 12 hours when kept on ice. When homogenates are frozen and stored in liquid nitrogen, subsequent thawing is frequently followed by loss of binding affinity for angiotensin II, and reduced selectivity for the octapeptide. During binding studies performed at 20°C, relatively little degradation of receptors is detectable during preincubation of the binding fraction for periods equivalent to those subsequently required during equilibrium binding studies.

2. *Localization of Receptor Sites.* The distribution of A II binding sites between the adrenal glomerulosa and fasciculata-reticularis layers has not been extensively studied, but the receptor sites appear to be most abundant in the glomerulosa layer, at least in the adrenals of the cow, rat and dog.

Further purification of the 1000-20,000 g adrenal fraction by discontinuous sucrose density gradient centrifugation has shown that the majority of the binding sites copurify with a vesicular particulate fraction which is enriched in alkaline pohsphatase, and plasma membrane enzymes including adenylate cyclase, 5'nucleotidase, and (Na^+,K^+)-dependent ATP'ase.[6] The mitochondrial fraction identified by electron microscopy and the presence of succinic dehydrogenase and cytochrome oxidase displays relatively low binding avidity for A II.[12] Isopycnic sucrose density gradient centrifugation of particulate adrenal cortex receptors (Figure 1) has likewise shown a close correlation between A II binding sites and plasma membrane enzymes including adenylate cyclase and sodium-potassium activated ATP'ase.[11] It is therefore most likely that the A II receptors are located in the plasma membrane fraction of the adrenal cortex homogenate. Other studies have suggested that A II becomes localized to the nuclei of muscle cells,[18] and that A II binding may be associated with adrenal mitochondria.[1] However, our data strongly suggest that the primary site of interaction of A II with specific receptors takes place in the plasma membrane.

3. *Binding Constants of Peptide-Receptor Interaction.* Scatchard plots derived from equilibrium binding and binding-inhibition data obtained at 20°C show the presence of a predominant set of high-affinity binding sites with Ka of approximately $10^9\ M^{-1}$, and frequently a second order of sites with Ka of about $10^8\ M^{-1}$. The initial binding of A II to receptors behaves as a second-order reaction with Ka of $2.4 \times 10^5\ M^{-1}\ sec^{-1}$. Dissociation of A II from prelabeled particulate adrenal receptors occurs as a biphasic process, with two components of half-life 4.5 and 23 minutes. The dissociation of cell-bound ^{125}I-A II was also relatively rapid and almost complete, again indicating the surface location of specific angiotensin binding sites in the target cell. The magnitude of the association constants obtained for A II binding sites in rat adrenal cortex was consistently somewhat higher than that of the bovine preparation, being commonly between 10^9 and $10^{-10}\ M^{-1}$.[6] The rate and extent of A II binding to receptor sites is significantly influenced by the sodium concentration of the incubation medium, and maximum binding activity is obtained when binding assays are performed in the presence of 120-140 mM NaCl.[8,11] The binding of A II to adrenal cortex receptors is also modulated by guanyl nucleotides including GTP and GMP-PNP, which significantly increase the dissociation rate constant and

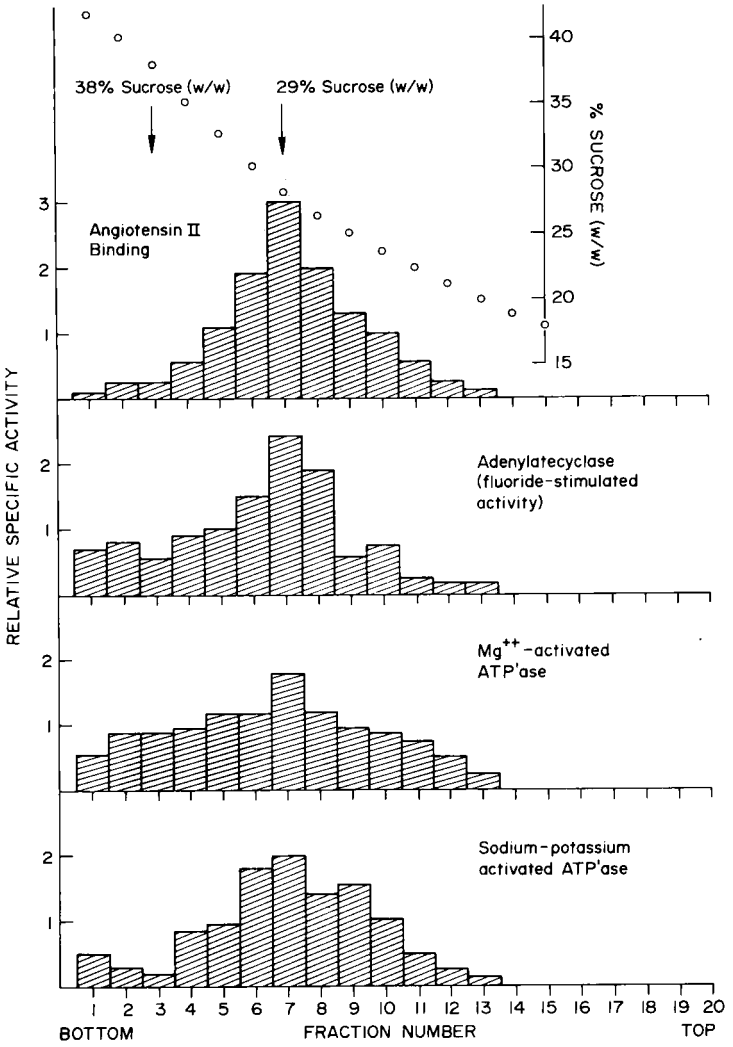

Figure 1: Isopyknic density gradient centrifugation of particulate bovine adrenal cortex receptors in 15-50% sucrose.[11] The peak of binding activity at 29% sucrose coincides with the peaks of adenylate cyclase and sodium-potassium activated ATP'ase corresponding to the position of plasma membrane particles. The arrow at 38% sucrose indicates the position of the mitochondrial fraction, confirmed by enzyme assays and electron microscopy.

equilibrium binding constant of the interaction between A II and specific receptor sites.[7]

Degradative Processes During Binding Studies

Preincubation experiments have shown that little or no receptor degradation occurs during incubation of adrenal particles at 20°C for up to 60 minutes. However, the free or unbound tracer angiotensin is rapidly degraded during incubation with subcellular particles from bovine or rat adrenal cortex. Degradation of tracer peptide is reduced by incubation at low temperature, in the presence of peptides such as glucagon and insulin, and sulfhydryl-protecting agents.[6] Although the rate of degradation of A II peptides is reduced by addition of carrier peptides and DTT, prolonged incubation periods may still be accompanied by effects attributable to significant degradation of the labeled peptide. Elution of labeled A II from adrenal binding sites at low pH, followed by rebinding studies with fresh adrenal particles, has shown that the receptor-bound angiotensin is not subjected to significant degradation.[6] By contrast, free A II in the incubation medium is relatively rapidly degraded. Such a change in the concentration of free angiotensin II during binding studies can vitiate the computation of binding constants, since all such calculations depend upon an accurate knowledge of the concentrations of bound and free hormone. When significant degradation occurs during binding studies, corrections for the measured degradative processes can be incorporated into computer calculations of binding constants and receptor concentrations.[11]

Specificity of Angiotensin II Binding

Apart from the nonspecific protective effects of certain peptides and proteins, true competition with tracer angiotensin II for binding to adrenal receptor sites is not observed with peptides unrelated to A II. Thus, no binding inhibition occurs with glucagon, insulin, ACTH and parathyroid hormone, or with a number of nonhormonal proteins and peptides.[6] In addition, the binding-inhibition potency of a variety of A II analogues and fragments is closely correlated with the biological activity of these compounds in smooth muscle and adrenal response systems. The val^5 and ile^5 forms of angiotensin are equivalent in terms of binding activity, and the C-terminal 2-8 heptapeptide formed by deletion of the N-terminal aspartic acid residue is equipotent

with the intact octapeptide. Angiotensin I and the synthetic tetradecapeptide display less than 5% of the activity of A II, and the activities of the 1-7 heptapeptide, 3-8 hexapeptide, 4-8 pentapeptide and 5-8 tetrapeptide are orders of magnitude below that of the intact octapeptide[6,19] (Figure 2). In the adrenal radioligand assay, A II analogues which are totally devoid of biological activity (*e.g.*, the phe^3, val^4, tyr^8 analogue of A II) do not exhibit detectable

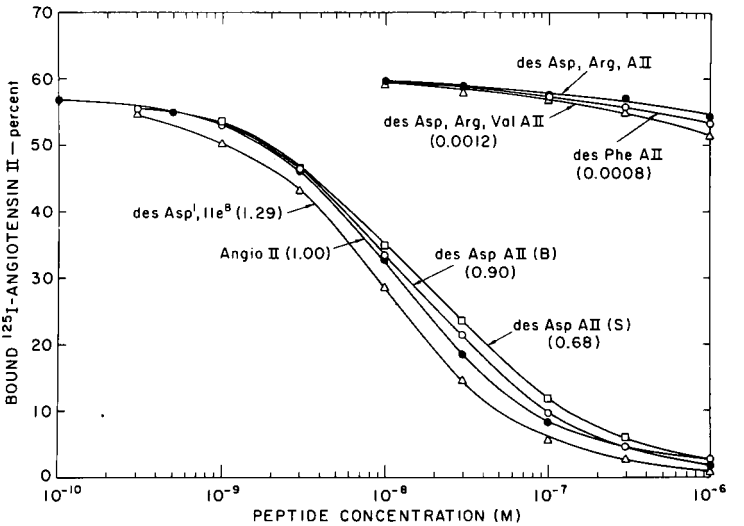

Figure 2: Binding-inhibition activity of A II and related peptides in the adrenal radioligand-receptor assay. The [Des-Asp^1, Des-Arg^2] and [Des-Phe^8] fragments exhibit negligible binding activity. The activity of highly purified [Des-Asp^1]-A II heptapeptide (B) was almost identical with that of A II.

binding-inhibition activity *in vitro*. By contrast, the highly potent A II agonist [Sar^1]-A II shows a several-fold increase in binding-inhibition activity upon adrenal cortex receptors. This increased binding affinity of the [Sar^1] derivative is consistent with its 10-fold higher activity upon smooth muscle contraction and aldosterone production by adrenal cells *in vitro*.[20] In all binding studies with angiotensin agonists, the relative binding-inhibition activities of the individual peptides have been directly proportional to their known biological activities upon aldosterone production by the adrenal glomerulosa *in vivo* and *in vitro*. The relative activities of A II and the 2-6 hexapeptide and

1-7 heptapeptide sequences upon aldosterone production by isolated adrenal cells are shown in Figure 3, and illustrate the negligible biological potency of these A II fragments which exhibit no significant binding to adrenal receptor sites.

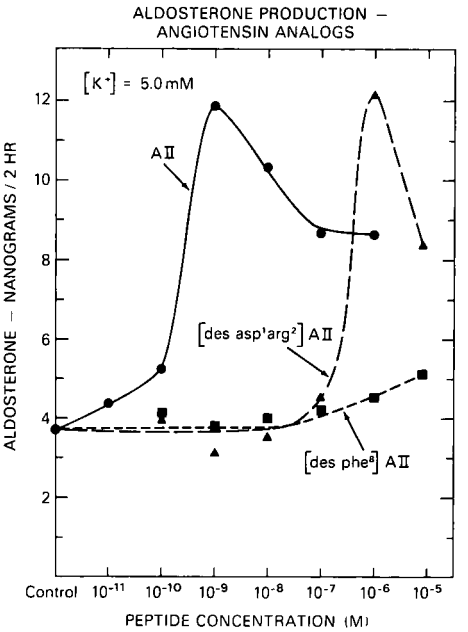

Figure 3: Aldosterone production by isolated canine adrenal cells, in response to A II and the [Des-Asp^1, Des-Arg^2] and [Des-Phe^8] fragments. The low biological potency of the fragments is commensurate with their low binding activity shown in Figure 2. The activity of [Des-Asp^1] A II in this system was identical with that of A II.

Radioligand-Receptor Assay of Angiotensin II Antagonists

The radioligand assay provides a precise and convenient method for evaluation and comparison of the binding-inhibition properties of A II antagonists. Replacement of the C-terminal phenylalanine residue by aliphatic residues including glycine, alanine, leucine, and isoleucine, has resulted in the formation of powerful competitive antagonists with relatively low agonist activity.[21] Just as the competitive binding activities of A II agonists have been found to be proportional to their biological activities, as described above, the binding-inhibition activity of a wide range of A II antagonists has

similarly been correlated with the activity of these compounds upon A II responses in smooth muscle and adrenal cortex.[19] Thus, antagonists formed by C-terminal substitution of A II with aliphatic residues exhibit binding-inhibition potencies in proportion to their known activities as antagonists of A II upon smooth muscle. In addition to the effect of such changes at the C-terminus upon the antagonist activity and binding potency of A II analogues, changes in the N-terminal portion of the molecule have also been shown to modify binding activity and biological potency. An example is the replacement of N-terminal aspartic acid with sarcosine, which causes a significant increase in both binding activity and antagonist activity of the 8-aliphatic peptides. Replacement of aspartic acid by guanidoacetic acid has a similar though more marked effect upon the binding activity of Ile^8 A II (Figure 4). Such enhancement of activity appears to be related to the basicity of the N-terminal portion of the molecule, and is completely abolished by substitution of acidic residues such as succinic acid at the N-terminus of A II. The enhancement of binding-inhibition potency by N-terminal sarcosine substitution is due to increased binding affinity of the modified peptide for the A II receptor site, and may also be influenced by decreased degradation of the peptide by angiotensinase A in the adrenal cortex.

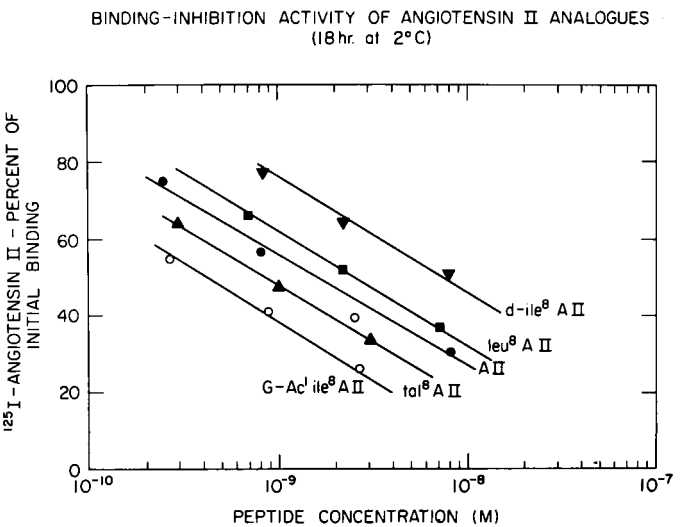

Figure 4: Inhibition of ^{125}I-A II binding to particulate bovine adrenal receptors in the presence of A II and competitive antagonists formed by C-terminal substitution with aliphatic amino acids.

Derivation of the relative binding potencies of angiotensin II agonists and antagonists in the radioligand-receptor assay permits comparison of the relative binding affinities of these peptides for their receptor sites in target tissue. Extension of the comparison of binding-inhibition potencies to the computation of relative binding affinities is valid if the radioligand-receptor studies are performed under saturation conditions. The most direct determination of intrinsic binding affinity of an individual peptide would be provided by homologous binding-inhibition studies, in which the labeled peptide is displaced by increasing concentrations of the corresponding unlabeled compound. However, since A II antagonists are most commonly employed to compete with A II for binding to receptors in target tissues, the relative binding potencies determined by competition with labeled A II are highly relevant to the antagonist activity of such analogues.

It is also important to emphasize that the binding-inhibition activity determined in radioligand-receptor assays can provide information only about the binding affinity of the receptor site for the peptide ligand, and *cannot* distinguish between the intrinsic agonist or antagonist activities of peptide analogues. For such a distinction, it is necessary to perform separate determinations of agonist and antagonist activity in an appropriate target cell response system. As long as the interpretation of binding-inhibition data is confined to the binding reaction between receptor and peptide hormone, the adrenal radioligand-receptor assay provides a direct and quantitative method for evaluation of the role of binding affinity in the action of both agonists and competitive antagonists upon angiotensin-responsive target tissues.

Correlation of Receptor Binding with Aldosterone Response to A II

The most significant evidence about the functional role of A II binding sites as "receptors" is provided by the correlations observed between binding and activation of steroidogenesis in the adrenal target cells. For this purpose, collagenase-dispersed cells from the canine zona glomerulosa were utilized for simultaneous determinations of A II binding and aldosterone production *in vitro*.[20] Such cells have been shown to possess high sensitivity to A II *in vitro*, responding to as little as 10^{-11} *M* A II and giving a maximum response to 3×10^{-10} *M* A II.[22] Binding studies with ^{125}I-A II and unlabeled peptide showed that the associ-

ation constant (Ka) of the receptor sites in intact glomerulosa cells was 10^9-10^{10} M^{-1}, commensurate with the affinity of the sites analyzed in particulate fractions of adrenal homogenate.

When increasing concentrations of labeled A II were incubated with dispersed adrenal cells, the progressive increase in cell binding was well correlated with the aldosterone response *in vitro*. Also, uptake studies with ^{125}I-[Sar^1]-A II showed that this more active agonist was bound with higher affinity than A II to isolated adrenal cells *in vitro*, and evoked aldosterone production at 10-fold lower concentrations than the native octapeptide. Conversely, the effects of progressive inhibition of receptor binding upon aldosterone production were analyzed in dispersed adrenal cells exposed to a maximal stimulating concentration of ^{125}I-A II, and increasing concentrations of the competitive antagonist, [Sar^1, Ala^8]-A II. The results of this study showed a parallel inhibition of both A II binding and aldosterone production in the presence of the inhibitor, as illustrated in Figure 5. Thus, in addition to the binding specificity for A II agonists and antagonists, the membrane receptors of the zona glomerulosa are coupled to steroidogenesis, and a close correlation exists between A II binding and the subsequent rate of aldosterone production *in vitro*. These observations provide direct evidence for the functional

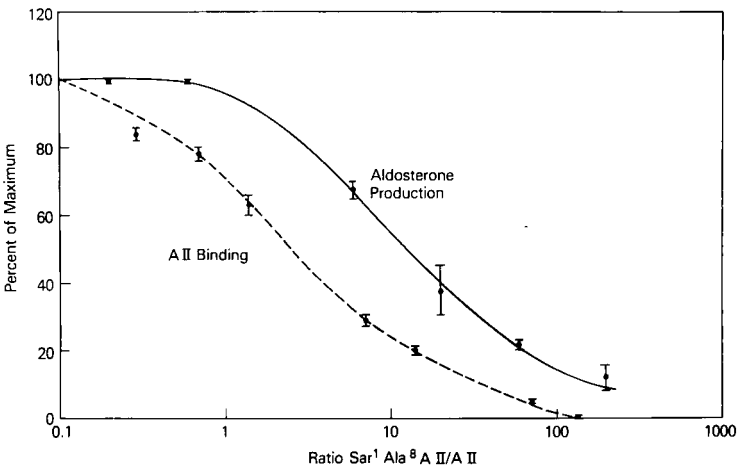

Figure 5: Inhibition of A II binding and aldosterone production by canine adrenal cells incubated with 10^{-9} M ^{125}I-A II and increasing concentrations of [Sar^1, Ala^8]-A II.

significance of the A II binding sites of the adrenal cortex, and confirm their identity as receptors with the capacity to regulate aldosterone production in response to occupancy by A II and its active analogues.

Acknowledgments

We are grateful to Dr. F. M. Bumpus for generously providing [Sar^1]-A II and antagonist analogues, and to Dr. A. Castellion, Norwich Pharmacal Co., for a gift of [Sar^1, Ala^8]-A II.

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ON THE SELECTIVE ANTAGONISM OF MYOTROPIC AND STEROIDOGENIC ACTIVITY OF ANGIOTENSIN II

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EARLIER WORK FROM OUR LABORATORIES described structure-activity relationships with the analogs of angiotensin II which eventually led to the development of potent antagonists of the pressor and myotropic response of angiotensin II.[1,2] These antagonists generated enthusiasm since it was long felt that these compounds could serve as useful tools in investigating the role of renin-angiotensin system in renal hypertension. The results obtained indicate that these antagonists may find clinical use. Present paper discusses: (a) critical evaluation of the antagonists of the pressor and myotropic response of angiotensin II with particular emphasis on their limitations, and (b) the requirements to stimulate or block the angiotensin II-mediated responses in adrenal medulla or adrenal cortex.

Antagonism of the Pressor and Myotropic Response of Angiotensin II

All the antagonists of the pressor and myotropic response of angiotensin II suffer from at least two limitations: (a) An initial pressor activity (12-16 mm Hg) was observed when these analogs, *e.g.*, [Sar^1, Ala^8]-, and [Sar^1, Ile^8]-angiotensin II, were infused in rats at a dose level of 250 ng/kg/min.[3] A part of this pressor activity has been shown to be due to the release of catecholamines from adrenal medulla,[3] and (b) the short *in vivo* half-life. The pharmacological half-life of [Sar^1, Ala^8]-angiotensin II has been reported to be 8.2 min in hypertensive patients.[4]

Although these side effects are not expected to be a major concern if these compounds are used as diagnostic agents or for short-term therapy, these could pose a serious problem if used in long-term therapy.

For increasing the *in vivo* half-life of these peptides we tried to take a lead from the mode of degradation of angiotensin II in human plasma. Since "angiotensinases" A, B and C cleave the peptide bonds in angiotensin II molecule between Asp-Arg, Tyr-Ile and Pro-Phe, respectively, it seemed apparent that the *in vivo* half-life of [Sar^1, Ile^8]-angiotensin II may possibly be prolonged if positions 1, 4, 5 and 8 were replaced with unnatural amino acids. Therefore, we synthesized a number of analogs with N-methylated amino acids. *In vitro* (rabbit aorta) and *in vivo* (infusion into rats) assays indicated that [1-N-Methylisoasparagine, 8-isoleucine]-angiotensin II was equipotent with [Sar^1, Ile^8]-angiotensin II in all respects. However, antagonistic activity of [Sar^1, Ile^8]-angiotensin II was reduced when position 1 was replaced with H, Pro, MePhe, Me_2Gly, MeAla, MeIle, D-aMeIle, or GdnAc-, or when chain-length was increased with a proline residue either at the N- or C-terminus,[3,5,6] or when this molecule was modified as in [Sar^1, MeTyr^4, Ile^8]-, [Sar^1, MeIle^5, Ile^8]-, [Sar^1, MeIle^8]-, [Sar^1, MeIle^5, MeIle^8]-angiotensin II. All these analogs also possessed varying degrees of initial pressure activity. Substitution of isoleucine in position 5 or position 8 with N-methylisoleucine significantly enhanced the initial pressor activity of [Sar^1, Ile^8]-angiotensin II. This effect was not diminished in adrenalectomized rats.

[Sar^1, Thr^8]-angiotensin II was synthesized[6] to study the effect of a polar group in position 8. Infusion studies in rats indicated that this analog was equipotent with [Sar^1, Ile^8]-angiotensin II with the additional advantage that the initial pressor activity of this analog was 50% that of [Sar^1, Ile^8] angiotensin II.[6] In order to get further information on the role of hydroxyl function in position 8, threonine was replaced with O-methylthreonine. Thus, [Sar^1, Thr(O$^\beta$Me)^8]-angiotensin II was found to be the most potent antagonist so far synthesized. It had a dose ratio of 62.52 ± 14.93 as compared to 26.91 ± 3.30 for [Sar^1, Thr^8]-, 23.63 ± 6.87 for [Sar^1, Ile^8]-, and 7.92 ± 1.21 for [Sar^1, Ala^8]-angiotensin II.

Inhibition of Angiotensin II-Induced Adrenal Catecholamine Release

We supplied all these analogs to Dr. M. Peach at the University of Virginia for testing on perfused cat adrenals. A comparison of myotropic activity of these peptides in smooth muscle and their ability to release catecholamines in adrenal medulla indicated[7] that most of these peptides are more potent as agonists in adrenal medulla than in vascular smooth muscle. For example, [Ile^8]-angiotensin II had 1% myotropic activity of angiotensin II in smooth muscle as compared to 25% catecholamine releasing effect in adrenal medulla. The latter activity was reduced to 3% when aspartic acid in position 1 was replaced with sarcosine.

In addition to the agonistic activity, these peptides are also antagonistic to angiotensin II-induced response in adrenal medulla. Analogs with branched aliphatic side chain in position 8 were good antagonists of angiotensin II-induced release of catecholamines. Although in smooth muscle, sarcosine in the 1 position enhanced the antagonistic properties of these compounds, no such effect was observed in adrenal medulla. pA_2 values of [Ile^8]-, and [Sar^1, Ile^8]-angiotensin II were 9.3 and 9.5, respectively.[7]

Synthesis of C-Terminal Heptapeptide Analogs of Angiotensin II as Specific Inhibitors for the Release of Aldosterone

It is well recognized that angiotensin II and aldosterone act together to regulate sodium and water balance. Further, angiotensin II is also one of the main factors which regulate the secretion of aldosterone from adrenal cortex. But the exact mechanism by which angiotensin II stimulates the biosynthesis or release of aldosterone is presently not understood.

Blair-West and his co-workers[8] observed that a naturally-occurring metabolite of angiotensin II, the C-terminal heptapeptide, which has less than 30% pressor activity of angiotensin II is at least as active as angiotensin II in stimulating the secretion of aldosterone from adrenal cortex in sheep. These results have now been confirmed by a number of investigators.[9-11] Further, as compared to angiotensin II-induced adrenal cortical stimulation, the [Des-Asp^1] angiotensin II-induced stimulation was resistant to inhibition with the octapeptide analogs [Sar^1, Ala^8]-, or [Sar^1, Ile^8]-angiotensin II.[9,10,12] For example, [Sar^1, Ile^8]-angiotensin II blocked the response due to [Des-Asp^1]-angiotensin II at

a molar dose ratio of 50:1, as compared to 2:1 molar dose ratio for angiotensin II-induced response.[9] It was therefore reasoned that if it is the heptapeptide which is responsible for adrenal cortical stimulation then the C-terminal substituted heptapeptides, *e.g.*, [Des-Asp^1, Ile^8]-angiotensin II[5] should be more potent for blockade of steroidogenesis than the octapeptide, *e.g.*, [Sar^1, Ile^8]-angiotensin II. In order to test this hypothesis the following heptapeptide analogs of angiotensin II were synthesized in which the C-terminal position was replaced with amino acid residues analogous to the antagonists of the pressor and myotropic response of angiotensin II.

H - [X] -Val-Tyr-Ile-His-Pro- [Y]

X = L-Arg, D-Arg, D-Ala, Gly, Ser

Y = Gly, Ala, Val, Ile, Leu, Ser, Thr(O^{β}Me)

In *in vitro* studies (adrenal cortical cell suspensions), [Des-Asp^1, Ile^8]-angiotensin II was found to be a better antagonist of angiotensin II-, or [Des-Asp^1]-angiotensin II-induced steroidogenesis than [Sar^1, Ile^8]-angiotensin. II.[13] In the *in vivo* experiments[12] (bilaterally nephrectomized, ACTH suppressed male mongrel dogs with isolated adrenal gland preparation) the results obtained, as exemplified with the octapeptide [Sar^1, Ile^8]-, and the heptapeptide [Des-Asp^1, Ile^8]-angiotensin II, indicated that at the dose levels (200 ng/kg/min) of [Sar^1, Ile^8]-angiotensin II, which abolished the pressor response of angiotensin II, the angiotensin II-stimulated aldosterone secretion was unaffected.[11] In contrast, the heptapeptide analog significantly inhibited aldosterone secretion without blocking the pressor response of angiotensin II.[11,13]

Discussion

The results obtained suggest that receptors for angiotensin II for vascular smooth muscle, adrenal medulla, adrenal cortex (and possibly in other tissues) are functionally different and that the chain length of the peptide plays a significant role in stimulation or blockade of the pressor and myotropic action of angiotensin II or its aldosterone-releasing effect.

The octapeptide [Sar^1, Ile^8]-angiotensin II is a potent antagonist of the pressor response of angiotensin II, but a poor antagonist of aldosterone secretion. Conversely, the

heptapeptide, [Des-Asp^1, Ile^8]-angiotensin II, is a potent antagonist of the secretion of aldosterone but a poor antagonist for the pressor action of angiotensin II. However, at high dose levels (>1 μg/kg/min), the heptapeptide analog also blocked the pressor action of angiotensin II. In the sodium-deprived rats, the administration of [Des-Asp^1, Ile^8]-angiotensin II was not associated with an acute increase in plasma renin activity, while treatment with [Sar^1, Ile^8]-angiotensin II resulted in a 6-fold increase in plasma renin activity.[13] Further, in the *in vivo* studies at equimolar doses the heptapeptide analog [Des-Asp^1, Ile^8]-angiotensin II showed less intrinsic activity[5,11,13] (pressor or steroidogenic) as compared to [Sar^1, Ile^8]-angiotensin II, and was also a good inhibitor of catecholamine release in adrenal medulla (M. Peach, unpublished results). Thus, these results suggest that: (a) [Des-Asp^1]-angiotensin II plays a significant role in the biosynthesis and/or secretion of aldosterone, and (b) C-terminal substituted heptapeptide analogs, *e.g.*, [Des-Asp^1, Ile^8] angiotensin II, appear to be more suitable for long-term administration than the corresponding octapeptides in the control of direct vasoconstrictor effect of angiotensin II or its aldosterone releasing effect.

The drastic decrease in antagonistic activity due to substitution in positions 4, 5 and 8 in [Sar^1, Ile^8]-angiotensin II with N-methylated amino acids, or the enhancement of the antagonistic activity due to the presence of O-methylthreonine in position 8 could be due to a number of factors, *e.g.*, change in size, conformation, binding properties with the receptor site, or mode of degradation in plasma. A detailed study of such parameters is important in order to determine the biologically active conformation of angiotensin II and its mode of interaction with the receptor site. A significant finding has been the preferential binding of angiotensin II, rather than angiotensin I, by the particulate receptor fraction prepared from bovine and rat adrenal cortex, and the location of angiotensin binding sites in microsomal membrane vesicles, rather than in mitochondria.[14-16] These observations have considerable potential for studying structure-activity relationships by measurement of relative binding affinities of angiotensin analogs having agonistic or antagonistic properties.

Acknowledgments

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CYCLIC ANGIOTENSIN

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DURING THE LAST FEW YEARS, a great deal of effort has been made in order to determine the conformation adopted by peptides in solution.[1] These studies have been carried out in the firm belief that precise knowledge of the conformational properties of a molecule would greatly help the understanding of its mode of action at the biological level.

Because the number of conformations accessible to peptides even short is enormous, conclusive results have been obtained only for the more constrained cyclic peptides. For example, the spatial structure of cyclic hexapeptides,[2,3] gramicidin S,[2,4] oxytocin[5] and actinomycin D[6] has been revealed by nuclear magnetic resonance spectroscopy complemented by various other techniques. The much more complicated cases of linear peptides, several of which present biological activity of the utmost importance, remains however a challenging problem.[7] Among those linear peptides, the potent vasoconstriction hormone, angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe) definitely deserves great attention.[8]

Experimental studies[9-11] suggest that angiotensin II is folded in solution, but the main question -- which folded conformation does it assume? -- remains unanswered. A recent theoretical analysis carried out in our laboratory has shown that a small number of folded conformers are highly probable. The course of the backbone of the most probable

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conformer is depicted in Figure 1A. Another type of structure met among the highly probable conformers is quasi-cyclic, like the one shown in Figure 1B. These results suggested to us to attempt the cyclization of angiotensin and to use the cyclic molecule as a probe to angiotensin conformation.

Asn^1, Val^5 angiotensin II (A II'), kindly given to us by Dr. Riniker (CIBA-GEIGY) was transformed into the dihydrochloride by lyophilization from dilute HCl. TLC showed no degradation of the peptide at this stage. The cyclization was carried out in dilute pyridine solution (10^{-2} *M*) using 1.5-fold excess of EEDQ. The reaction was followed by TLC on silica plates (Merck) developed in the solvent ethyl acetate-butanol-acetic acid-water (2:1:1:1). After 6 hours, all the A II' has disappeared as judged by the absence of positive ninhydrin and Pauly spot migrating at the same R_f.

The reaction mixture was evaporated to dryness under vacuum; the resulting oil was taken up in 10% acetic acid and chromatographed on a Sephadex G-15 column. The fractions absorbing in UV and giving positive Pauly spots were pooled and lyophilized. The mixture of peptides was chromatographed on prepacked silica gel columns (Merck) eluted in the solvent used in TLC. After repeated chromatography, a product was obtained that was ninhydrin negative but Pauly and Sakaguchi positive. It showed only a single spot in TLC. This product was finally chromatographed on Sephadex G-15 in 10% acetic acid giving a symmetrical UV peak. Cyclization was indicated by the following criteria: (1) no reaction with ninhydrin; (2) enzymatic cleavage with trypsin gave only one spot in different solvents; (3) the elution volume was the same as that of A II'; (4) the amino acid analysis after acid hydrolysis was the same as that of A II'; (5) the UV spectrum was similar to that of A II'.

The biological activity was tested on the vascular response in the anasthetized dog. As was predictable, from the absence of the carboxyl terminal group, the cyclized A II' showed no activity in this test at dose levels up to hundred-fold those where A II' was active (0.1 μg/kg *iv*). To test the possibility of an antagonist activity, the peptide was injected into the vein of the dog together with A II'. The doses were respectively 10 μg/kg and 0.1 μg/kg. There was, at this dose, no inhibition of the response of A II'. These observations confirm the previous results showing that a free carboxyl group is essential for the activity of the peptides derived from angiotensin.

The next step was to investigate the effect of the cyclization upon the conformational state of A II'. The CD

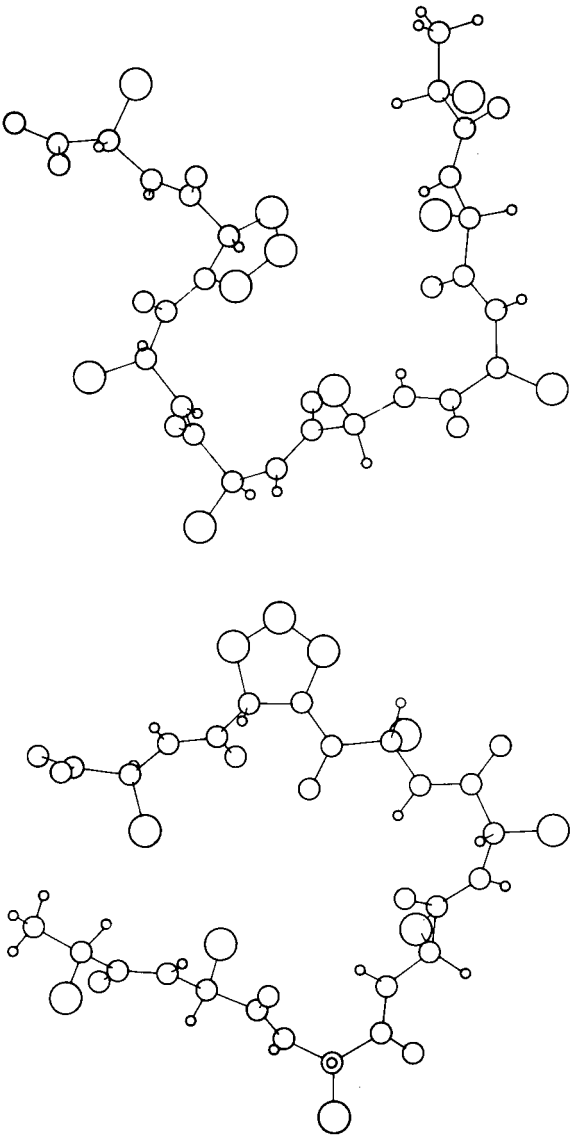

Figure 1: Projections of the backbone conformation of two angiotensin conformers.
A) The most probable conformer, as was determined by our energy calculations.[12] It contains one β-turn at the level of Val_5His_6, but no C_{10} hydrogen bonded ring because of the presence of the pyrrolidine ring of Pro_7.
B) A quasi-cyclic conformer, devoid of any β-turn.

spectra, in aqueous solution of linear and cyclic A II', are shown in Figure 2. The important feature is the absence for the cyclic molecule of the negative band observed for the linear peptide at 222 nm. Aromatic side-chains chromophores as well as $n \rightarrow \pi^*$ transitions of the peptide groups can contribute to the ellipticity in these regions. Little is known about the former contribution. On the other hand, secondary structures associated with negative bands in the 220 nm region can be of various types (α-helices, β-pleated sheets, β-turns...) Our theoretical analysis[12] as well as experimental results[13] suggest that β-turns are the most probable secondary structure present in linear A II'. If this is true, the difference between the CD spectra of linear and cyclic angiotensin could be interpreted as reflecting the disappearance of β-turns when going from the linear to the cyclic molecule.

This interpretation goes along with the results of our energy calculations: most of the highly probable quasi-cyclic conformers of angiotensin are stabilized by the

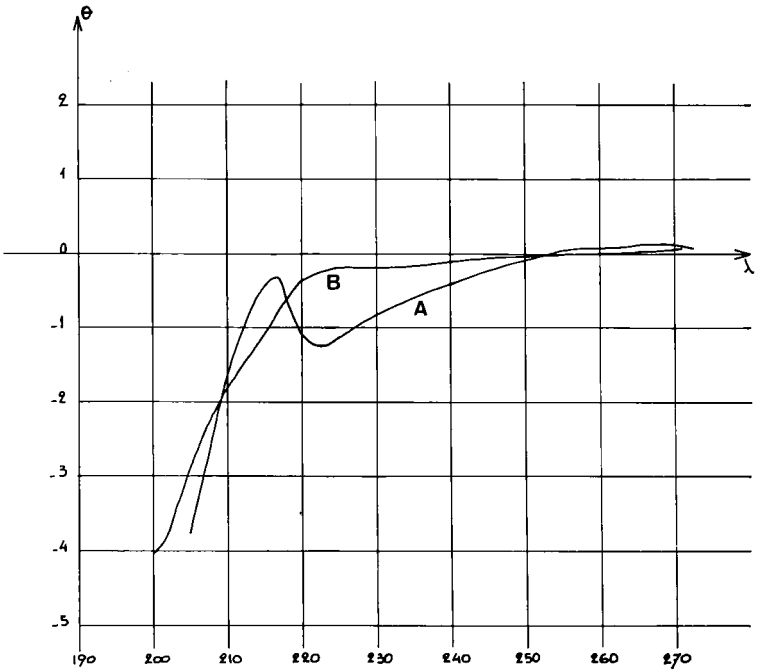

Figure 2: CD spectra of linear (A) and cyclic angiotensin (B) in water at pH 6.0 and 25°C. The spectra were made on a Cary 61 dichrograph. The ellipticity Θ is given in 10^4 degree·cm^2·decimole^{-1}, and the wave length λ in nm.

formation of C_5 or C_7 hydrogen bonded rings, and do not contain any β-turn. The cyclization of angiotensin in a conformation like the one represented in Figure 1B would lead to a square conformation labeled $(C_7C_5)_4$, similar to the one proposed by Blout *et al.*[14] for *cyclo*-(Pro-Gly)$_4$.

Thus, if our interpretation is correct, and not misled by the presence of side-chain contributions to the ellipticity in the 220 nm region, our results suggest that for cyclic octapeptides the geometrical and energetical properties of the backbone favor square conformations while it is well known that rectangular shapes are preferred for the cyclic hexa- and decapeptides.[2,4] For linear octapeptides, on the other hand, there is competition between square conformers and rectangular conformers, these containing β-turns. In the case of linear angiotensin, the latter seems to be more probable.

In summary, numerous experimental results suggest that angiotensin is a folded molecule in solution. Theoretical conformational analysis indicated among the most probable conformations of angiotensin a group of quasi-cyclic ones with significant probability of occurrence. Thus it appeared interesting to cyclize the molecule to compare conformational and biological properties of the two peptides. Cyclization with the aid of EEDQ in pyridine followed by column chromatography on silica gel and gel filtration gave a ninhydrin-negative product with positive reaction to the Pauly, Sakaguchi and chlorine/tolidine reagents. Cleavage by trypsin gave only one product. The UV spectrum was similar to that of angiotensin.

The product was inactive as agonist or antagonist on the vascular response in the anaesthetized dog. The CD spectrum of cyclic angiotensin in water appears quite different to that of the linear molecule. The contribution in the 220 mμ region is very weak compared to angiotensin and appears in the form of two positive shoulders at 210 and 225 mμ. Such a change in the CD spectrum could be attributed either to a variation of intensity of the $n\rightarrow\pi^*$ band of the peptide chromophores or to a different contribution of the aromatic side chains (Tyr 4 - His 6 - Phe 8).

This preliminary comparative study of linear and cyclic angiotensin provides a first hint of the possible difference between the overall topology of the two peptides. Work in progress will hopefully allow us to define with more precision the conformational properties of various angiotensin analogs, including cyclic ones, and to correlate these properties with biological activities.

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ANGIOTENSIN AS A TOOL TO STUDY EXCITATION-CONTRACTION COUPLING IN SMOOTH MUSCLE

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PREVIOUS STUDIES HAVE SHOWN THAT the responsiveness of isolated rat uterine smooth muscle to angiotensin II (A-II)[1,2] and an inhibitor analog, [Phe^4-Tyr^8]-angiotensin II (P_4T_8)[3] is markedly influenced by both the pH and Ca^{++} concentration of the bath fluid. For example, at pH 7.4 and a $[Ca^{++}]_0$ of 0.5 *mM* the sensitivity of rat uterus (RU) to A-II is relatively high and the responses to repeated identical doses are consistent. However, at pH 6.8 and $[Ca^{++}]_0$ of 0.2 *mM* (Both low Ca^{++} and low pH were required) the tissue is less responsive and successive identical doses result in progressively smaller responses--that is the tissue now exhibits tachyphylaxis (TACHY) to the hormone. Therefore, a model system was proposed in which it was suggested that the pH effect was due to protonation of the histidine residue of the A-II molecule while the ability of Ca^{++} to prevent tachyphylaxis was attributed to a direct interaction with a Ca^{++} binding site on or near the receptor.[1,2] Therefore, the following studies were designed to investigate more closely the role of Ca^{++} in the interaction of A-II and its receptor substance in smooth muscle.

Isolated smooth muscle preparations were carried out as described previously[4] on duplicate tissues from the same animal. When drugs, ions or pH were tested for their ability to modify responsiveness of the tissues to various agonists, they were preincubated for 30 minutes with one muscle while the duplicate remained in the control solution throughout to insure that no major spontaneous changes in sensitivity occurred. All responses have been normalized as a percent of the maximum contraction with the best control response being taken as 100%.

Interactions of the kind suggested by our earlier work[1-3] almost certainly occur at the extracellular membrane since A-II and P_4T_8 are relatively large cationic molecules which in all probability would not penetrate intracellularly. This is supported by the findings (Table I) that several drugs and ions, whose main pharmacological effects are thought

Table I

Effect of Ca^{++} Antagonists on Angiotensin II-Induced Contraction of Rat Uterus

Tissue	*Treatment*	*Ca^{++} (mM)*	*ID_{50} Antagonist (M)*	*ED_{50} Angiotensin (M)*
Rat uterus ($5.6\,mM\ K^+$)	Control	0.45	-	1.2×10^{-9}
Rat uterus ($5.6\,mM\ K^+$)	Verapamil	0.45	5.5×10^{-7}	1.0×10^{-8}
		0.18	2.0×10^{-7}	7.0×10^{-9}
Rat uterus ($5.6\,mM\ K^+$)	SKF 525-A	0.45	2.3×10^{-6}	1.5×10^{-8}
Rat uterus ($5.6\,mM\ K^+$)	Tetracaine	0.45	4.2×10^{-5}	$1.7 \times 10^{-}$
Rat uterus ($5.6\,mM\ K^+$)	$LaCl_3$	0.45	2.1×10^{-4}	1.3×10^{-8}
Rat uterus ($5.6\,mM\ K^+$)	$MnCl_2$	0.45	2.4×10^{-3}	7.5×10^{-9}
Rat uterus ($151\,mM\ K^+$)	Control	0.18	-	1.9×10^{-9}
Rat uterus ($151\,mM\ K^+$)	Verpamil	0.18	1.1×10^{-8}	1.0×10^{-8}
Rabbit aorta ($5.6\,mM\ K^+$)	Control	0.45	-	2.2×10^{-9}
Rabbit Aorta ($5.6\,mM\ K^+$)	Verapamil	0.45	1.1×10^{-4}*	5.0×10^{-9}

Each value represents an average of 3-10 individual experiments. Inhibitors were pre-incubated with the tissue for 30 min before testing. ID_{50} = dose necessary to reduce maximum response to 50% of control value. ED_{50} = dose of A-II to give 50% maximum contraction at the ID_{50} concentration of the inhibitor

*No significant inhibition seen even at this dose.

to be due to antagonism of transmembrane Ca^{++} movements, block A-II induced contractions of rat uterus (RU). In contrast, A-II induced contractions of the smooth muscle of the rabbit aortic strip (RAS) are remarkably resistant to agents like verapamil (Table I) and SKF 525-A.[4,5] If in fact the mechanism of action of these compounds is to antagonize movement of Ca^{++} from the extracellular membrane into the myoplasm, then A-II induced contractions appear to have a strong dependence on an extracellular Ca^{++} pool in RU while in RAS they appear to utilize predominantly, if not exclusively, an intracellular Ca^{++} pool. This property is not shared by a variety of other agonists including norepinephrine, histamine, serotonin, potassium chloride[5] and acetylcholine.[4] Therefore, A-II could be a very useful tool to study effects of drugs or experimental maneuvers on specific Ca^{++} pools in smooth muscle.

With respect to RU certain other observations suggest that the Ca^{++} site (pool?) observed in the tachyphylaxis experiments may represent the link between agonist/receptor combination and membrane depolarization. Foremost is the fact that placing RU in an isotonic KCl solution abolished the ability of low pH and low Ca^{++} to produce A-II TACHY (Figure 1). As shown, application of A-II to depolarized RU (151 *mM* KCl, 50 *mM* NaCl, 0.2 *mM* $CaCl_2$ at pH 7.4) results

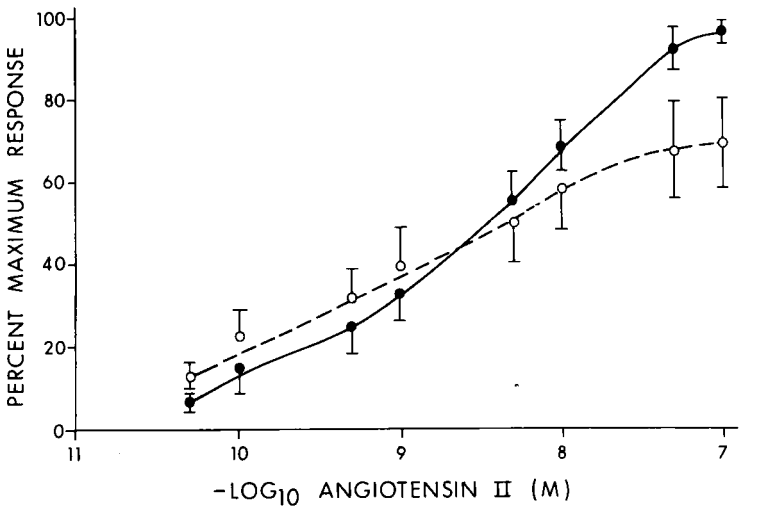

Figure 1: Effect of pH on A-II responses of depolarized rat uterus. ●——● = Response in the presence of 150 *mM* KCl, 50 *mM* NaCl, 0.2 *mM* $CaCl_2$ at pH 7.4; o——o = Response under identical conditions at pH 6.4.

in good contractile responses of this tissue. There is, however, no significant change in the response to A-II when the pH is lowered to 6.4 (Ca^{++} still at 0.2 *mM*). This is in contrast to the normally polarized preparation which rapidly develops TACHY under these conditions.[1,2] These data imply, therefore, that the ability of A-II to develop TACHY is dependent on the membrane potential of the smooth muscle membrane. It is interesting that in RU Ca^{++} currents are believed to be responsible for a considerable portion of the membrane electrical events following stimulation.[6-8] It should be pointed out that the response of the depolarized RU to A-II is still Ca^{++} dependent as indicated by the ability of low concentrations [ID_{50} of 5×10^{-7} *M*] of verapamil to inhibit the tissue (Table I).

Based on the results of these studies, as well as previous work, the working model of the A-II contraction shown in Figure 2 is proposed. In this model the interaction of A-II with its receptor in a normally polarized membrane would result in the generation of a Ca^{++} dependent electrical response mediated by a Ca^{++} binding site designated as the $Ca^{++}{}_1$ site. Release from the $Ca^{++}{}_1$ site would then in turn release Ca^{++} from a second store, ionophore, or carrier system designated the $Ca^{++}{}_2$ site. The number of steps or the mechanism of this excitation-contraction coupling remains a mystery. It is the $Ca^{++}{}_2$ mechanism which we postulate would ultimately result in the contractile event. As before, the mechanism by which this $Ca^{++}{}_2$ site generates contractile activity is not known. We have no evidence to judge whether this $Ca^{++}{}_2$ site represents the intracellular (cytoplasmic organelle) Ca^{++} pool in rat uterus although for the purposes of our model building it is considered as another membrane component.

Therefore, in considering mechanisms by which A-II induced contractions may be inhibited, we propose the following scheme. Since in a depolarized rat uterus the electrical component of the system is now eliminated as is the ability of elevated H^{+} concentration to result in A-II tachyphylaxis, it is proposed that the interaction of the imidazole side chain of A-II is with the $Ca^{++}{}_1$ site involved in the generation of electrical activity in response to applied A-II. Confirmation of this hypothesis must await direct measurement of the electrical events occurring in the membrane under various experimental conditions. In contrast to the tachyphylaxis experiments, verapamil was an effective inhibitor in both normal and depolarized muscles, and therefore clearly interferes with some common step in the pathway distal to the membrane excitation. Its site of

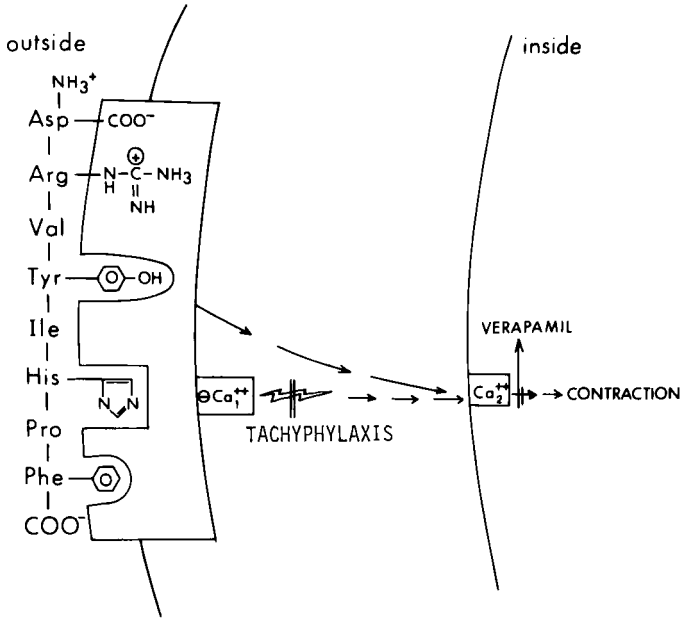

Figure 2: Hypothetical model of A-II/receptor interaction in rat uterine smooth muscle.

action is tentatively considered to be the hypothetical Ca^{++}_2 mechanism.

Acknowledgments

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APPROACHES TO MECHANISM OF SMOOTH MUSCLE ACTION OF SUBSTANCE P, ELEDOISIN AND PHYSALAEMIN, USING THEIR COOH-TERMINAL SEQUENCES AND ACYL DERIVATIVES

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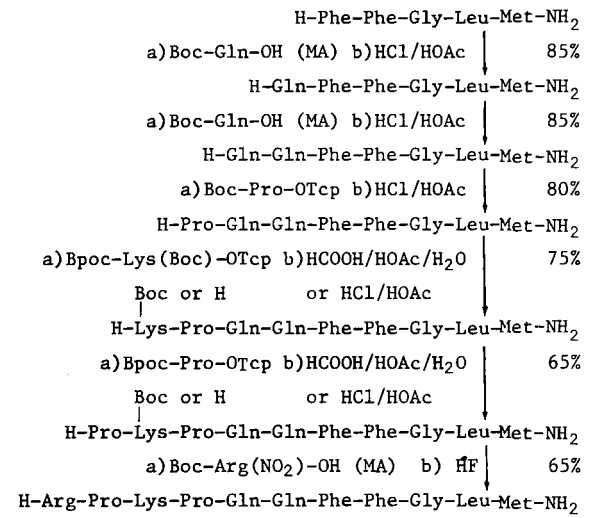
SUBSTANCE P PEPTIDES WERE SYNTHESIZED[1] by stepwise chain elongation, Scheme I. On guinea pig ileum and rat colon a stepwise prolongation of pentapeptide results in different



Formation of pyroglutamyl peptides during purification.

Scheme 1: Synthesis of substance P.

trends of biological activity, receptor affinity and intrinsic efficacy.

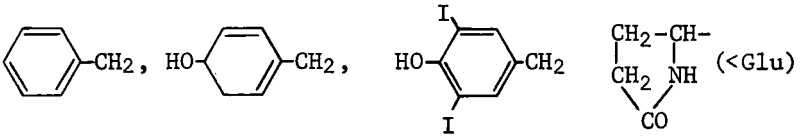
For acylation of COOH-terminal eledoisin and physalaemin peptides we used mixed anhydrides or 2.4.5-trichlorophenyl esters of several carbonic acids in DMF, see Table I. The smooth muscle activity of three series of peptides is summarized in Table II. A relation between biological results and hydrophobicity of acyl substituents may be seen. The proteolytic degradation of acyl derivatives is retarded. They are suitable for binding studies. H-Acetylphysalaemin hexapeptide was used for some investigations about nonspecific and specific binding to cell membrane fractions, see Table III.

Table I

Acylation of Penta- and Hexapeptides of Eledoisin, Physalaemin and Substance P

a) R-CO-O-$C_6H_2Cl_3$ (2,4,5)	+ H-Phe-Ile-Gly-Leu-Met-NH_2
b) R-CO-O-C_4H_9 (sec)	+ H-Phe-Tyr-Gly-Leu-Met-NH_2
	+ H-Phe-Phe-Gly-Leu-Met-NH_2
	+ H-Ala-Phe-Ile-Gly-Leu-Met-NH_2
	+ H-Lys(Boc)-Phe-Ile-Gly-Leu-Met-NH_2
	+ H-Lys(Boc)-Phe-Tyr-Gly-Leu-Met-NH_2
	+ H-Gln-Phe-Phe-Gly-Leu-Met-NH_2

R = H_3C, H_3C-CH_2, F_3C, $ClCH_2$, $BrCH_2$, ICH_2, $H_5C_2OCOCH_2$,



Estimation of Affinity and Intrinsic Efficacy on Guinea Pig Ileum[5]

In the presence of peptides in the organ bath, especially compounds 7 and 8, the dose response curves were shifted to higher concentrations. With a distinct peptide content in the bath solution the maximal effect begins to drop, *i.e.*, all reserve receptors are occupied. Evaluation and determination of K^X was done after Furchgott,[6] who used

Table II

Biological Activity of COOH-Terminal Peptides of Eledoisin, Physalaemin, Substance P and Some of Their Acyl Derivatives on Ileum and Colon

		A_{Il}+)	K^{x}_{Il}	e_{Il}	A_{Co}	ME_{Co}
1.	H-Phe-Ile-Gly-Leu-Met-NH_2	25.5	1115	11 ± 2	290	73 ± 7
2.	CH_3CO----------	0.7	14	20 ± 4	86	56 ± 4
3.	CH_3-CH_2CO----------	0.48	--	-	--	
4.	$BrCH_2CO$----------	0.4	21	53 ± 12	50	77 ± 4
5.	C_6H_5-CH_2CO----------	0.4	--	-	--	-
6.	HO-C_6H_5-CH_2CO----------	0.15	18	40 ± 8	12	44 ± 2
7.	HO-$C_6H_2I_2$-CH_2CO----------	0.2	64	81 ± 22	23	38 ± 5
8.	Lys----------	1.0	191	320 ± 55	12	100
9.	$BrCH_2CO$-Lys----------	0.3	26	102 ± 33	5	89 ± 4
10.	Ala----------	1.4	495	170 ± 40	8	90 ± 4
11.	H-Phe-Tyr-Gly-Leu-Met-NH_2	5.1	1330	30 ± 11	230	70 ± 6
12.	$BrCH_2CO$----------	0.45	31	64 ± 14	29	39 ± 3
13.	Lys----------	0.45	270	396 ± 98	10	96 ± 4
14.	CH_3CO-Lys----------	0.12	--	-	--	-
15.	R = Phe-Phe-Gly-Leu-Met-NH_2	35	314	28 ± 6	350	67 ± 4
16.	$BrCH_2CO$--R	0.4	69	90 ± 19	24	44 ± 4
17.	H-Gln--R	1.0	13	29 ± 7	9	72 ± 4
18.	H-Gln-Gln--R	0.56	51	113 ± 25	42	82 ± 4
19.	H-Pro-Gln-Gln--R	0.36	40	113 ± 33	24	86 ± 4
20.	H-Lys-Pro-Gln-Gln--R	0.21	56	207 ± 30	14	85 ± 3
21.	H-Pro-Lys-Pro-Gln-Gln--R	0.66	14	30 ± 8	41	68 ± 6
22.	Subst. P = H-Arg-Pro-Lys-Pro-Gln-Gln--R	0.49	12	15 ± 3	24	75 ± 4

Table II Notes

A_{Il} = ED_{50} on guinea pig ileum x $10^8 \cdot M^{-1}$.

K^x_{Il} = Apparent dissociation constants of peptides for the isolated organ x $10^8 \cdot M^{-1}$.

e_{Il} = Intrinsic efficacy (degree of effect in coupling of receptor complex).

A_{Co} = ED_{50} on rat colon x $10^8 \cdot M^{-1}$.

ME_{Co} = Maximal effect in percent of compound 8 = 100.

+) = The maximal effect on ileum was in all cases 100 ± 4%, except for compound 7 with 88%. The standard deviations for A_{Il} and A_{Co} from about 10 experiments are in a magnitude of 25%.

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irreversible inhibitors for (acetylcholine-) receptor occupation. Comparison of equieffective concentrations before (A) and after blockade (A') in a plot 1/A against 1/A' results in

$K^x = \frac{\text{slope} - 1}{\text{intercept}}$. The receptor occupation is $y = \frac{1}{1 + \frac{K}{A}x}$

The intrinsic efficacies e = 1/y are calculated from y_{50}, *i.e.*, at 50% occupation of residual receptors.

Summarized Discussion and Conclusions

--Substance P, eledoisin, physalaemin and their peptides act on smooth muscles by the same mechanism.

--One may distinguish among five components of molecular mechanism:

1. transport and distribution into receptor region.
2. elimination and metabolism.
3. binding to discriminators on cell surface (affinity).
4. coupling to intracellular effectuation (intrinsic efficacy).
5. regeneration of the whole receptor system.

Table III

Preliminary Biochemical Results about Interactions of Eledoisin, Physalaemin and Substance P Peptides with Subcellular Fractions from Guinea Pig Ileum

A. Microsomes from whole organ, a model system for degradation studies.
B. Microsomes from a smooth muscle preparation.
C. Plasmamembrane preparation from smooth muscles obtained by gradient centrifugation.

Rate of Irreversible Inactivation (%)[a]	*A*	*B*	*C*
Lys-Phe-Ile-Gly-Leu-Met-NH_2 (I)	96.5±1	90±1	85.5±2
Acetyl-I	50 ± 5	<10	<10
Bromoacetyl-I	78 ± 2	-	18 ± 6
Acetyl-Lys-Phe-Tyr-Gly-Leu-Met-NH_2 (II)	76 ± 3	-	<15
Glu-Gln-Phe-Phe-Gly-Leu-Met-NH_2	68 ± 3	-	-
H-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	82 ± 2	-	-
Rate of Reversible Inactivation (%)[a] *of II (15 min) and Nonspecific Binding of* ^{3}*H-II*[b] *(15 min at* 1.10^{-7} *M)*			
[pMol/mg protein]	4.0±0.3[a] ∿7	-	53±3[a]
Maximal Specific Binding of ^{3}*H-II*[c] [pmol/mg protein]	-	0.15-0.3[d]	0.8-1.4[d]
5'-Nucleotidase Activity [nmol P/mg·min]	-	16-65[d]	140-320[d]

[a] The rate is nearly independent from concentration of peptides in the range of 10^{-7} to 10^{-5} *M*. Incubation 60 min, 37°C with 0.1 mg protein/ml, biological assay of residual peptides after liberation of masked peptides by acidification to pH 2.2. n = 6 - 10.

[b] Specific radioactivity of ^{3}H-acetyl-peptide was 290 mCi/mmol.

[c] 25°C, 15 min saturation with $2 \cdot 10^{-8}$ *M* ^{3}H-II, liberation of radioactivity by a 10- to 1000-fold excess of II.

[d] Scope of values from several preparations.

--These components would be influenced differently by structure variations.

--Degradation studies with free and acylated peptides show that aminopeptidases play an important role in inactivation by smooth muscle membranes.

--Enhancement of activity by acylation is not only due to protection against degradation (a carbamoyl-pentapeptide is inactive and depends on hydrophobicity of RCO).

--The rat colon shows a much lower sensibility to these peptides than the guinea pig ileum.

--On the rat colon, in contrast to the ileum, most peptides are partial agonists; therefore the maximal contraction represents the intrinsic efficacy.

--Enhancement of biological activity by acylation is due to an increase of affinity in both organs.

--COOH-terminal lysin enhances the intrinsic efficacy.

--Stepwise prolonged COOH-terminal sequences of substance P show maximal intrinsic efficacy for the nonapeptide, affinity maxima for hexapeptide and undecapeptide.

--The enrichment of specific binding proportional to 5'-nucleotidase demonstrates the existence of a high affinity binding site in cell membranes of smooth muscles of guinea pig ileum.

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STUDIES OF BIOACTIVE ANALOGS OF PARATHYROID HORMONE

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THE PRIMARY STRUCTURES OF THE complete bovine and porcine parathyroid hormones[1-3] and a partial sequence of the human hormone[4] (Figure 1) have now been elucidated. Some of the major sequence changes in the NH_2-terminal region of the porcine molecule include the substitution of serine for alanine at position 1 and the replacement of one methionine, at position 18, by a leucine residue. Like the porcine hormone and unlike the bovine, the human has an NH_2-terminal serine. Unlike the porcine, the human retains two methionine residues, thus making it similar to the bovine molecule in this respect. Peptides consisting of the NH_2-terminal 34 residues of the bovine and human molecule have been synthesized by solid phase methods and shown to be highly active; essentially 100% on a molar basis of the biological potency of the native bovine hormone has been shown *in vivo* and *in vitro*[5] (insufficient human hormone is available for test *in vivo*, but the synthetic peptide is equipotent to native *in vitro*).

Since oxidation of methionine residues within the biologically active core of the molecule to the sulfoxide or sulfone results in loss of biological activity,[5,6] and conventional iodination techniques employ oxidative conditions,

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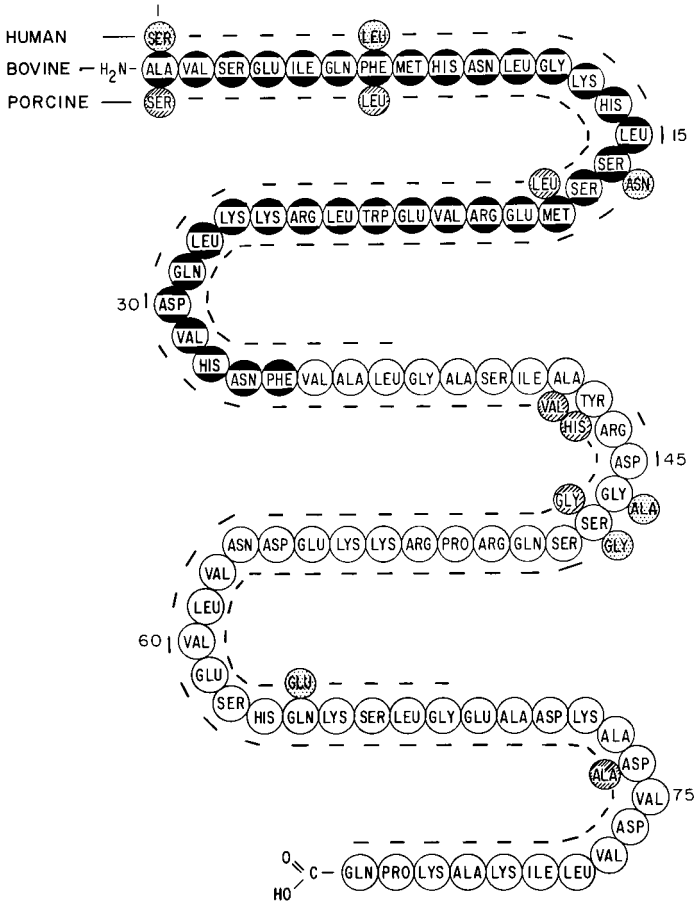

Figure 1: Amino acid sequence of bovine parathyroid (bPTH) (continuous chain). Substitutions found in the porcine hormone are represented by the cross-hatched circles, and known substitutions found in the human sequence are represented by the stippled circles. The NH_2-terminal 34 residues, represented by the dark circles, contain virtually all of the biological potency of the bovine molecule. (Modified from H. T. Keutmann *et al.*, ref 12).

the resulting loss of activity has hampered the development of biologically useful tracers for studies of parathyroid hormone metabolism and binding to target-tissue receptors. Consequently, an analog of the bovine NH_2-terminal tetratriacontapeptide in which the methionine residues at positions

8 and 18 were replaced by sterically similar norleucine residues was synthesized. This alteration resulted in an analog resistant to oxidation. A tyrosine was substituted for phenylalanine at position 34, permitting iodination to a high specific activity. The molar ratio of incorporated iodine to hormone was approximately 1:1 (as confirmed by amino acid analysis). When tested in the *in vitro* rat renal adenylyl-cyclase assay, this analog exhibited at least 69% of the biological activity of the native sequence both before and after iodination by the conventional chloramine-T method (Table I).[7] Evidently, methionine residues are not essential for hormonal activity, even though oxidation results in loss of activity. The biologically active, highly radioactive analog is useful for investigation of hormone metabolism and hormone-receptor interaction *in vitro*.[7]

Table I

Biological Activity of Synthetic Bovine Parathyroid Hormone Analogs Before and After Iodination

Peptide Number	*Residue Number*				*Potency*		*Potency %*
	1	*8*	*18*	*34*	*Avg.*	*Range*	
I	Ala	Met	Met	Phe	5400	(3900-8000)	100
II	Ala	Nle	Nle	Tyr	4100	(3400-4900)	76
III	Ala	Nle	Nle	^{125}I	3700	(2500-5300)	69
IV	Ala	Met	Met	Tyr	7500	(6000-9400)	139
V	Ala	Met-SO_2	Met-SO_2	^{125}I	<150		< 3

We previously investigated the biological activity of fragments of the bovine hormone successively shortened at the NH_2- and COOH-ends of the NH_2-terminal tetratriaconta-peptide. The minimum active sequence appears to comprise residues 2 through 27.[8]

Two synthetic analogs of the bovine hormone with NH_2-terminal modifications that rendered them inactive in the *in vitro* renal adenylyl-cyclase assay were effective inhibitors of PTH action in this system--these were [desamino-Ala^1] bPTH-(1-34) and bPTH-(3-34).[9] In contrast, the biologically inactive analogs bPTH-(13-34), with extensive deletion of the NH_2-terminus, and bPTH-(1-26), with extensive deletion at the COOH-terminus, did not exhibit antagonist activity. This suggests that shortening at the COOH-terminus results

in peptides of decreasing potency due to decreasing capacity to bind to the target-tissue receptor. In contrast, a small deletion at the NH_2-terminus results in a peptide that still binds to the renal receptor, albeit with reduced affinity, but can no longer stimulate adenylyl cyclase. Extensive amino-terminal deletions result in loss of both biological potency and receptor binding.

A critical role of the NH_2-terminal residue in the *in vitro* assay was suggested by previous studies.[8,9] The human peptide, with serine at position 1, was significantly less potent than the bovine analog, with alanine at position 1, *in vitro* in the rat renal adenylyl-cyclase assay (Table II).

Table II

Biological Activity *In Vitro* of Analogs of Bovine and Human Parathyroid Hormone

PEPTIDE	RAT		COW		DOG		MAN		CHICK	
	U/mg	Mol %	U/mg	Mol %	U/mg	Mol %	U/mg	Mol %	U/mg	Mol %
bPTH-(1-34)	6700	111	6000	99	1200	20	7000	116	5200	86
hPTH-(1-34)	1300	22	1400	23	370	6	1500	25	1800	30
[Ser^1]bPTH-(1-34)	1600	27	2200	36	440	7	1700	28	2100	35
[Ala^1]hPTH-(1-34)	4100	68	4200	69	830	14	4900	81	5400	89

The relative role of serine *vs* alanine in the bovine and human tetratriacontapeptides was further examined.[10] Both of these peptides and synthetic [Ser^1]-bPTH-(1-34) and [Ala^1]-hPTH-(1-34) were assayed *in vitro* in membranes[11] prepared from bovine, canine, chick, and human kidneys, and in rat membranes, to identify any possible species differences. In both homologous and heterologous hormone-membrane assay systems, the human analog was less potent than the bovine. Furthermore, substitution of serine for alanine in the bovine analog reduced potency in each membrane system, whereas substitution of alanine for serine in the human hormone enhanced its potency, indicating that the low potency *in vitro* of the human peptide reflected the presence of serine rather than alanine at position 1. Peptides with alanine at position 1 were full agonists in all membranes, whereas peptides with serine at position 1 were full agonists only

in mammalian membranes and partial agonists in the chick, even in [Ser^1]-bPTH-(1-34). On the other hand, the human and bovine synthetic peptides hPTH-(1-34) and bPTH-(1-34) were of equivalent potency *in vivo*.[12]

Several conclusions seem possible. The similarity in the order of relative potencies in membranes prepared from both mammalian and submammalian species suggests conservatism in the evolution of PTH receptor-cyclase complexes. These studies further emphasize the striking importance of the NH_2-terminal residue of PTH in determining the biological activity of the hormone, at least in the *in vitro* adenylyl-cyclase assay. The evidence that these analogs are equipotent in the *in vivo* assay leaves unanswered the question whether requirements for binding to bone and renal receptors are similar.

Recently, we have examined the role of co-factors in the *in vitro* adenylyl-cyclase assay, particularly the role of GTP. In view of recent reports that guanine nucleotides enhance the response of adenylyl-cyclase systems to peptide hormones,[13] the activity of several bPTH analogs with NH_2-terminal deletions was studied[14] in the presence and absence of guanylylimidodiphosphate (GMP-PNP), an analog of GTP whose terminal phosphate is apparently not readily cleaved.[13] In the absence of GMP-PNP, the analog bPTH-(2-34) was a partial agonist, capable of stimulating adenylyl-cyclase activity to only 30% of that achieved by a maximal dose of bPTH-(1-84). Both [desamino-Ala^1]-bPTH(1-34) and bPTH-(3-34) were virtually inactive (Figure 2).

In the presence of the guanine nucleotide, each synthetic peptide demonstrated a moderate increase in potency relative to the intact native bovine hormone (1-84). More strikingly, however, the intrinsic activity of the partial agonists bPTH-(2-34) and especially [desamino-Ala^1]-bPTH-(1-34) was enhanced. If only a small quantity of cyclic AMP needs to be generated *in vivo* for hormonally mediated events to occur, and if GTP or a similar co-factor is indeed operative *in vivo*, these observations may help to explain some of the discrepancies between *in vivo* and *in vitro* activity demonstrated by some of the synthetic analogs.[12,15]

These studies with synthetic analogs of PTH have therefore already provided considerable information regarding the mechanism of action of PTH at the molecular level. In addition, the role that methionines play in the biologically active region of the molecule has been clarified, and a sulfur-free radioactive, biologically active synthetic analog, ^{125}I-labeled-[Nle^{8,18},Tyr^{34}]-bPTH-(1-34), has been prepared, which should prove helpful in further exploring

the nature of parathyroid hormone metabolism and interaction with renal-cell membranes.

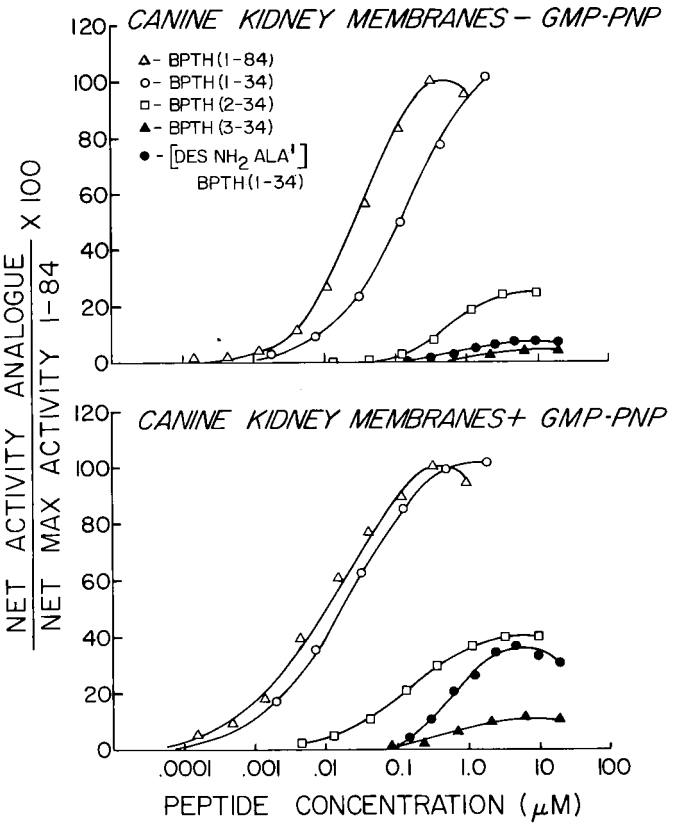

Figure 2: Dose-response curves of native bPTH and synthetic analogs of bPTH in canine renal cortical membranes in the absence of (upper) and in the presence of (lower) 10^{-6} *M* GMP-PNP. Response is expressed as adenylyl-cyclase activity above basal stimulated by each analog, divided by maximal adenylyl-cyclase activity above basal stimulated by bPTH-(1-84).

Acknowledgment

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N,N'-DIPROTECTED α,ω-AMINOALKANOYL-LYSINE DIPEPTIDE BRONCHODILATORS

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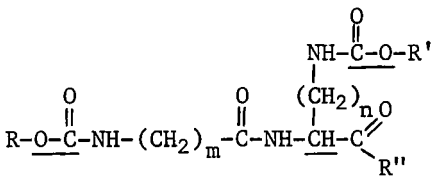
SOME EARLY STUDIES INDICATED that the antifibrinolytic drug ε-aminocaproic acid (εAcp) could also inhibit immunologic reactions[1-5] including lethal anaphylaxis.[6] Although some of these findings were later refuted[7,8] we were prompted to synthesize[9] small peptides of εAcp as potential therapeutic agents in the treatment of hypersensitive and allergic disorders. During the synthesis of dipeptide H-εAcp-Lys-OH, it was discovered that the amino-protected intermediate Z-εAcp-Lys(Z)-OH (I) possessed *in vitro* bronchodilator activity while the free dipeptide was void of activity.

A structure-activity study of I was undertaken in which the structure of the molecule was varied at the sites indicated in II. Syntheses were generally effected by DCC coupling of the appropriately substituted and/or protected amino acid components followed by saponification, aminolysis or other common peptide reactions leading to the desired compound.



(II)

In vitro test data are given in Tables I and II which summarize active and inactive compounds. Generally, activity was favored by R and R' being mono-substituted benzyl groups, m = 2,3,5,7, n = 4, R'' = OH, urethan connecting groups and an L-configuration. The N^{ε}- and retro- isomers of I were inactive.

Table I

In Vitro Active Bronchodilator Compounds (II)[a,b]

$\overline{C}$ No.	R	R'	m	n	ED_{50}
	Aminophylline				*20.5 γ/ml*
1.	Bzl	Bzl	5	4	77
2.	3-CH_3OBzl	Bzl	5	4	52
3.	4-CH_3OBzl	Bzl	5	4	38
4.	2-ClBzl	Bzl	5	4	15
5.	3-ClBzl	Bzl	5	4	29.5
6.	4-ClBzl	Bzl	5	4	15.8
7.	4-BrBzl	Bzl	5	4	7.5
8.	4-FBzl	Bzl	5	4	A[a]
9.	Bzl	4-CH_3Bzl	5	4	18.5
10.	Bzl	2-ClBzl	5	4	20.5
11.	Bzl	3-ClBzl	5	4	21.5
12.	Bzl	4-ClBzl	5	4	15.0
13.	Bzl	2-BrBzl	5	4	63
14.	Bzl	3-CH_3OBzl	5	4	50
15.	Bzl	3,4-Cl_2Bzl	5	4	31
16.	Bzl	Bu	5	4	67
17.	Bzl	4-FBzl	5	4	A[a]
18.	Bzl	Bzl	2	4	32.5
19.	Bzl	Bzl	3	4	22
20.	Bzl	Bzl	5	4	77
21.	Bzl	Bzl	7	4	18
22.	Bzl	Bzl	5	3	A[a]

[a]Active compounds ≥50% the activity of aminophylline at 60 γ/ml in relaxing spiral strip of guinea pig trachea hung in oxygenated Tyrode's solution.

[b]Urethan connected protecting groups and R" = OH generally required for activity.

Table II

In Vitro Inactive Bronchodilator Compounds (II)

C No.	*R*	*R'*	*R''*	*m*	*n*
23.	Bzl	Bzl	NH	5	4
24.	Bzl	Bzl	$NHNH_2$	5	4
25.	Bzl	Bzl	$NH(CH_2)_5CO_2CH_3$	5	4
26.	Bzl	Bzl	$NH(CH_2)_5CO_2H$	5	4
27.	H[a]	Bzl	OH	5	4
28.	3-BrBzl	Bzl	OH	5	4
29.	2,6-Cl_2Bzl	Bzl	OH	5	4
30.	3,4-Cl_2Bzl	Bzl	OH	5	4
31.	1-Adamantyl	Bzl	OH	5	4
32.	Et	Bzl	OH	5	4
33.	Bu	Bzl	OH	5	4
34.	Bu^t	Bzl	OH	5	4
35.	Bzl	dl-$C_6H_5CH(CH_3)$	OH	5	4
36.	Bzl	2,6-Cl_2Bzl	OH	5	4
37.	Bzl	Bzl	OH	5	2
38.	Bzl	Bzl	OH	1	4
39.	Bzl	Bzl	OH	4	4
40.	Bzl	Bzl	OH	6	4
41.	H[a]	H[a]	OH	5	4

[a]Urethan $-\overset{O}{\overset{\|}{O}}-C-$ removed.

Table III

Distribution of Z-β-Ala-l-^{14}C-Lys(Z)-OH in the Guinea Pig at 100 mg/kg[a] IV

Time	*DPM/g Tissue or Fluid*								
min	*Heart*	*Lung*	*Kidney*	*Liver*	*Brain*	*Muscle*	*Fat*	*Blood*	*Urine*
30[a]	780	1385	10,000	17,000	210	2300	440	2000	127,000
60	416	660	4,800	7,800	117	790	329	660	
120	360	486	3,987	4,500	113	770	270	510	317,000

[a]Bronchodilator activity, 96% bronchoconstriction reduction, observed at this concentration and time interval, returned to control in 38 min.

Active compounds were further evaluated *in vivo* with the modified Konzett-Rössler[10] preparation of the guinea pig in which compounds were tested for their ability to block bronchospasm produced by histamine. At an IV dose of 10 mg/kg, only compound 7 exhibited any activity and this was 33% of that of aminophylline at this dosage. Activity in compounds 16 and 18 was observed at higher doses of 50-100 mg/kg, the overall potencies relative to aminophylline being ca. 1/4 and 1/3, respectively.

In order to determine whether the low *in vivo* activity of these protected dipeptides was due to lack of transport, poor distribution, or rapid degradation, radiolabeled 18, Z-β-Ala-1-^{14}C-Lys(Z)-OH was synthesized to determine these pharmacokinetic parameters. Results (Table III) from studies in the guinea pig revealed that poor distribution was the main factor contributing to low *in vivo* activity, *i.e.*, after IV administration of the compound at 100 mg/kg, much accumulated in the liver and kidneys and much was excreted in the urine after 0.5-1 hour. Lung levels did attain sufficient concentration to produce the bronchodilator activity observed and examination of lung, liver and urine extracts showed the parent protected dipeptide to be largely intact, *i.e.*, >85% after 10 min (lung, liver and urine) and >95% (urine) and *ca.* 35% (liver) after 40 min.

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OVINE PLACENTAL LACTOGEN: STRUCTURAL AND FUNCTIONAL RELATIONSHIP WITH GROWTH HORMONE AND PROLACTIN

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IN 1962, JOSIMOVICH AND MACLAREN[1] first described a lactogenic hormone from the human placenta with immunochemical properties similar to those of human growth hormone (hGH). Since then it has been shown that human placental lactogen (hPL; human chorionic somatomammotropin, hCS) and hGH are very similar in primary structure, with over 85% of their amino acid residues identical.[2-4] Because hPL also shares many biological properties of prolactin, the structural relationships within this superfamily[5] of protein hormones are of particular interest as they reveal structure-function relationships and mechanisms of biochemical evolution.

Although evidence exists for placental lactogens in many other species, including the monkey, rat, mouse, goat, sheep, cow, guinea pig, and hamster, subprimate forms have not been adequately characterized. We have undertaken the isolation and characterization of placental lactogen from the sheep[6-7] in order to elucidate its chemical and biological properties as they relate to those of pituitary growth hormone and prolactin. In addition, the sheep provides a model system for investigation of the physiological role of placental lactogen in pregnancy.[8]

Ovine placental lactogen (oPL) has been purified from placental cotyledons obtained surgically and stored at -20°C by the procedure shown in Table I. Total protein was determined by the method of Lowry and lactogenic activity by a radioreceptor assay[9] using rabbit mammary receptor, lactoperoxidase-labeled ^{125}I-human or ovine prolactin tracer and

Table I

Isolation of Ovine Placental Lactogen

Step	*Total Protein (mg)*	*Specific Activity*
Placental cotyledons (400 g)		
Homogenized and extracted with 0.1 *M* ammonium bicarbonate pH 9.5	3310	.014
pH 6.5 supernatant	2850	.021
35-65% ammonium sulfate precipitate	304	.148
Chromatography on Sephadex G-150	52	.610
Chromatography on DEAE cellulose	16.3	.890
Chromatography on carboxymethyl cellulose	5.5	.990

All steps carried out at 4°C.

hPL standards. Approximately 400 g of partially thawed tissue were homogenized and extracted overnight at 4°C with 0.1 *M* ammonium bicarbonate, pH 9.5. After removal of a precipitate at pH 6.5, the supernatant was fractionated with ammonium sulfate. The precipitate forming between .35 and .65 saturation was dissolved in 0.1 *M* ammonium bicarbonate and applied directly to a column, 5 x 125 cm, of Sephadex G-150 eluted with 0.1 *M* ammonium bicarbonate, pH 8.3. Material with activity in the mammary radioreceptor assay was pooled and rechromatographed on a column, 0.9 x 30 cm, of DEAE cellulose eluted with .01 *M* Tris-HCl, pH 9.0 and a linear gradient to .05 *M* sodium chloride. The major active fraction was subjected to final purification on a column, 0.9 x 20 cm, of CM-cellulose eluted with .01 *M* ammonium acetate, pH 5.5 and a linear gradient from 0.1 *M* to 0.3 *M* sodium chloride.

The purified oPL, obtained in an overall yield of approximately 12%, migrated as a single band during electrophoresis in 7.5% polyacrylamide gels with .05 *M* sodium phosphate buffer, pH 7.2, containing 0.1% SDS. The molecular weight of oPL, estimated by SDS gel electrophoresis to be approximately 23,000 daltons, is, within experimental limits, the same as both ovine growth hormone (oGH) and

ovine prolactin (oPR). Polyacrylamide disc gel electrophoresis of oPL at pH 9.0 revealed two bands with mobilities similar to those of the multiple bands of purified oGH but quite distinct from those of oPR.

The amino acid composition of purified oPL, determined with a Beckman 121M analyzer from multiple 24, 48 and 72 hour acid hydrolysates and expressed as assumed integral number of residues, is shown in Table II. The composition of oPL (second column) shows identity with that of oGH in the number of methionine residues, and is also identical with that of oPR in the number of the highly conserved residues, cysteine and tryptophan. oPL is also identical with hGH in the number of threonine residues and with hPR in the number of aspartic acid, cysteine, methionine, isoleucine, and tryptophan residues.

Table II

Comparison of the Amino Acid Composition of oPL, oGH, oPR, hPL, hGH and hPR

Amino Acid	*oGH*	*oPL*[a]	*oPR*	*hGH*	*hPL*	*hPR*
Aspartic Acid	16	19	22	20	22	19
Threonine	12	10	9	10	12	9
Serine	12	16	15	18	18	15
Glutamic Acid	25	24	22	27	25	28
Proline	8	10	11	8	5	9
Glycine	10	15	11	8	7	9
Alanine	14	13	9	7	6	12
Half Cystine	4	6[b]	6	4	4	6
Valine	7	13	10	7	7	9
Methionine	4	4	7	3	6	4
Isoleucine	7	10	11	8	7	10
Leucine	22	13	22	26	25	25
Tyrosine	6	4	7	8	8	6
Phenylalanine	13	7	6	13	11	6
Tryptophan	1	2[c]	2	1	1	2
Histidine	3	4	8	3	7	7
Lysine	13	14	9	9	9	10
Arginine	13	10	11	11	11	11
Total	191	194	198	191	191	197

[a]Based on 24, 48 and 72-hour hydrolysates.

[b]Determined as S-aminoethyl cysteine.

[c]Determined by the method of Edelhoch.

Preliminary immunochemical characterization has been carried out by Ouchterlony immunodiffusion in agar gels, using anti-oPL serum produced in rabbits. In this system, oPL shows a cross-reaction of partial identity only with oGH, and no cross-reactivity with oPR, hPL, hGH and hPR. High titer rabbit anti-oPL has also been used to develop a sensitive radioimmunoassay for measurement of plasma oPL levels.

In the mammary radioreceptor assay for prolactin,[9] oPL is equipotent with hPL and parallels the activity of oPR in displacing ^{125}I-hPR from the receptor (Figure 1). By contrast, oGH is inactive in this assay. oPL also stimulates a lactogenic response *in vivo* in the rabbit intraductal assay and *in vitro* in the mouse mammary explant assay.[7] In the radioreceptor assay for growth hormone[10] oPL displaces ^{125}I-hGH from liver membrane receptors parallel to, but with only 20% of the potency of hGH (Figure 2). The potency of oPL in this assay is nevertheless much greater than that of hPL, suggesting that the former is more growth hormone-like than the latter in its biological activity.

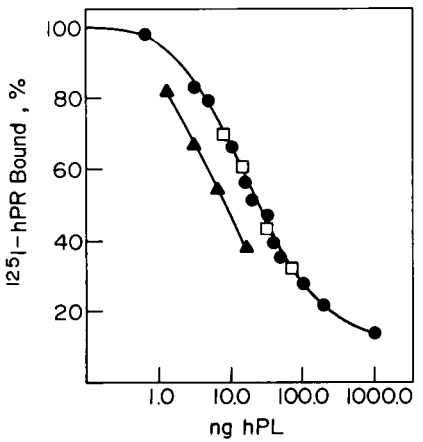

Figure 1: The displacement of peroxidase-labeled ^{125}I-hPR from rabbit mammary tissue membrane receptors by oPL (□), hPL (●), and oPR (▲). Adapted from ref. 6.

The physico-chemical similarities of oPL and oGH combined with its functional similarity to oPR indicate an evolutionary position for oPL intermediate between oGH and oPR in this superfamily of somatrotropins and mammotropins. Dayhoff *et al.*[5] have constructed an evolutionary tree for this group of proteins based on an estimate that the divergence of the growth hormone and prolactin lines by gene duplication occurred approximately 350 million years ago and that placental lactogen developed by gene duplication from the primate growth hormone line in the relatively recent past, just before the human-rhesus monkey divergence.

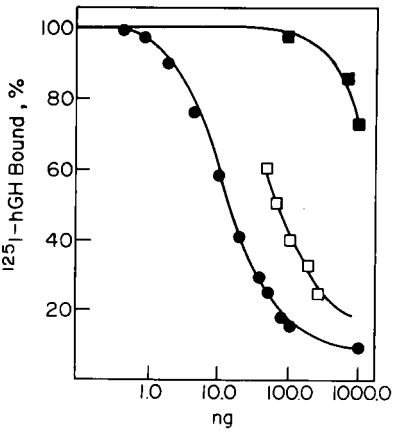

Figure 2: The displacement of peroxidase-labeled ^{125}I-oGH from rabbit liver membrane receptors by oPL (□), oGH (●), and hPL (■). Adapted from ref. 6.

In order to further delineate the evolutionary tree of the growth hormone-prolactin superfamily (Figure 3) it now becomes necessary to consider the placement of the subprimate placental lactogens as exemplified by oPL. While it has seemed most reasonable to expect that oPL might have developed from the growth hormone line by an earlier gene duplication than that leading to hPL (Figure 3, left), recent chemical data suggest that unlike the primate hormones the subprimate placental lactogens may have arisen from the prolactin line (Figure 3, right). Elucidation of the correct evolutionary scheme is dependent on sequence studies now in progress.

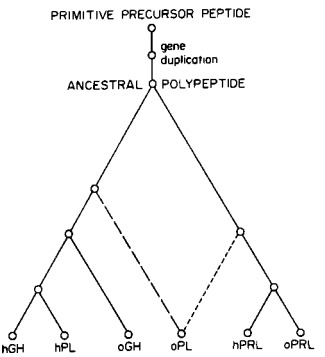

Figure 3: An evolutionary tree of the growth hormone-prolactin superfamily. Alternate hypothetical evolutionary paths to oPL from the growth hormone branch (left) and the prolactin branch (right) are indicated by broken lines. Branch lengths are not drawn to scale.

Acknowledgments

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PHARMACOLOGICAL AND CHEMICAL PROPERTIES OF UROTENSIN I AND II PEPTIDES FROM THE CAUDAL NEUROSECRETORY SYSTEM OF BONY FISH

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THE UROPHYSIS IS THE HORMONE storage-secretion organ of the caudal neurosecretory system of bony fish. Pharmacological investigations on urophysial extracts have established the presence of several biologically active principles, and these have been classified under the name "urotensins,"[1,2] *i.e.*, urotensin I, a rat hypotensive principle; urotensin II, a fish smooth muscle stimulating principle; urotensin III, a Na^+ movement influencing activity; and urotensin IV, a hydroosmotic activity. Standard units of activity have been defined.[1,2] At the present time it is not known whether or not all of these biological activities originate from separate chemical substances. However, two of these principles, namely urotensin I and II, have been separated and partially purified in our laboratory from a teleost fish, the white sucker, *Catostomus commersoni*.[2-6]

Pharmacology

Urotensin I has a long-acting blood pressure (b.p.) lowering effect in mammals and a weak pressor activity in fish (eel). After iv injection in the conscious rat of doses of 200 mU/kg body weight, there is an initial sharp fall in blood pressure followed by a gradual return to the pre-injection blood pressure over a period of about 1 hour (Figure 1). With doses of 500 mU/kg, the blood pressure remains low for up to 4 hours. Similar effects have also been observed in the sheep (10 day old lamb) and in the

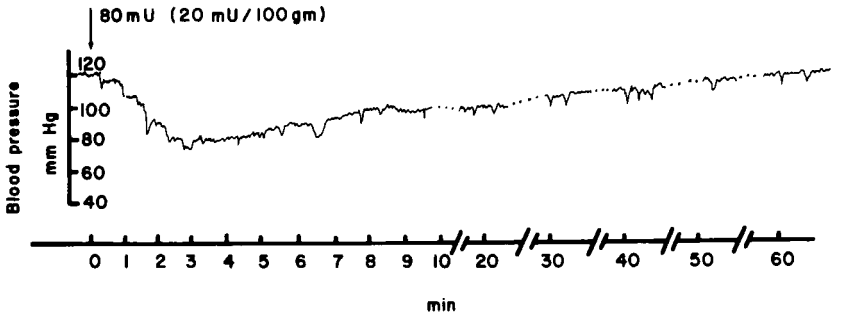

Figure 1: Effect of partially purified urotensin I (IV, 200 mU/kg body weight) on blood pressure in the conscious rat.

squirrel monkey. The effect of urotensin I is dependent on the pre-injection blood pressure in the animal, since a given dose causes a greater fall in b.p. in congenitally hypertensive rats than in normotensive animals (Figure 2). Subcutaneous injection of urotensin I (dose 500 mU/kg) has been found to lower b.p. in normal or in hypertensive rats for periods in excess of 24 hours.

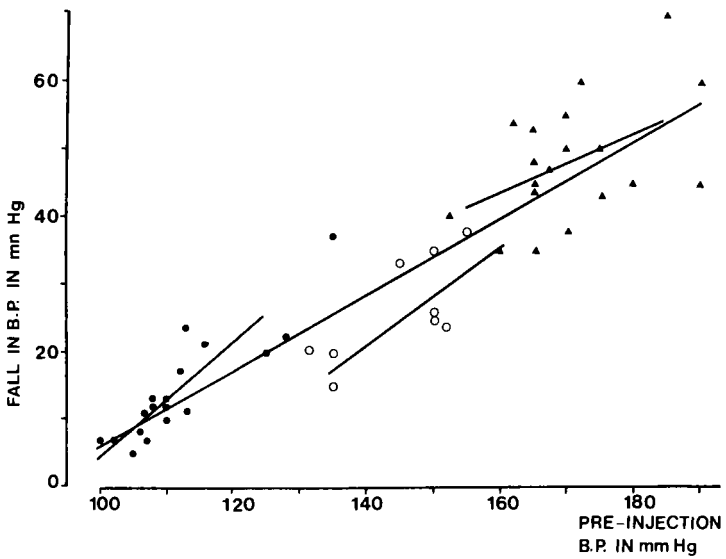

Figure 2: Effect of urotensin I on blood pressure in normotensive and hypertensive rats (● normotensive ♂ and ♀ ; ○ hypertensive ♀ ; ▲ hypertensive ♂).

Urotensin II stimulates the contraction of fish smooth muscle (*i.e.*, isolated trout intestine or rectum) and causes elevation of blood pressure in fish (eel), but has no effect in the equivalent mammalian systems.

Chemistry

Urotensins I and II have been isolated from acetone-dried urophyses using two extraction procedures: (1) homogenization in 0.25% acetic acid followed by heating in a water bath for 3 min and (2) homogenization in 0.1 *N* HCl followed by heating in a water bath for 15 min. After centrifugation the supernatants were applied to gel filtration chromatography columns. Two species of urotensin I (hypotensive principle) of apparently different molecular weight were obtained depending on the original extraction method used (Figure 3). These have been designated as urotensin I and urotensin I_s. Thus, extraction with hot 0.25% acetic acid or with 0.1 *N* HCl at room temperature produced exclusively urotensin I, whereas heating in 0.1 *N* HCl caused conversion to urotensin I_s.

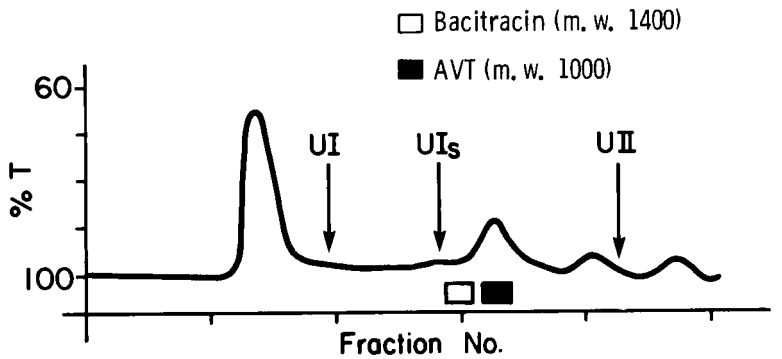

Figure 3: Chromatography of urophysial extracts on Biogel P-4. The molecular form of urotensin I was dependent on the procedure used to extract active principles from acetone-dried urophyses: 0.25% acetic acid (heated 3 min) or 0.1 *N* HCl (room temperature) produced exclusively urotensin I; 0.1 *N* HCl (heated 3 min) produced both urotensin I and I_s; 0.1 *N* HCl (heated 15 min) or 0.25% acetic acid (heated 3 min, pH adjusted to 1 with HCl, heated a further 15 min) produced exclusively urotensin I_s.

The molecular weights were estimated from the elution profile by comparison to peptide standards as *ca.* 3000 for urotensin I and 1600 for urotensin I_s. Due to adsorption, urotensin II was retarded on Biogel columns under these conditions (Figure 3) and its molecular weight could not be estimated.

Urophysial extracts were also investigated by polyacrylamide gel electrophoresis in the presence of SDS and 8 *M* urea.[7] Urotensin I and II were identified in homogenized gel slices by bioassay and their molecular weights estimated by calibrating the gels with peptides of known molecular weight (Figure 4). In accord with our findings by gel filtration chromatography, both urotensin I and I_s were observed on SDS-polyacrylamide gels. Two species of urotensin II were also observed by this technique. However, for urotensin II the β-mercaptoethanol present in the sample buffer favored the formation of a higher molecular weight species, suggesting the possibility of disulfide interchange. Indeed, the biological activity of urotensin II is abolished in the presence of β-mercaptoethanol, indicating that the presence of a disulfide bridge might be essential for biological activity.

Molecular weight estimates for the urotensins from gel filtration and SDS-polyacrylamide gel electrophoresis experiments are given in Table I. It appears that heating of urotensin I (mol wt, *ca.* 3000) in the presence of dilute HCl produces a fragment, urotensin I_s (mol wt, *ca.* 1600) which retains the full biological activity of the parent peptide. The two urotensin II species, on the other hand, may represent either monomer and dimer or open and closed chain forms of the peptide.

Table I

Comparison of Molecular Weights of Urotensins Estimated by SDS-8 Urea Polyacrylamide Gel Electrophoresis and Biogel Chromatography

Peptide	*SDS Gel Electrophoresis*	*Gel Filtration**
Urotensin I	2300 - 2700	3000
Urotensin I_s	1200 - 1500	1400 - 1700
Urotensin II	2000 - 2400	-
Urotensin II	1100 - 1400	-

*Biogel P-2 and P-4.

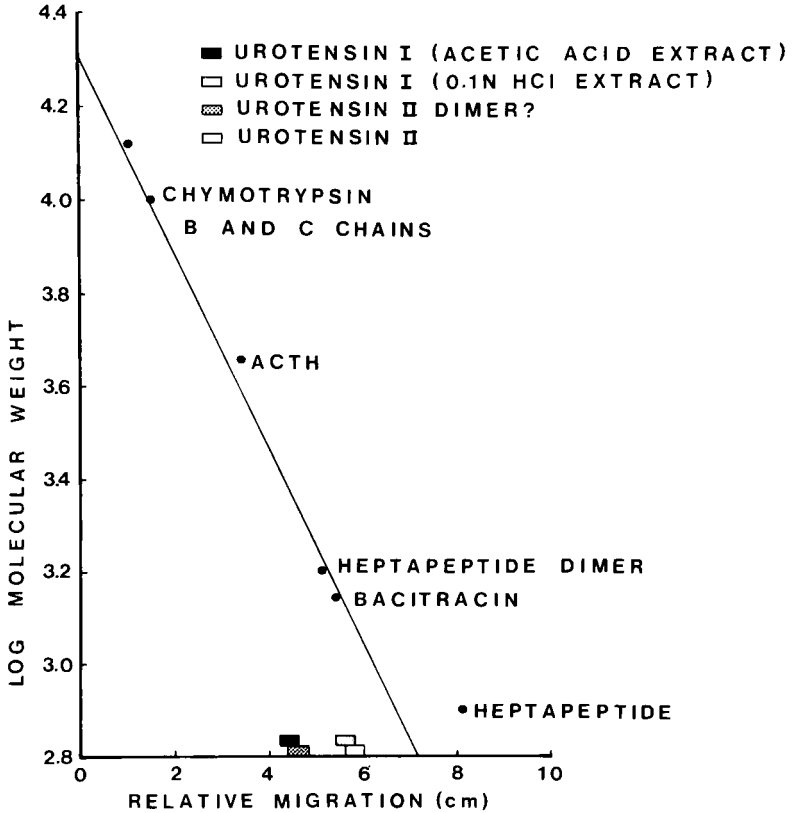

Figure 4: SDS-8 *M* urea polyacrylamide gel electrophoresis of urophysial extracts. Monomer concentration was 12.5% and the acrylamide:bisacrylamide ratio was 10:1.[7] Molecular weights of peptides used to calibrate gels were: chymotrypsin B-chain, 13,999; chymotrypsin C-chain, 10,200; ACTH, 4550; bacitracin, 1,400; heptapeptide dimer and monomer (ref 8), 1,600 and 800. The standard heptapeptide, which migrated at a faster rate than would be expected on the basis of its molecular size, was omitted from the calibration. (From ref 6, reprinted with permission of the National Research Council of Canada.)

Urotensin I_s and II have been purified by gel filtration and ion exchange chromatography. Examination of the urotensin I_s and II fractions from ion exchange columns by paper electrophoresis and TLC indicated that the urotensin II peptide was highly purified whereas the urotensin I_s peptide was contaminated with varying amounts of several other

peptides. Treatment of these fractions with a number of proteolytic enzymes, followed by bioassay, has given some information on the structure of these peptides (Table II). Trypsin, chymotrypsin and staphylococcal protease rapidly destroy the biological activity of both urotensin I_s and II, indicating the presence of basic, aromatic and acidic amino acid residues, respectively, cleavage at which causes

Table II

Effect of Proteolytic Enzymes on the Biological Activities of Urotensin I_s and Urotensin II

*Enzyme**	*Urotensin I_s*		*Urotensin II*	
	Incubation Time	*Residual Activity*	*Incubation Time*	*Residual Activity*
Trypsin	5 hr	0	3 hr	0
	16 hr	0	16 hr	0
α-Chymotrypsin	5 hr	0	3 hr	0
	16 hr	0	16 hr	0
Staphylococcal protease	5 hr	40%	3 hr	50%
	16 hr	0	16 hr	0
Leucine aminopeptidase	5 hr	55%	3 hr	20%
	16 hr	50%	16 hr	0
Carboxypeptidase A	5 hr	25%	3 hr	15%
	16 hr	0	16 hr	0
Carboxypeptidase B	5 hr	100%	3 hr	0
	16 hr	90%	16 hr	0

* Incubations were carried out in 0.1 *M* tris-HCl, pH 7.8. [E] = 5×10^{-7} *M* for urotensin I and 10^{-6} *M* for urotensin II. Estimated [S] : [E] ratios were 20:1 for urotensin I_s and 10:1 for urotensin II. Under similar assay conditions, leucine aminopeptidase and carboxypeptidase A reduced the pressor activity of arginine vasopressin to 50% and 95%, respectively, of its original activity after 16 hours.

loss of activity. Carboxypeptidase A inactivates both urotensins, indicating free COOH-termini for both peptides. Leucine aminopeptidase (LAP) inactivates urotensin II, indicating a free NH_2-terminus. Urotensin I_s, which retains 50% of its activity after prolonged treatment with LAP, may have a blocked NH_2-terminus.

The amino acid composition of highly purified urotensin II obtained after ion exchange chromatography indicate that the peptide contains 23 amino acids, *i.e.*, $Asp_2Thr_1Ser_2Glu_1$ $1/2\text{-}Cys_4Gly_3Ala_2Val_2Tyr_2Phe_2Lys_2$.

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THE MECHANISM OF LIPID BINDING BY THE PLASMA LIPOPROTEINS: SYNTHESIS OF MODEL PEPTIDES

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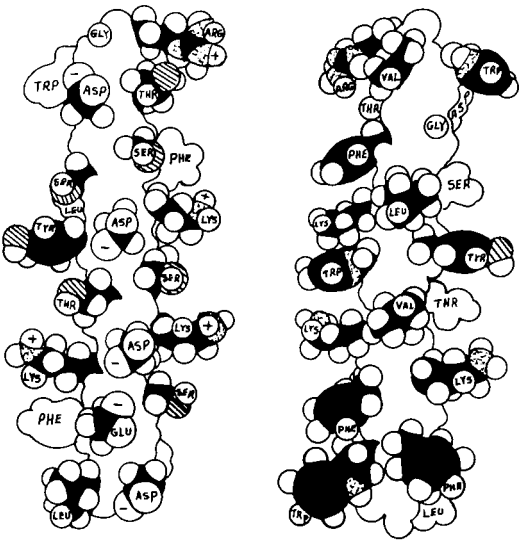
THE NATURE OF THE INTERACTION between the protein and lipid constituents of cell membranes and plasma lipoproteins is currently a topic of considerable interest. Although there are several models for the structure of membranes and plasma lipoproteins,[1] there is little information which describes, at the molecular level, the mechanism of interaction between these two constituents. Segrest *et al.*[2] have proposed that the plasma lipoprotein-proteins have a unique structure, which might account for their lipid-binding properties. From studies of Corey-Pauling models of apoproteins of known sequence and from circular dichroic studies, it was suggested that these molecules contain helical segments which are amphipathic or two-faced, one being polar, the other nonpolar (Figure 1). In this model the acidic residues are distributed along the center of the polar face and alternate with the basic amino acids which are positioned along the two lateral edges which separate the polar and nonpolar faces. Because of this distribution of amino acids, it was suggested that close steric contact of the oppositely charged amino acids and the polar head groups of the phospholipid might be possible, although no evidence was presented to support polar interactions. The fatty acyl chains of the phospholipid could interact with the hydrophobic face of the helix.

In order to test the potential contributions of the hydrophobicity of the nonpolar face and the distribution of charged amino acids on the helix to phosphatidylcholine



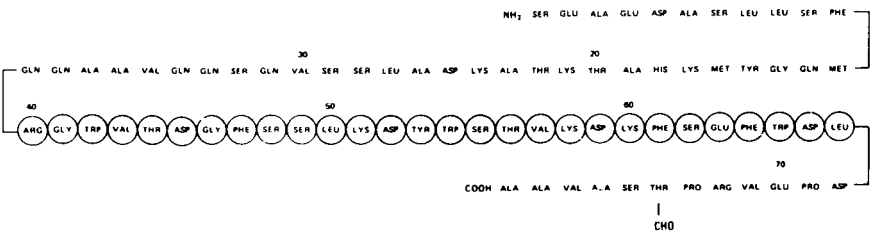


Figure 1: The amino acid sequence and the CPK model of the amphipathic helical region of apolipoprotein C-III from the human plasma very low density lipoproteins.

binding, we have synthesized three model peptides (Figure 2) and tested them for their ability to bind dimyristoyl phosphatidylcholine (DMPC). Each of the three model peptides exhibits the amphipathic feature of two-sidedness when placed in an α-helical conformation. In addition, each contains pairs of oppositely charged amino acids in a typical topographical relationship as illustrated in the space-filling model for peptide III shown in Figure 3. Each of the three model peptides is distinguished by a relative mean hydrophobicity as estimated using the hydrophobicity scale

Ala-Ser-Leu-Lys-Asp-Ser-Leu-Ser-Asp-Lys-Trp-Lys-Asp-Ser-Leu-Ser-Asp-Lys-Leu-Ser

I

Val-Ser-Ser-Leu-Lys-Asp-Ala-Ala-Ser-Ser-Leu-Lys-Asp-Ser-Phe-Ser

II

Val-Ser-Ser-Leu-Lys-Asp-Tyr-Trp-Ser-Ser-Leu-Lys-Asp-Ser-Phe-Ser

III

Figure 2: The model peptides synthesized to test the role of hydrophobicity in phospholipid binding.

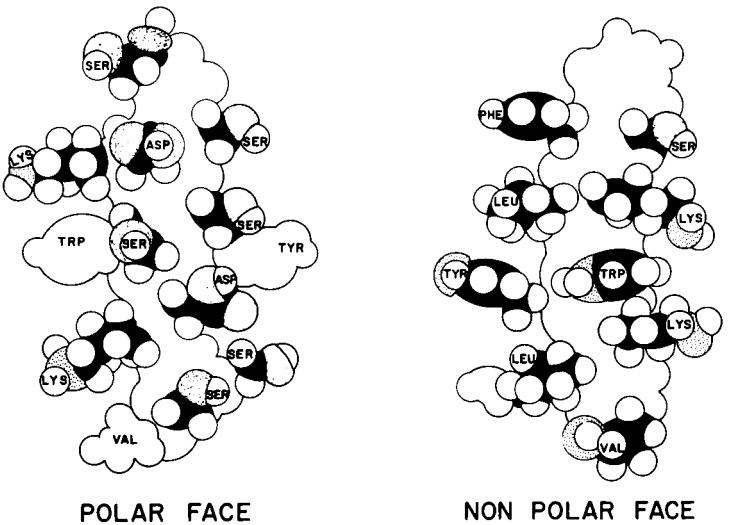


Figure 3: A drawing of the CPK model of synthetic Peptide III showing the polar and nonpolar faces of the amphipathic helix.

derived by Bull and Breese[3] from surface tension measurements on amino acid solutions. Model peptide I contains 20 amino acid residues, four of which are leucine and tryptophan, and which lie on the nonpolar face. Based on the relative hydrophobicities of the amino acids, the calculated hydrophobicity of this peptide is 829. Model peptide II contains leucine, two alanines, valine and phenylalanine on its hydrophobic face. The calculated average hydrophobic index of this peptide is 791. Model peptide III differs from II only in that tyrosine and tryptophan have been substituted

for the two alanine residues to raise the calculated hydrophobicity to 1031. In the present report, we describe the phospholipid-binding properties of these three model peptides.

Experimental Procedures

The peptides used in this study were synthesized by an improved solid-phase technique,[4] using a Schwarz-Mann automated synthesizer. They were purified by chromatography on Bio-Gel P-10 and Dowex 50 and judged homogeneous by their behavior during purification, their amino acid composition and by thin layer chromatography. The amino acid compositions of the peptides are shown in Table I. The interaction of these model peptides with DMPC was studied by methods previously described for the human apoproteins,[5] including circular dichroism, intrinsic tryptophan fluorescence, and flotation of apoprotein-phospholipid complexes by density gradient ultracentrifugation.

Table I

Amino Acid Composition of Model Peptides

	Peptide I	*Peptide II*	*Peptide III*
Asp	4.01 (4)	1.97 (2)	2.10 (2)
Ser[1]	5.65 (6)	5.64 (6)	5.68 (6)
Ala	1.00 (1)	2.00 (2)	0 (0)
Val	0 (0)	0.92 (1)	1.00 (1)
Leu	4.05 (4)	2.08 (2)	2.04 (2)
Tyr	0 (0)	0 (0)	1.00 (1)
Phe	0 (0)	0.97 (1)	1.02 (1)
Lys	4.11 (4)	1.98 (2)	1.97 (2)
Trp[2]	(1)	(0)	(1)

[1]Uncorrected for destruction.
[2]Undetermined.

Results

The circular dichroic spectra of model peptides I and II were negligibly changed in the presence of DMPC, while peptide III showed a significant increase in its ellipticity. This spectral change corresponded to an increase in α-helicity from 15% to 28% (Table II). As a further test of phospholipid binding, the intrinsic tryptophan fluorescence maximum

Table II

Fluorescence and Helicity of the Synthetic Peptides

Peptide	*Without PC*		*With PC*	
	λ	*% Helix*	λ	*% Helix*
I	353	10.0	351	10.0
II	---	13.7	---	12.4
III	353	15.4	347	28.3

was determined for the two model peptides containing tryptophan, peptides I and III. Peptide III exhibited a blue shift in the maximum from 353 nm to 347 nm in the presence of DMPC, while peptide I did not show a significant change (Table II). Density gradient ultracentrifugation of the peptide/DMPC mixtures was performed (Figure 4). Peptides I and II sedimented to a density greater than 1.090 g per ml while approximately 40% of peptide III was associated with the DMPC lipid band at a density between 1.063 and 1.090 g per ml.

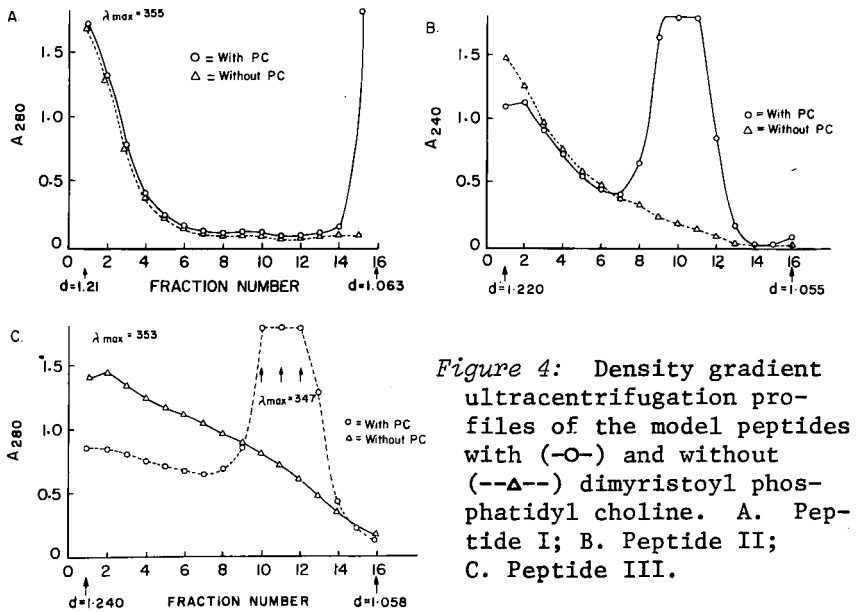


Figure 4: Density gradient ultracentrifugation profiles of the model peptides with (-O-) and without (--Δ--) dimyristoyl phosphatidyl choline. A. Peptide I; B. Peptide II; C. Peptide III.

Discussion

These findings suggest that model peptide III contains the structural determinants necessary for phospholipid binding while peptides I and II do not. It is important to emphasize that all three of the model peptides contain acidic and basic amino acid residues in a sequence which permits their steric juxtaposition. However, only the peptide with the highest degree of hydrophobicity, *i.e.*, peptide III, interacted with DMPC. We conclude therefore from these results that the hydrophobic index of the nonpolar face of the helix plays an important role in the binding of phosphatidylcholine. Stoffel *et al.*[6] have recently provided evidence from NMR measurements for the role of hydrophobic binding between apoproteins and phospholipids. The role of the juxtaposed, oppositely charged amino acids is currently under study.

Acknowledgments

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PEPTIDE ALDEHYDE INHIBITORS OF THE FIBRINOGEN-THROMBIN REACTION

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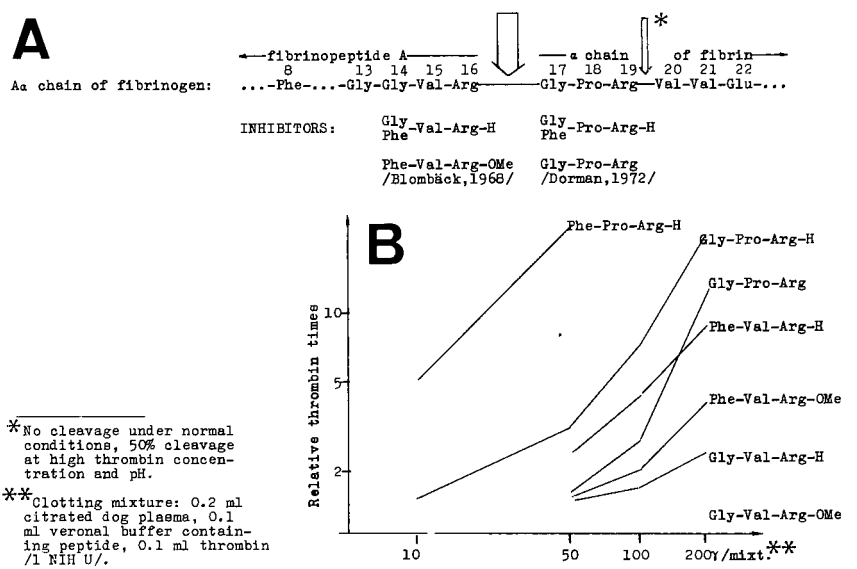
RECENTLY WE HAVE PREPARED TRIPEPTIDE aldehydes[1] as H-Gly-Val-Arg-H, H-Gly-Pro-Arg-H and the Phe[1] analogs corresponding to the COOH-terminal or fibrinopeptide A and the NH_2-terminal fragment of fibrin α chain. These relate to the known peptide inhibitors of thrombin, *e.g.*, H-Phe-Val-Arg-OMe[2] and Gly-Pro-Arg[3] (Figure 1A). Of these, contrary to expectation, the Pro-containing compounds which resemble the tripeptide sequence succeeding the susceptible Arg-Gly bond displayed higher activity in prolonging the thrombin time of plasma than the Val analogs, Figure 1B. The same experiments proved that replacement of the NH_2-terminal Gly by Phe was advantageous in both groups of peptides.

For increasing the binding capacity of the most potent H-Phe-Pro-Arg-H, we decided to substitute Phe by D-amino acid residues, *i.e.*, D-Ala, D-Val, D-Ale, D-Phe, while the Pro-Arg-H portion, which seemed to be essential, was kept unchanged. The activity of these compounds, shown in Figure 2, is rising from the D-Ala derivative to the D-Phe analog as if the -terminal residue would be involved in a hydrophobic interaction with some Phe side chain of thrombin. Similarly, the activity of H-Phe-Val-Arg-H could be enhanced by that kind of substitution too, but both H-D-Ala-Val-Arg-H and H-D-Phe-Val-Arg-H were less active than the corresponding Pro-peptide aldehydes.

According to Dixon plots[4] H-D-Phe-Pro-Arg-H is a non-competitive inhibitor while H-D-Phe-Val-Arg-H seems to be rather a competitive one like hirudin. The K_i values for the three substances determined from the plots were about 7.5×10^{-8}, 8.8×10^{-7} and 2.42×10^{-9} *M* , respectively.



*No cleavage under normal conditions, 50% cleavage at high thrombin concentration and pH.

**Clotting mixture: 0.2 ml citrated dog plasma, 0.1 ml veronal buffer containing peptide, 0.1 ml thrombin /1 NIH U/.

Figure 1: (A) Thrombin sensitive region of the Aα chain of fibrinogen. (B) The relative thrombin time of peptide inhibitors corresponding to (A).

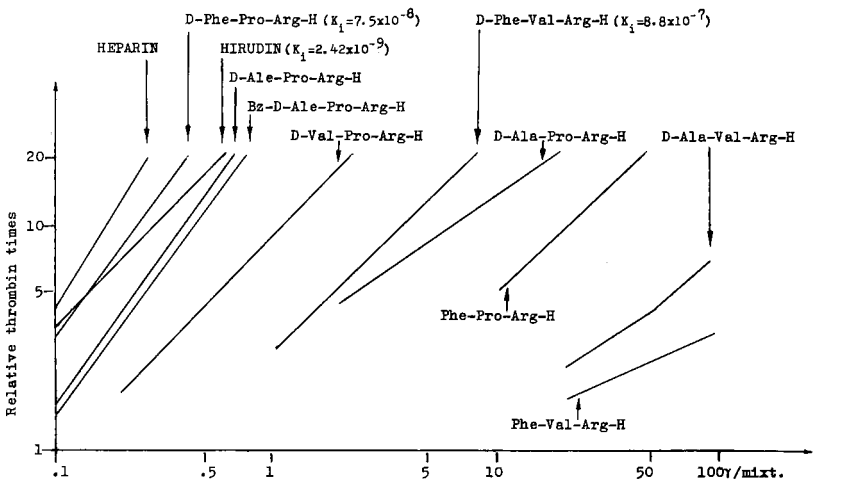


Figure 2: Relative thrombin time of peptide aldehydes containing D-amino acid residue at the NH_2-terminus.

Reaction Mixtures

0.2 ml fibrinogen (A: 3.1 mg/ml,
B: 0.775 mg/ml)

0.1 ml thrombin (5 NIH U/ml)

0.1 ml veronal buffer containing inhibitor

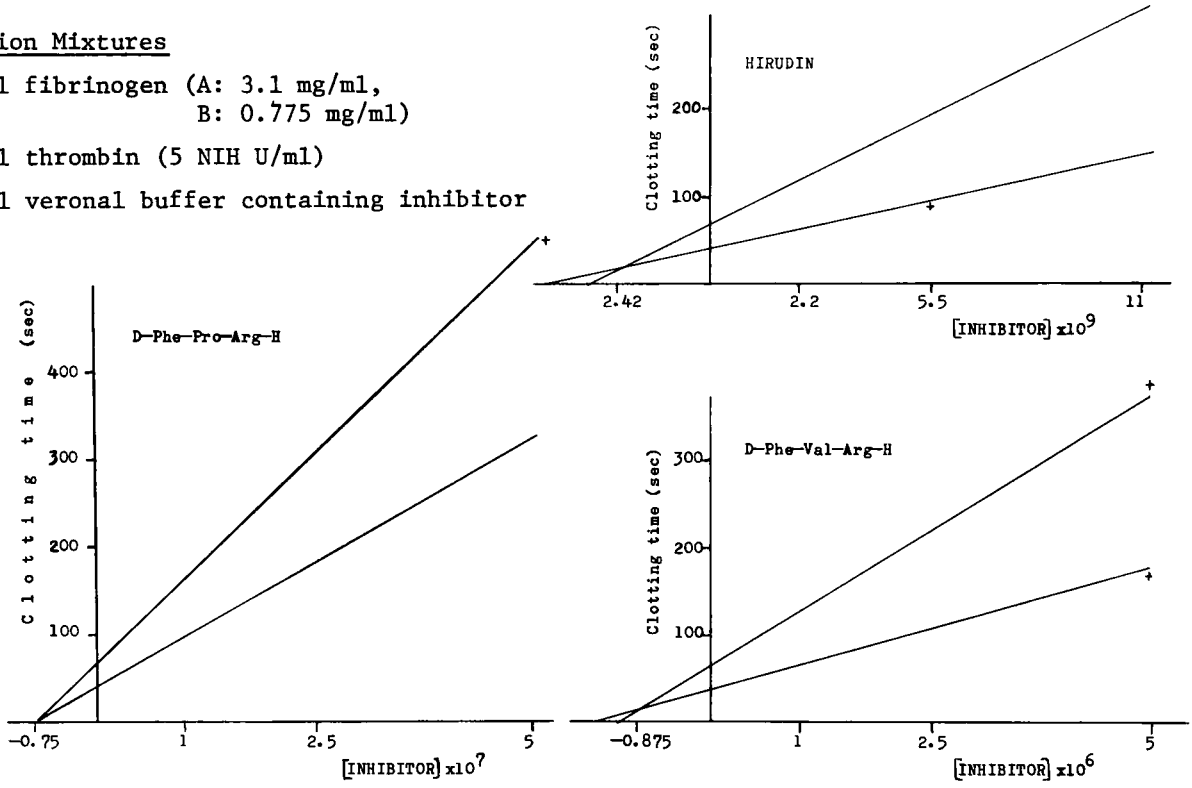


Figure 3: Demonstration of thrombin inhibition by H-D-Phe-Pro-Arg-H, H-D-Phe-Val-Arg-H and hirudin using Dixon plots (ref 4).

For obtaining more information about the way of action of these Pro and Val peptides, the thrombin times were also determined in a "reversed" method, *i.e.*, the peptide inhibitor was added to thrombin and this mixture was reacted with plasma. While the usual assay gives information about the ability of a certain peptide to bind to thrombin in the presence of its own substrate, fibrinogen, the reversed method provides an opportunity to study the action of fibrinogen on the thrombin-inhibitor complex. Compounds were tested in a concentration at which thrombin time determined in the usual way had doubled. Data presented in Table I indicate that, in general, enhanced activities were obtained with the reversed method, and a particularly high increment per cent was found among the Pro-peptide aldehydes. The significance of the penultimate Pro residue is well demonstrated by comparing the increments of 1127, 43 and 33% obtained for H-D-Phe-Pro-Arg-H and the corresponding Val and Gly analogs, respectively. These results can hardly be explained by the difference in the ability of Pro, Val and Gly side chains to participate in hydrophobic bonding. It is also remarkable that increment per cent considerably drops on acylation of the free NH_2-terminal (*cf.* 1127 *vs.* 87% obtained for H-D-Phe-Pro-Arg-H and for its benzoyl (Bz) derivative, respectively) whereas both compounds show a similar activity in the usual assay.

Table I

Relative Thrombin Times of Peptide Aldehydes Determined in the Usual and Reversed Way

Peptide	*conc. γ/ mixt.*[a]	*Relative Thrombin Times in Usual and Reversed Method*[b]	*Increment percent*
Gly-Pro-Arg-H	20	1.8 - 2.7	50
D-Ala-Pro-Arg-H	0.5	2.1 - 4.8	128
D-Val-Pro-Arg-H	0.2	2.2 - 12.7	477
D-Ale-Pro-Arg-H	0.15	2.0 - 19.0	850
D-Phe-Pro-Arg-H	0.05	2.2 - 27.0	1127
Bz-D-Phe-Pro-Arg-H	0.2	2.0 - 3.7	85
Gly-Val-Arg-H	200	2.0 - 2.0	0
D-Ala-Val-Arg-H	25	2.1 - 2.3	19
D-Phe-Val-Arg-H	0.5	1.9 - 2.7	42
D-Phe-Gly-Arg-H	2	2.0 - 2.7	35

[a] As in Figure 1.

[b] Usual method: /plasma+peptide/ clotted by thrombin, reversed method: /peptide+thrombin/ reacted with plasma.

In order to explain these facts we supposed that the P_2 subside of substrates and inhibitors, *e.g.*, the penultimate Pro and Val residue in our aldehydes, is also determinant in enzyme complexing.

We also suppose that successively acting binding sites of thrombin are involved in the fibrinogen-fibrin conversion. In thrombin complexing, the Val-Arg portion of fibrinogen is probably the primary site, Figure 4A (although 8-Phe and 22-Glu have also been supposed to be involved). Therefore the complementary site of thrombin is called Val-Arg binding site. Since inhibitors with Val-Arg or X-Arg (X = other than Pro) terminals are also to bind to this site, their thrombin complexes can be disrupted by the native substrate, fibrinogen, to a certain equilibrium at once, *i.e.*, the formation of such complexes hardly depends on the presence of fibrinogen (see H-D-Phe-Val-Arg-H or H-D-Phe-Gly-Arg-H in Table I: low increment per cent). The second, that is the Pro-Arg-binding site of thrombin can solely be operational in the activation of prothrombin nd Factor XIII, where the fragments to be cleaved are Ile-Pro-Arg-Ser and Val-Pro-Arg-Gly, Figure 4A. In the fibrinogen-fibrin conversion, this binding site could combine with the nascent Gly-Pro-Arg terminal of fibrin α chain (Figure 4B) and bring about a conformational change or "opening up" in the molecule $(\alpha,B\beta,\gamma)_2$ required for its end-to-end aggregation followed by the X-Arg binding site-mediated removal of fibrinopeptide B. The Pro-Arg binding site seems to be accessible even in the fibrinogen-thrombin complex since that can form rather strong connection with free Pro-peptide aldehydes (high activity of H-D-Phe-Pro-Arg-H in the normal thrombin time assay). The free Pro peptide-thrombin complex cannot

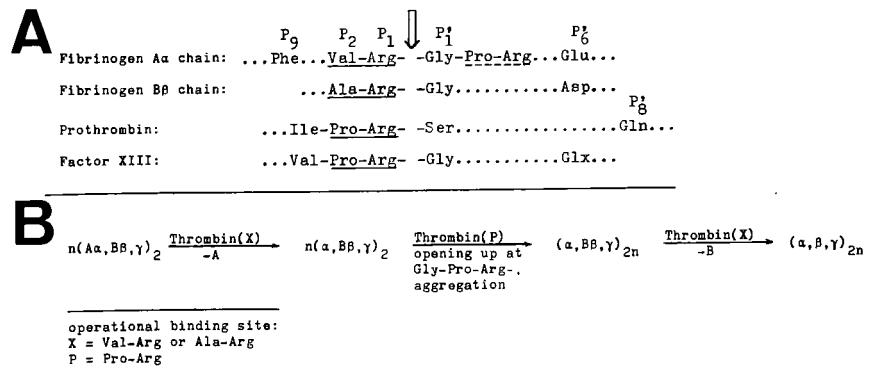


Figure 4: (A) Thrombin sensitive region of clotting factors. (b) Action of thrombin on fibrinogen.

be easily decomposed by fibrinogen, which is otherwise attached to the Val-Arg binding site (see free Pro-peptide aldehydes in Table I: high increment per cent).

The acyl derivative, however, being too large to fit into the place of the Gly-Pro-Arg portion of the α chain, can be expelled from its thrombin complex (see Bz-D-Phe-Pro-Arg-H in Table I: low increment per cent). These results may indicate the close vicinity of the two binding sites.

Peptide aldehydes were obtained from the corresponding δ-lactams (Figure 5) by $LiAlH_4$ reduction. To minimize the danger of racemization at lactam-formation, Arg(Z)-δ-lactam was used as starting material. Otherwise, the conventions of classical peptide synthesis were followed.

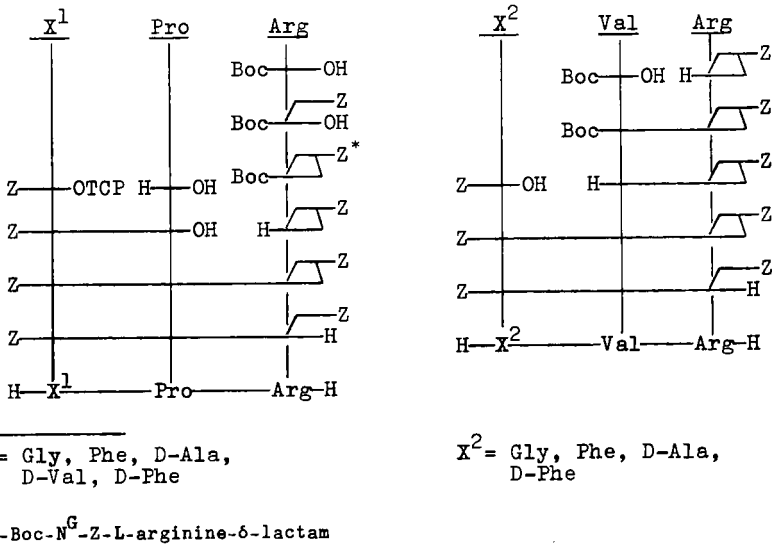


Figure 5: Synthesis of tripeptide aldehydes.

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BIOSYNTHESIS OF β-MSH AND ACTH

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THE AMINO ACID SEQUENCE OF β-MSH, which has 18 residues, is present in the central region of a 91 residue polypeptide termed β-lipotrophic hormone (β-LPH).[1] The biosynthesis of β-MSH could take place by one of two routes: the hormone might be formed by assembly of the constituent amino acids or it could be formed from β-LPH by the action of proteolytic enzymes. If β-LPH is the precursor of β-MSH, release of β-MSH would be accompanied by release of the adjacent fragments of the precursor molecule. In our experiments we have conducted a search for the "missing" fragments of the prohormone.

Pituitary glands were collected from 1200 pigs and after homogenization a salt precipitate (Fraction D) was obtained as described by Li[1] (Figure 1).

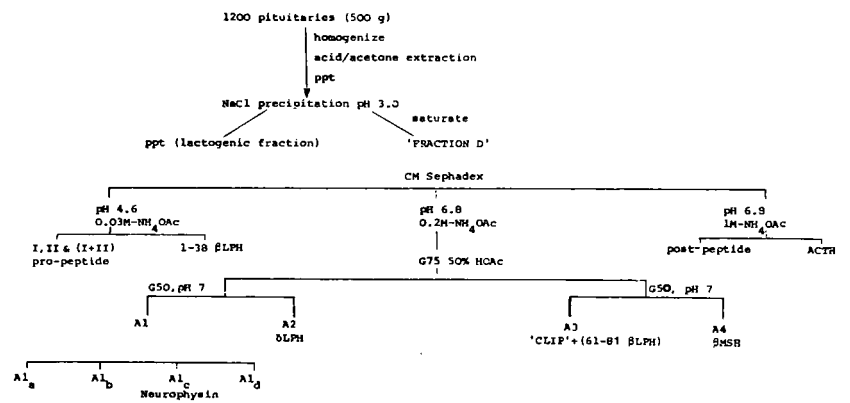


Figure 1: Protocol for fractionation of porcine pituitary glands.

The mixture of polypeptides was separated into three fractions by stepwise elution on CM-Sephadex (Figure 2). The first emerged in 0.02 *M* ammonium acetate at pH 4.5, the second in 0.2 *M* NH_4OAc at pH 6.8, and the third in 1 *M* NH_4OAc at the same pH. The 0.2 *M* fraction was resolved further by gel filtration on Sephadex G-75 to give two broad peaks. Each peak was subjected to chromatography on Sephadex G-50 to give the profiles shown in Figures 3a and 3b.

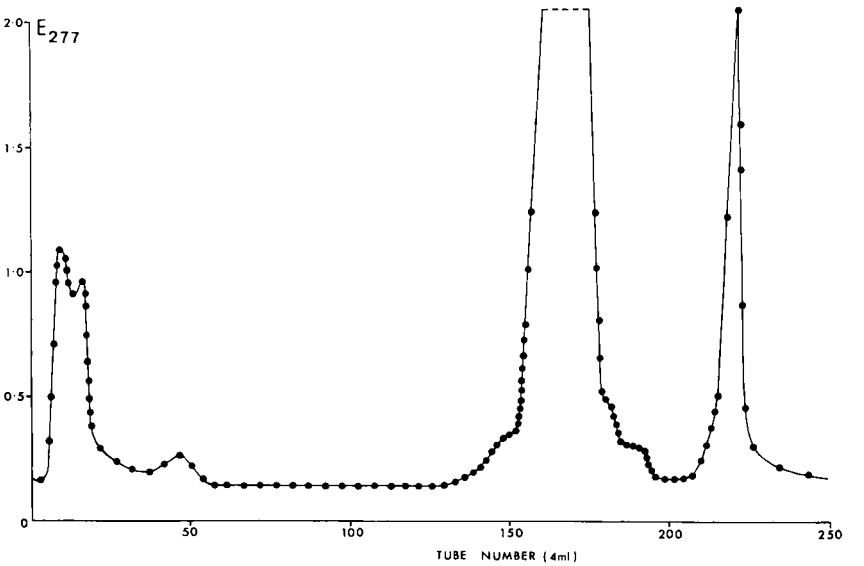
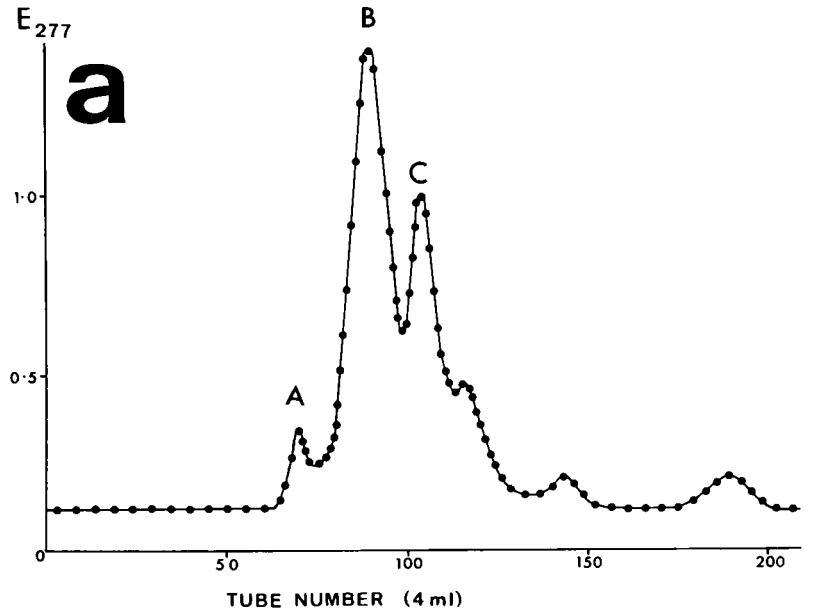


Figure 2: Resolution of Fraction D on carboxymethyl Sephadex. Column dimensions, 60 x 1.5 cm.

Peak B (Figure 3a) after gradient elution from C-25 provided homogeneous β-LPH. Peak C, after purification on A-25, provided pure β-LPH, which comprises the first 58 residues of β-LPH. Peak E (Figure 3B) contained the COOH-terminal fragment of ACTH (the corticotrophin-like intermediary peptide, CLIP[2]) together with residues 61-87 of β-LPH, which were separated on Sephadex A-25. The last peak from the G-50 column (Peak F) contained pure β-MSH.

From the CM-Sephadex column, the 1 *M* salt eluate provided ACTH together with residues 61-91 of β-LPH, and these were subsequently resolved on G-50. Finally the 0.02 *M* ammonium acetate eluate was resolved on Sephadex A-25, and



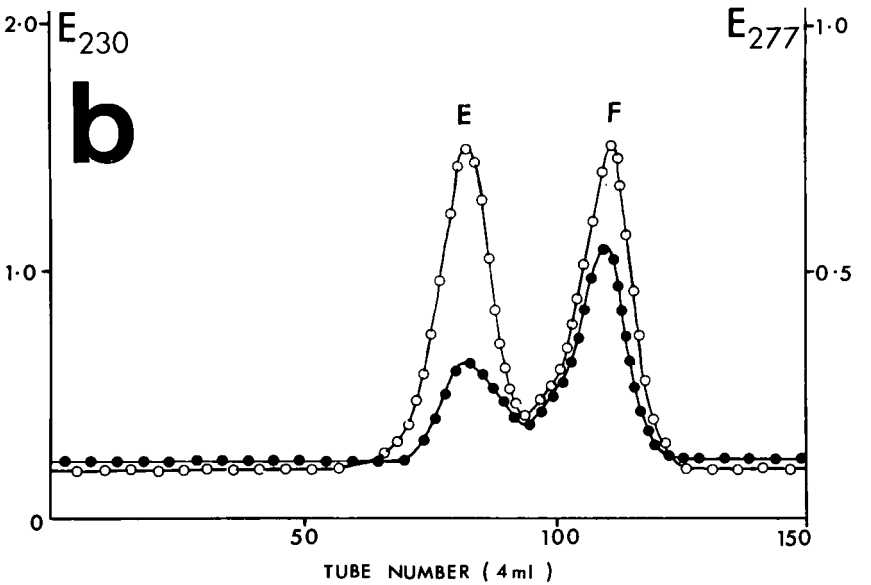


Figure 3: (a) Gel filtration on Sephadex G-50 of first peak from Sephadex G-75; column dimensions 150 x 2.0 cm. (b) Gel filtration on Sephadex G-50 of second peak from Sephadex G-75; column dimension 150 x 1.2 cm; —o—o— E_{230}, —●—● E_{277}.

this provided residues 1-38 of LPH (Figure 4, Peak D). In addition, there were two peptides in Peak A which could not be identified with any published sequence. The yields of the homogeneous peptides isolated from the pituitary glands are given in Table I.

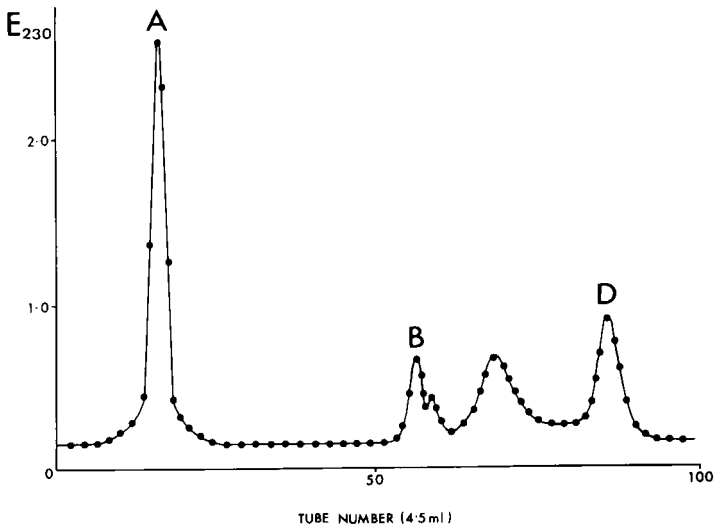


Figure 4: Chromatography of 0.02 *M* NH_4Ac fraction on Sephadex A-25, pH 7, gradient 0 to 1 *M* NaCl. Column dimensions, 50 x 1 cm.

Table I

Fragments Isolated from the Pituitary Gland of the Pig

pro-βMSH Residue No.	*Fragment*	*Yield (Umole)*	*pro-ACTH Residue No.*	*Fragment*	*Yield (Umole)*
1-91	β-LPH	1.1	1-38	pro-peptide	2.2
1-58	δ-LPH	1.7	1-19		6.8
1-38		0.7	20-38		6.8
41-58	β-MSH	1.6	44-82	ACTH	8.0
61-87		0.7	61-82	CLIP	2.3
61-91		0.9			

To summarize, we have isolated residues 1-91 (intact β-LPH), residues 1-58 (γ-LPH), residues 1-38, residues 41-58 (β-MSH), residues 61-87, and residues 61-91. The presence of this series of fragments in the pituitary gland is strong evidence that β-MSH is formed from β-LPH; its release is accompanied by the release of the contiguous fragments of the prohormone.

It is of interest that β-LPH is only weakly active as a lipolytic agent; in contrast, β-MSH is highly potent. β-LPH is therefore a prohormone rather than a hormone in its own right, and is more suitably renamed "Lipolytic Prohormone." The pituitary gland contains large amounts of this prohormone and β-LPH therefore serves as a storage form of β-MSH. The enzymic release of hormone from the prohormone must be under physiological control and this may be one point at which the levels of the ciruculating hormone are regulated *in vivo*.

The enzymes that activate the prohormone cleave the peptide chain in the region of the lysyl lysyl sequence at positions 39-40 and the lysyl argininyl sequence at 59-60, either in between or on the COOH-side of the paired basic residues. Our isolation of the fragment containing residues 61-91 strongly implies that the cleavage on the COOH-terminal side of β-MSH takes place at the carboxyl side of Arg-60. If the cleavage were to occur between the basic residues, removal of arginine by aminopeptidase action would be necessary to produce the fragment 61-91. In this context, we have found that amino peptidase M action *in vitro* degrades this peptide by the removal of the NH_2-terminal amino acids and would not stop with the removal of arginine.

The same cleavage at Arg-Tyr was observed when β-LPH was digested by trypsin *in vitro* and trypsin digestion (pH 8, 37°, substrate C 0.2 μmol/ml, enzyme C 0.5 μg/ml) of two synthetic model peptides representing the activation regions of the prohormone to β-MSH shows the same specifications as discussed, *i.e.*,

$$\text{Lys-Asp-Lys-Arg-Tyr-Gly} \xrightarrow{K_1} \text{Lys-Asp-Lys-Arg} + \text{Tyr-Gly}$$

$$(K_1 = 7.7 \times 10^{-2}\text{min}^{-1})$$

$$\text{Ala-Glu-Gly-Lys-Asp-Glu-Gly} \xrightarrow{K_2} \text{Ala-Gly-Lys} + \text{Lys-Asp-Glu-Gly}$$

$$(K_2 = 4.0 \times 10^{-4}\text{min}^{-1})$$

Trypsin cleavage on the NH_2-terminal side of β-MSH was much slower and this is in line with the fact that γ-LPH accumulates in the gland. Moreover, trypsin digestion of the synthetic peptide Ala-Glu-Lys-Lys-Asp-Glu-Gly, which overlaps the first activation region, was found to take place in between the lysine residues and not on the carboxyl side of the second lysine. Since the sequence of β-MSH commences with aspartic acid and not lysine, it is clear that the pituitary enzyme involved in the activation of β-LPH cleaves on the carboxyl side of Lys-40 and differs from trypsin. Finally, the absence of basic amino acids at the COOH-terminus of the pituitary fragments points to the presence of an enzyme with the specificity of carboxypeptidase B.

The two peptides present in Peak A of Figure 4 were isolated from pituitary in large quantity and have been sequenced (Figure 5).

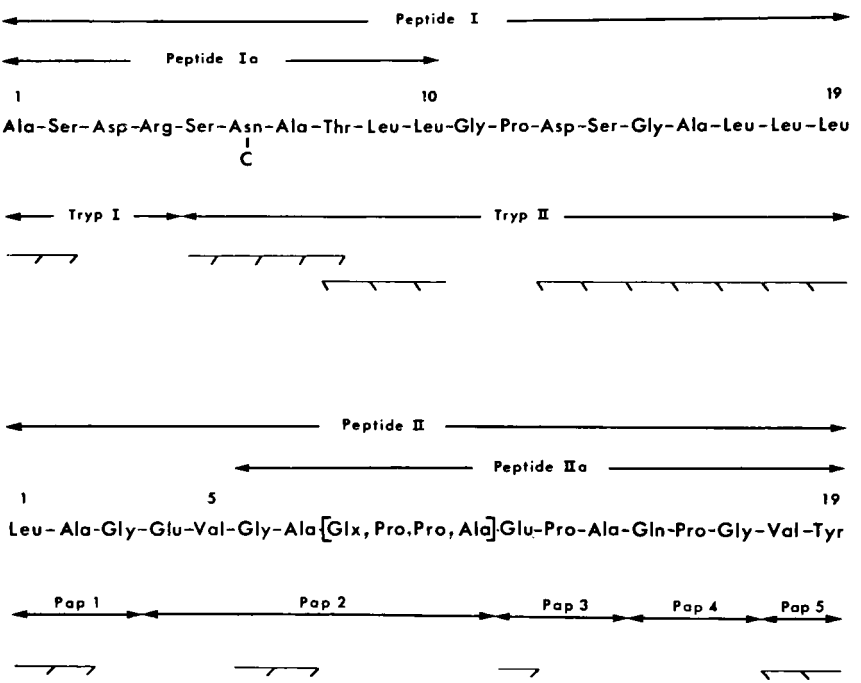


Figure 5: Amino acid sequences of Peptides I and II from Peak A, Figure 4.

The primary structures were determined by the isolation of fragments formed by trypsin and papain and by stepwise degradation with the Edman reagent and carboxypeptidase A. An oligosaccharide which included five glucosamine residues was attached at position 6 in Peptide I.

A longer peptide from Peak B of Figure 4 was also isolated which contains the complete sequences of Peptide I and Peptide II. This 38-residue peptide contains no cystine, methionine, isoleucine, phenylalanine, tryptophan, lysine or histidine and in this respect resembles the NH_2-terminal region of the prohormone to β-MSH. Two of its fragments present in the pituitary, terminated in leucyl-leucine and the third in valyl-tyrosine, which indicates that they have been released by an enzyme with a specificity more like chymotrypsin than trypsin. One of the fragments, Peptide IIa, was formed in the pituitary by cleavage of a valyl-glycyl bond, the same specificity that is involved in the release of the COOH-terminus of α-MSH. This glycopeptide may form the NH_2-terminal region of a prohormone to α-MSH or ACTH.

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METABOLISM OF ANGIOTENSIN IN VASCULAR SMOOTH MUSCLE[1]

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PEPTIDE HORMONES UNDERGO RAPID metabolic degradation presumably by proteolytic enzymes present in most organs and tissues. Incubation of peptides with purified proteolytic enzymes has been generally used to identify the point(s) of attack of each enzyme in the peptide chain (see ref 1, for angiotensin II). Thus, a number of possible angiotensin II metabolites have been identified, synthesized and purified.[2,3]

Because of differences in charge and size, the fragments were separated from each other by chromatographic and electrophoretic techniques. These compounds have been used as standards for the identification of metabolites resulting from the degradation of angiotensin II (A II) in plasma,[4,5] homogenates of tissues,[5,6] isolated organs,[7] intact animals,[8] and in man.[9]

One of the most important points which remains to be elucidated is whether metabolism occurs during the various phases of biological actions (*e.g.*, during the development, the plateau phase or the relaxation of smooth muscles *in vitro*). Because peptides are active in very small concentrations, the possible interference of metabolism with the biological effects can be studied only if labeled preparations of peptides, with high specific activity and full biological effects, are available. [^{3}H]-Angiotensin II ([H^3]-A II) as prepared with the method of Morgat *et al.*[10]

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[1]We thank the Medical Research Council of Canada for financial support.

[2]Associate of the M.R.C. of Canada.

[3]Fellow of the Canadian Heart Foundation.

possesses these properties: its specific activity is 56.3 Ci/mmoles, its biological activity is 100%, and its physico-chemical properties are very similar to those of synthetic Hypertensin (CIBA-GEIGY). This compound has been used in the present experiments to measure the metabolism of angiotensin in a vascular smooth muscle (rabbit aorta) under three experimental conditions: (A) in homogenates of aortae, (B) in aorta strips incubated in Krebs solution, and (C) in aorta strips after immersion in oil, according to the method of Kalsner and Nickerson.[11] The experimental protocols are summarized below.

A. Homogenates of rabbit aorta: (1) 200 mg of tissue in 1.8 ml of H_2O; grinding for 2 min at 4°C. (2) Dilution with H_2O 1:2, leave for 1/2 hr at 4°C. (3) Homogenization for 15 min in a Potter-Elvehjem. (4) Centrifugation for 10 min at 10,000 g. (5) Dilution with 1:2 of supernatant with concentrated (2x) Krebs; pH of mixture (eventually adjusted) = 7.0. (6) Addition of [^{3}H]-A II to obtain 50 ng/ml. (7) Incubation at 37°C for 5, 15 and 60 min. (8) TLC: 1st development in *s*-Butanol + 3% NH_3, 2nd development in Butanol: acetic acid: H_2O. (9) Plates divided in segments of 1 cm: scraping and suspension of resin for counting by liquid scintillation.

B. Rabbit aorta strips incubated in Krebs: (1) Strips of 30-50 mg (ref 12) equilibrated for 90 min in 2 ml of Krebs at 37°C, oxygenated with 95% O_2 - 5% CO_2; wash at intervals of 10 min. (2) Addition of [^{3}H]-A II (50 ng/ml): incubation for 30, 60, 120 or 180 min. (3) TLC (as in A). (4) Manipulation for liquid scintillation counting (as in A).

C. Rabbit aorta strips: oil immersion technique:[11] (1) Strips of 30-50 mg prepared as indicated in B. (2) Equilibration for 90 min in 10 ml of oxygenated Krebs at 37°C, under tension (2 g); wash at 10 min intervals. (3) Addition of [^{3}H]-A II (50 ng/ml): contraction recorded isometrically. (4) At plateau of contraction, immersion of tissues in mineral oil. (5) Removal of tissues for analysis of metabolites: a) plateau of contraction; b) 50% relaxation; c) 100% relaxation. (6) Wash in 0.5 ml of acetic acid (1 *M*) at 4°C for 30 min. (7) TLC (as in A). (8) Manipulation for liquid scintillation counting (as in A).

The choice of the systems A, B and C was determined by the following considerations:

1. Angiotensin does not enter into the cells and is probably metabolized by proteolytic enzymes localized in the biophase or at the cell surface. Therefore, the degradation of angiotensin by homogenates of tissues may provide an erroneous picture of the actual metabolism occurring under physiological conditions, if metabolism by homogenates results from the action of intracellular enzymes.

2. The fraction of angiotensin II reaching the extracellular space of isolated tissues, *e.g.*, vascular smooth muscle, is probably very small, because of the limited extent of this space and also because angiotensin diffuses slowly into tissues (Regoli *et al.*, unpublished). Therefore, when aorta strips are suspended in a relatively large volume of fluid (as in B), long periods of time may be required to accumulate measurable amounts of metabolites. The disadvantages of using long incubation periods for this type of studies are obvious.

3. The oil immersion technique[11] provides adequate conditions to measure the metabolism of the small fraction of peptide determinant for the biological action. This technique has already been successfully used by Kreici *et al.*[13] to estimate the rate of inactivation of oxytocin and analogs in uterus and by Rioux *et al.*[14] to compare the disposition of various angiotensins in rabbit aortae.

The results obtained with the experimental approaches A, B and C are exemplified by Figure 1. Incubation for 15 min in the presence of diluted homogenates of rabbit aorta inactivates a large percentage (90%) of angiotensin. TLC shows typically (Figure 1a) heptapeptide 1-7, resulting from the action of carboxypeptidase, tetrapeptide 1-4, resulting probably from the action of a chymotrypsin-like enzyme and a large amount of tyrosine which may derive from the action of aminopeptidase or of several other unidentified enzymes. A small trace of heptapeptide 2-8, which is difficult to distinguish from the spot of angiotensin II and a small amount of hexapeptide 3-8 are also found.

The fluid in which aorta strips were immersed and incubated for 180 min does not contain detectable amounts of metabolites: actually 100% of labeled angiotensin II is recovered unaltered (Figure 1b). It must however be pointed out that no tension was applied on the tissues in this type of experiment, although the dose of angiotensin was active. Diffusion of AT_{II} into the biophase and of fragments out of it may have been considerably reduced.

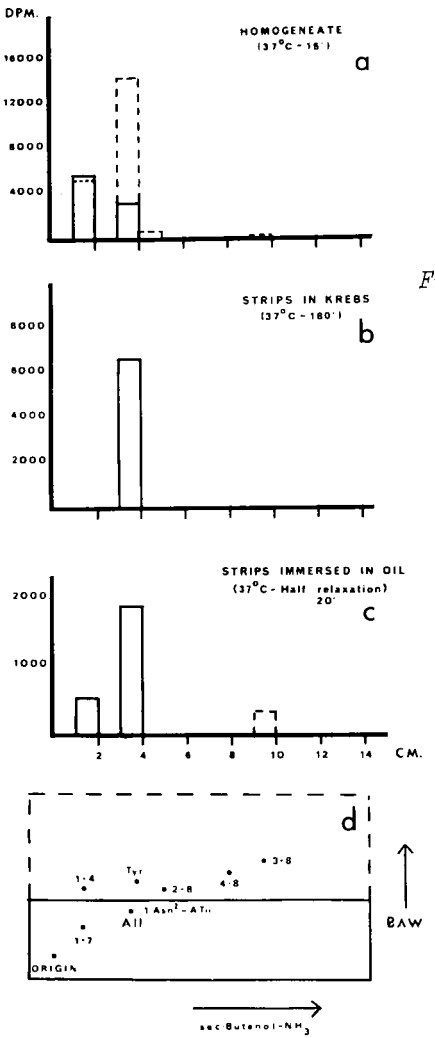

Figure 1: Radioactive products identified by TLC (see text. (a) after incubation at 37° of [^{3}H]-A II with an homogenate of rabbit aorta, for 15 min; (b) with strips of rabbit aorta for 180 min; and (c) with strips of aortae immersed in oil and allowed to relax to 50% of initial contractions, during 20 min. (d) development of chromatograms with two solvent systems (*s*-butanol-NH_3 and BAW). Positions of fragments are indicated: products migrating above the horizontal line are shown in the histogram by broken line (*e.g.*, tetrapeptide 1-4 and Tyr in (a) and hexapeptide 3-8 in (c). Ordinate: radioactivity (disintegration per min, DPM) Abcissa: migration distance (CM) of radioactive spots from the origin (d).

Aorta strips immersed in oil and allowed to relax to 50% of the initial contraction (in approximately 20 to 30 min) metabolize approximately 42% of angiotensin II, and two major metabolites appear in the chromatogram (Figure 1c). These are: heptapeptide 1-7, which indicates the presence of active carboxypeptidase, and hexapeptide 3-8, which usually results from successive splitting of the first and second amino acid by an aminopeptidase. In this particular

experiment no heptapeptide 2-8 was detected; however, this compound migrates very closely with angiotensin II and a precise separation of the two peptides is not always feasible. It is therefore possible that heptapeptide 2-8 is present in small quantities, but further experiments are required to elucidate this point.

The oil immersion technique has been used to measure metabolism during two phases of the biological action: the plateau of contraction and the relaxation. These studies have been carried out to answer two important questions: (A) Does metabolism interfere with the development or the maintenance of the biological effect? (B) Is the metabolism involved in terminating the action of the peptide?

The results of these experiments are summarized in Table I.

Table I

Radioactive Products Obtained in Rabbit Aorta Strips, Immersed in Oil to Correlate Various Phases of Biological Activity with Metabolism

	*Radioactive Products**					
	AII	*1-7*	*Tyr*	*2-8*	*3-8*	*Recovery*
Plateau of Contraction	69.3±3.0	5.6±0.2	6.7±1.5	trace	4.1±1.5	85.7
50% Relaxation	57.9±2.6	9.9±1.5	4.3±0.8	trace	10.4±1.4	82.5
100% Relaxation	55.8±2.5	19.2±2.9	6.9±0.8	trace	3.1±0.0	85.0

Results are expressed in % (means ± S.E.) of radioactivity extracted from TLC plate, excluding origin and solvent front (which usually contain 10-15% of total).

*Fragments 1-4 and 4-8 were not detected.

When the tissue is taken out for analysis immediately after immersion in oil, in order to estimate the concentration of metabolites which are present during the plateau phase of the contraction, a large part of radioactivity behaves as [^{3}H]-A II. However, small amounts of heptapeptide 1-7 and traces of heptapeptide 2-8 and hexapeptide 3-8 are detected. This indicates that metabolism occurs during the plateau and possibly also during development of the contraction.

At half relaxation 58% of AT_{II} is still present; however, a significant fraction has been broken down to heptapeptide 1-7, hexapeptide 3-8 and possibly some heptapeptide 2-8. At the end of the relaxation, the fraction of unaltered angiotensin is reduced and the concentration of a heptapeptide 1-7 is increased (Table I). From these results, it emerges that a carboxypeptidase and to a minor extent and aminopeptidase intervene in the degradation of angiotensin by rabbit aortae.

These conclusions are supported by the results shown in Figure 2.

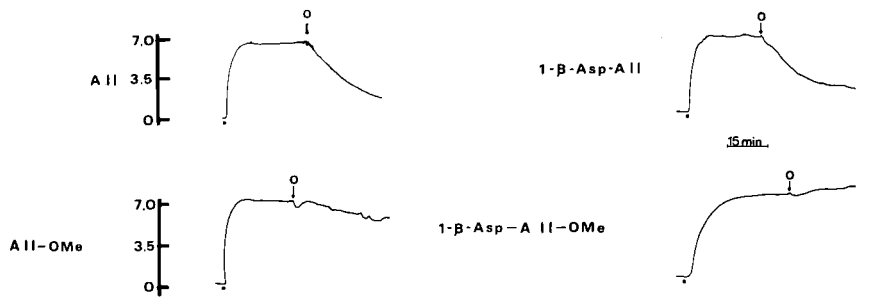

Figure 2: Isotonic recording of contractions of rabbit aorta strips elicited with 2.2 x 10^{-9} *M* A II; 4.6 x 10^{-9} *M* [βAsp^1]-A II; 16.5 x 10^{-9} *M* A II-OMe; and 84.6 x 10^{-9} *M* [βAsp^1]-A II-OMe. Krebs was substituted with mineral oil at the point indicated by 0, to measure relaxation. Ordinate: contraction in centimeters. Abcissa: time.

Three analogs of angiotensin were prepared to prevent the action of the two enzymes, *i.e.*, [βAsp^1]-A II, which according to Regoli *et al.*[5] resists degradation by aminopeptidase; A II-OMe which does not bind to carboxypeptidases, because it lacks the free terminal COOH group[15] and the doubly modified [βAsp^1]-A II-OMe being protected against

both enzymes. As indicated in Figure 2, a stable plateau of contraction is generally maintained for at least 15 to 20 min when rabbit aorta strips are contracted with the four compounds. When the tissues are immersed in oil, those contracted with A II relax relatively rapidly, while the rate of relaxation is progressively reduced in tissue contracted with [βAsp^1]-A II and A II-OMe. In the presence of [βAsp^1]-A II-OMe the aorta strips do not show relaxation but a small contraction, which persists as long as the tissue is immersed in oil.

In conclusion, the oil immersion technique appears to be adequate for measuring metabolism of peptides by intact tissues *in vitro*. Vascular smooth muscles inactivate very rapidly the fraction of angiotensin II which causes contraction. The demonstration of two fragments, heptapeptide 1-7 and hexapeptide 3-8 suggests that carboxypeptidases and aminopeptidases may be the two most important enzymes participating in the degradation of angiotensin II by a rabbit aorta. The study reveals also that a large fraction of labeled A II is not accessible to enzymatic degradation. Further experiments are needed for a comprehensive evaluation of the metabolism and fate of angiotensin in vascular smooth muscles.

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HYDROLYSIS AND CYCLIZATION OF L-ASPARTYL-L-PHENYLALANINE METHYL ESTER IN BLOOD PLASMA *IN VITRO*

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DURING EXPERIMENTS ON THE chromatographic determination of esters of amino acids and peptides, we have studied H-L-Asp-L-Phe-OMe, a dipeptide ester introduced by R. H. Mazur and associates as a potential sugar substitute.[1,2] The ultimate metabolic conversion of the compound to Asp, Phe, and MeOH in the monkey has been studied in detail by Oppermann *et al.*[3,4] The present experiments were designed to ascertain whether we could measure the extent of esterase and dipeptidase action on the dipeptide ester when it is added to blood plasma *in vitro*. Part of the aim was methodological, since there are a number of potentially physiologically active amino acid and peptide esters for which chromatographic methods might be useful. Amides are the subject of similar research, as in the recent study by Walter *et al.*[5] of the fate of H-Pro-Leu-Gly-NH_2 in human blood plasma.

The chromatographic system that we have selected is a 70-min analysis on a Durrum D-500 amino acid analyzer. The procedure is the same as is used with that instrument for the analysis of protein hydrolysates; for the present purpose the procedure can be employed to gain useful information on deproteinized blood plasma, even though a few overlapping peaks are present on the elution curves. Advantages in time and convenience make it practical to use the more rapid elution system rather than a slower procedure, which can give higher resolution with physiological fluids and which is available[6] when needed. The principles of the

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chromatography are similar to those utilized for earlier amino acid analyzers[7-11] combined with the high-pressure capability and the automation of the Durrum design.

The type of elution pattern obtained when human blood plasma is thus analyzed is shown in Figure 1A. The analysis of the same plasma after incubation for 20 min at 37° with added H-L-Asp-L-Phe-OMe is given in Figure 1B; the chromatogram provides measurement of the amounts of the substrate and the products of its hydrolysis. The peak from the dipeptide ester emerges at the position of histidine, but the relatively small area of the histidine peak in the control can be subtracted to give the net value for the area of the ester peak. The action of an esterase in the plasma yields Asp-Phe, which emerges at the isoleucine position; the area of the isoleucine peak is subtracted. Free Asp and Phe are measured by the increases in the amounts of those amino acids.

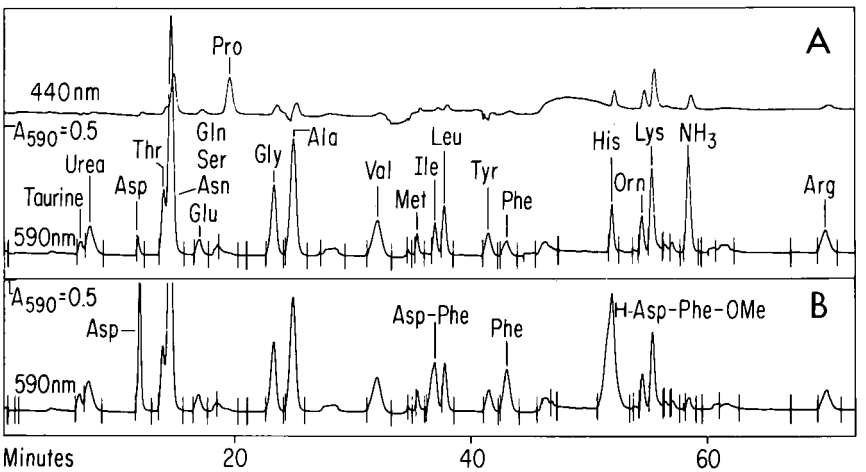

Figure 1: Chromatographic analyses of human blood plasma. The analyses were performed on a Durrum D-500 amino acid analyzer with 20 µl of deproteinized solution corresponding to 16.7 µl of undiluted plasma. The principal amino acids to be expected in blood plasma are known from the earlier studies of its composition by ion exchange chromatography.[13-14] A: Control analysis on the plasma; the integrations range from 7.59 nanomoles for alanine to 1.66 for arginine. B: The same plasma 20 min after the addition of 22.1 nanomoles of H-L-Asp-L-Phe-OMe per 16.7 µl of plasma. With A as the baseline, the residual dipeptide ester and the Asp-Phe, Asp, and Phe arising from its enzymic hydrolysis are quantitatively determined. (The variations of NH_3 in these sensitive analyses are not significant; it is difficult not to pick up variable traces of NH_3 in submitting small samples of plasma to acid precipitation.)

When a control experiment was run in which H-L-Asp-L-Phe-OMe was incubated at 37° in 0.1 *M* phosphate buffer at pH 7.3, there was a progressive decrease in the peak from the dipeptide ester and no formation of Asp, Phe or dipeptides. In this manner the rate of nonenzymic formation of the ninhydrin negative diketopiperazine (Table VI in ref 1) was determined; this cyclization gives H-L-Asp-L-Phe-OMe a half-life in plain aqueous solution of about 45 min at neutral pH and 37°.

Since the samples for analysis are stored in the refrigerated sample-loader of the Durrum instrument in pH 2.2 buffer at 10° for varying periods of time prior to analysis, the rate of diketopiperazine formation was measured under this condition and found to be 0.42% per hr. This small correction can be applied in the determinations of the recoveries of H-L-Asp-L-Phe-OMe in the analytical procedure. The ninhydrin color factor, with the ninhydrin-hydrinantin-DMSO reagent,[12] per 10 nmoles was 19,600 for H-L-Asp-L-Phe-OMe under conditions which gave 25,000 for Asp; the lower value for the dipeptide ester is a result of diketopiperazine formation during the chromatography, particularly at pH 6 and 70°, but the reproducibility of this yield permits accurate determinations of the ester. The corresponding color factor for Asp-Phe is 26,700.

The results of such analyses at different times are plotted in Figure 2 for rat plasma. The procedure provides a measure of three reactions that H-L-Asp-L-Phe-OMe can undergo in the plasma: (1) hydrolysis by an esterase to yield Asp-Phe, (2) hydrolysis of the resulting Asp-Phe by a peptidase to give Asp and Phe, and (3) hydrolysis of the initial ester by a peptidase to give Asp and H-Phe-OMe (calculated from the relative yields of Asp and Phe, since H-Phe-OMe is retarded beyond Arg, and not determined). In addition, the formation of ninhydrin-negative plus any protein-bound products can be calculated by difference. In rat blood plasma the dipeptide ester, present initially at about 1 μmole per ml, decreased to about half in 2-1/2 min. At 12 min the ester was 98% gone, 60% had appeared as Asp, 54% as Phe (indicating 6% H-Phe-OMe), and 30% as Asp-Phe. Diketopiperazine formation was not more than 8% (98-60-30).

In human blood plasma the esterase action was slower; the dipeptide ester decreased to about half in 20 min. At 120 min the ester was 99% gone, 54% had appeared as Asp, 50% as Phe, and only 3% as Asp-Phe. The difference calculation gave a limit of 42% (99-54-3) on possible diketopiperazine formation. When the diketopiperzine was added to human blood plasma, there was no detectable hydrolysis to Asp and Phe in 120 min.

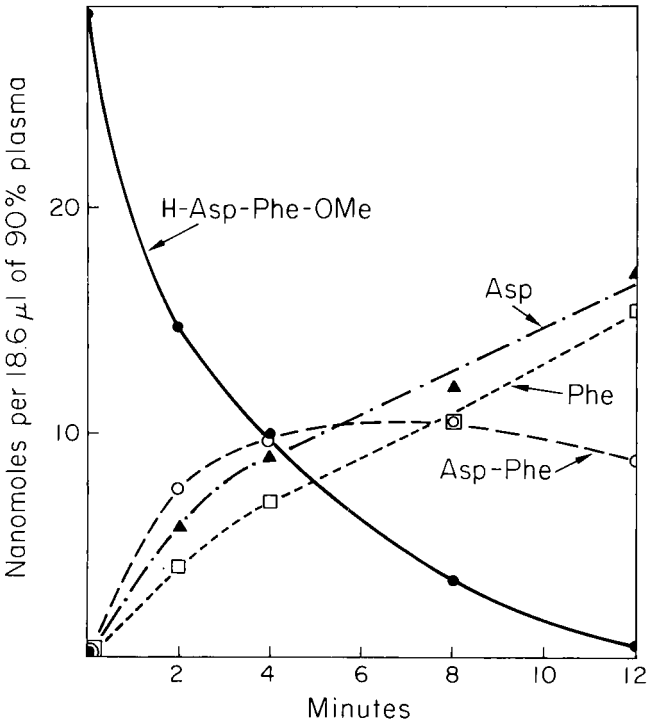

Figure 2: Course of enzymic hydrolysis of H-L-Asp-L-Phe-OMe during incubation of the ester in rat blood plasma at 37°. The nanomoles of each component were calculated from chromatograms of the type illustrated in Figure 1 for human plasma.

The products not accounted for as ninhydrin-positive ones exceed the amount of diketopiperazine to be expected from the measurement of the half-life of H-Asp-Phe-OMe in the plasma-free control. This observation leaves room for enzymic actions other than the three hydrolyses measured directly in these experiments. In regard to possible diketopiperazine formation, since Oppermann *et al.*[3] find that in the monkey the products of the metabolism of H-L-Asp-L-Phe-OMe and an equimolar mixture of L-Asp, L-Phe, and MeOH are indistinguishable, either diketopiperazine formation is minimal *in vivo* or there is a role for diketopiperazinases from some source in the metabolic process.

Experimental Section

Chromatographic System. The general procedure recommended by the Durrum Instrument Corp. for the analysis of protein hydrolysates on their D-500 instrument was employed (1st buffer pH 3.25, 2nd buffer pH 4.25), with the 3rd buffer adjusted to pH 6.0 to move histidine away from the buffer breakthrough point. The Milton Roy pump setting was 95. A temperature change from 55° to 70° was made 31 min 15 sec after the injection of the sample. The reaction coil temperature was 125°. A two-pen recorder measured the output at 0-2 A full scale for the photometer at 590 nm and at 0-1 A for the photometer at 440 nm.

Calibration. The color factors for H-L-Asp-L-Phe-OMe (aspartame, Searle, Lot No. 5270-8; moisture 2.4%, ash 1.0%) and L-Asp-L-Phe·H_2O (Cyclo, Lot No. D-1711) were measured on samples that were checked by elementary analysis. The amino acid calibration was made with Beckman/Spinco amino acid calibration mixture Type 1 at a load of 10 nanomoles per peak; the amino acid analyses are reproducible to 100 ± 3%.

Blood Plasma Samples. Heparinized plasma was used. The control solution, incubated at 37°, was 0.9 ml of plasma to which 100 µl of 0.1 *M* phosphate at pH 7.4 were added. For the incubation experiments with the dipeptide ester, a solution of 40 to 50 mg of H-L-Asp-L-Phe-OMe in 10 ml of the same buffer was freshly prepared and 100 µl of this solution were added to 0.9 ml of the plasma to give an initial concentration of about 500 µg of aspartame per ml. To obtain an accurate zero time value for the amount of H-L-Asp-L-Phe-OMe in these experiments, 100 µl of the solution were added to 0.9 ml of the pH 2.2 buffer used for loading samples for the analyzer; 20 µl were taken for chromatographic analysis and the area of the dipeptide ester peak was corrected for the dilutions characteristic of the deproteinization procedure for plasma defined below. The enzymic action can be so rapid, particularly with rat plasma, that it is difficult to obtain a sample fast enough to give a correct zero time value after plasma and ester are mixed.

At the desired time intervals, 100 µl aliquots were withdrawn from the plasma samples and added immediately to a disposable micro centrifuge tube, 0.4 ml capacity (A. H. Thomas, Cat. No. 2591 D15), freshly rinsed with water and acetone and air dried, that contained 10 µl of a 50% (w/v) solution of sulfosalicylic acid.[6,15,16] The mixture was stirred repeatedly with a fine glass rod over a period of 5 min and the tube was centrifuged for 10 min on a Beckman

Microfuge Model 152. Exactly 75 µl of the supernatant solution were withdrawn to a second cleaned tube with a 75 µl Drummond Microcap attached to a Manostat Accropet. To bring the pH to about 2.2, 5 µl of 4% NaOH were added. The mixture was centrifuged as above and about 50 µl withdrawn to a third tube. Exactly 20 µl were added with a 50 µl Hamilton syringe to a Durrum sample holding unit, that contained buffer at pH 2.2, and the sample was washed in twice with 20 µl of pH 2.2 buffer. In this procedure the 20 µl sample contains the supernatant solution from 18.6 µl of the 90% plasma taken for deproteinization. (When the analyzer is being employed for study of the amino acid composition of blood plasma *per se*, the load is usually 40 µl[6] corresponding to 37.2 µl of undiluted plasma, and one 20 µl wash is used.)

The solution of the diketopiperazine added to the plasma was prepared by keeping a solution of H-L-Asp-L-Phe-OMe at 37° overnight at pH 7.3; chromatographic analysis showed that the conversion was complete since no ninhydrin-positive constituents were present. Each experiment with the dipeptide ester was conducted in duplicate on separate plasma samples.

Acknowledgments

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SECTION V

BRAIN SPECIFIC PEPTIDES

PITUITARY PEPTIDES AND MEMORY

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THE CONCEPT THAT THE PITUITARY GLAND affects nerve function through the release of "neuropeptides" is of recent origin.[1] The importance of the pituitary gland in acquisition and maintenance of conditioned avoidance behavior has been derived from studies in partially and fully hypophysectomized rats. Extirpation of the anterior pituitary gland seriously interferes with acquisition of a shuttlebox avoidance response[2] while removal of the posterior pituitary, which does not so much affect acquisition, markedly reduces the ability to maintain the behavior.[3] Such abnormalities could be restored by treatment with adrenocorticotrophic hormone (ACTH) from the anterior lobe, melanocyte stimulating hormore (MSH) from the intermediate lobe or vasopressin from the posterior lobe of the pituitary as well as analogues of these polypeptides.[4,5]

Lysine-8-vasopressin (LVP) not only normalizes extinction behavior of posterior lobectomized rats, but it also amends the impaired acquisition of shuttlebox avoidance behavior of hypophysectomized rats.[6]

In intact rats, vasopressin facilitates acquisition of a pole jumping avoidance response but the effect is minor probably due to the insensitivity of the measure.[7] Extinction of the avoidance response is a more suitable parameter to measure the effect of vasopressin analogues. Originally we used pitressin, a rather crude posterior pituitary preparation. It dramatically increased resistance to extinction of a shuttlebox avoidance response.[8] This effect was of a long-term nature which lasted for weeks after discontinuation of the treatment. These studies suggested that

posterior pituitary principles may be involved in long-term memory processes. The behaviorally active principle in the posterior pituitary appeared to be vasopressin since oxytocin in similar amounts was without effect.[9] Subsequent experiments indicated that the behavioral effect of vasopressin is independent of its vasopressor and antidiuretic activities. Desglycinamide-lysine-8-vasopressin (DG-LVP), which was isolated from hog pituitary material,[5] is practically devoid of classical endocrine activities.[10] It effectively normalizes avoidance acquisition of hypophysectomized rats and results in long-term resistance to extinction of active and passive avoidance behavior.[11]

Attempts to determine the active core of the vasopressin molecule which contains the essential requirements for the behavioral effect were only partially successful due to the variation in purity of available compounds. Their influence was tested on extinction of a pole jumping avoidance response.[9] Briefly, rats were trained to jump onto a pole in response to the conditioned stimulus (CS) which was a light on top of the box, to avoid the unconditioned stimulus (US) of electric footshock. Ten trials a day were given with an intertrial interval of 60 seconds presented in a predetermined sequence for 3 consecutive days. Peptides were injected subcutaneously at the end of the 3rd acquisition session. Extinction sessions in which the CS was presented only were run on days 4, 5 and 8 of the experiment. Of every peptide tested, the amount needed to induce 6 or more positive responses on day 8 (3rd extinction session) was determined. Saline-treated rats never scored more than 2 positive responses in this extinction session. If the criterion was not reached with a dose of 5 μg of the peptide per rat, it was considered inactive although the number of positive responses might differ significantly from saline-treated controls when this or higher amounts were used.

Arginine-8-vasopressin (AVP) appeared to be the most potent peptide, followed by LVP. Removal of the glycinamide (DG-LVP and DG-AVP) decreased the potency to approximately 50 percent. Oxytocin and vasotocin were equally potent and possessed circa 20 percent of the activity of AVP and LVP. Pressinoic acid amide had retained only 10 percent of the behavioral potency, while rats treated with 5 μg of $(H\text{-}Cys\text{-}Pro\text{-}Leu\text{-}Gly\text{-}NH_2)_2$ failed to achieve the criterion as formulated above (Table I). Since oxytocin is as active as vasotocin, the ring structure seems to be more important than the tail for the behavioral effect of vasopressin. Nevertheless, removal of the C-terminal part of the molecule leads to a drastic decrease in behavioral potency. It may

Table I

Effect of Various Related Peptides on Resistance to Extinction of a Pole Jumping Avoidance Response

		Order of Potency
Arginine-8-vasopressin	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2	1
Lysine-8-vasopressin	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2	2
Desglycinamide-Lysine-8-vasopressin	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-OH	3
Desglycinamide-Arginine-8-vasopressin	H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-OH	4
Oxytocin	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	5
Arginine-8-vasotocin	H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH_2	6
Pressionic acid amide	H-Cys-Tyr-Phe-Gln-Asn-Cys-NH_2	7

be that it protects the active moiety against metabolic degradation en route to the central nervous system (CNS). Indeed, if pressinoic acid amide was administered via one of the lateral ventricles, only twice as much AVP was needed to induce an equipotent resistance to extinction of the pole jumping avoidance response. In addition, both peptides were hundreds of times more active when given through this route than following subcutaneous administration.[12] This suggests that vasopressin which is produced in the hypothalamic supraoptic and paraventricular nuclei may be released into the CSF and in this way transported to septal and/or hippocampal structures which may be the sites of action in the brain.[13] Morphological observations point to connections between neurosecretory cells and the ependyma of the infundibular recess of the third ventricle. Neuronal processes containing granules with neurophysin and vasopressin were found not only close to the portal capillary loops but also protruding into the third ventricle.[14] To further substantiate this hypothesis, specific antisera against vasopressin were administered into one of the lateral ventricles and the effect was determined on the retention of a one-trial passive avoidance behavior. This situation uses the innate response of rats to prefer darkness to light.[15] The apparatus consists of a dark box connected with an illuminated elevated platform. Rats are placed on the platform and latency to enter the dark box is recorded. One trial is given on day 1, and 3 trials on day 2. At the end of the 3rd trial on day 2, the animals received an electric footshock of 0.5 mA for 3 sec. Retention trials were run at 4, 24 or 48 hr after the learning trial. Vasopressin antiserum was given immediately after the learning trial and compared to oxytocin antiserum or normal rabbit serum.[16] Whereas avoidance latency of rats treated with either oxytocin antiserum or rabbit serum were maximal (300 sec) animals treated with vasopressin antiserum failed to avoid. Thus, the presence of vasopressin antiserum in the CSF prevents the formation of passive avoidance behavior. The intravenous injection of 100 times as much vasopressin antiserum, which effectively neutralizes circulating vasopressin as indicated by the absence of vasopressin in the urine and the marked increase in urine production, did not affect the behavior. These results therefore support the notion that vasopressin released from its production sites into the CSF rather than circulating vasopressin is responsible for the behavioral effect. Studies by Zaidi and Heller[17] corroborate this view. Antidiuretic activity is increased in the eyeplexus blood of rats during retention testing of passive avoidance

behavior. The rate of increase is related to the intensity of the shock to which the animals are exposed during the learning trial.[18] It is conceivable that the release of vasopressin into the CSF is similarly increased.

The physiological role of vasopressin in memory processes has also been demonstrated in rats with hereditary hypothalamic diabetes insipidus (DI), a homozygous variant of the Brattleboro strain, which lacks the ability to synthesize vasopressin. Their heterozygous littermates have a relatively normal water metabolism but a disturbed release of vasopressin,[19] while the original homozygous variant is normal in this respect. Severe memory impairment can be observed in rats with hereditary hypothalamic diabetes insipidus in the same one-trial passive avoidance situation as described above.[20] Absence of passive avoidance behavior in these rats is found 24 hr after the learning trial even when a high intensity (1 mA) shock of long duration (10 sec) is used as the aversive stimulus. In contrast, heterozygous DI or homozygous normal rats exhibit passive avoidance behavior already after much milder shock intensities. Treatment of DI rats immediately after the learning trial with 1 µg of AVP or DG-LVP restores passive avoidance behavior. Interestingly, avoidance latencies of homozygous and heterozygous DI rats as well as of homozygous normal animals are indistinguishable if tested immediately after the learning trial. These observations indicate that memory rather than learning processes are disturbed in the absence of vasopressin. The fact that homozygous DI rats acquire a shuttle-box multiple trial avoidance response albeit slower than heterozygous DI and homozygous normal rats, is in accord with this hypothesis.[21] In addition, the central administration of vasopressin antiserum similarly fails to affect avoidance latency when rats are tested within 2 hr after the learning trial.[16]

The site of the behavioral effect of vasopressin is in midbrain limbic structures as derived from lesion- and microinjection studies in various parts of the brain. Although microinjection in mesodiencephalic areas in particular at the parafascicular thalamic level increases resistance to extinction of the pole jumping avoidance response, bilateral lesions of this area failed to prevent the behavioral effect of systemically administered vasopressin. Lesions in rostral septal and dorsal hippocampal structures, however, abolished the effect of vasopressin.[13,22,23] Thus, structures which may be the site or mediate the consolidation of learned responses seem to be involved in the behavioral effect of vasopressin. Our results suggest that

vasopressin may act on septal and hippocampal structures to facilitate the storage of recently acquired information in response to specific stimuli.

It has recently been shown that rhythmic slow activity (RSA) during paradoxical sleep (PS) episodes contains substantially lower hippocampal theta frequencies in homozygous DI rats as compared to heterozygous DI and homozygous normal rats. Differences were found in all spectral parameters and the averaged peak frequency of homozygous DI rats is approximately 1 Hz lower than that of controls. Thus, PS produces an inferior quality of RSA in the absense of vasopressin. Administration of DG-AVP enhanced the generation of higher frequencies in homozygous DI rats towards nearly the level found in normal controls (Urban and de Wied, in preparation). As mentioned before, homozygous DI rats have severe memory deficits. Interestingly, PS deprivation leads to consolidation deficits.[24-26] It might be, therefore, that the impaired memory of diabetes insipidus rats is due to the low quality of PS found in these animals. Drugs which facilitate memory functions increase theta frequency in the post learning period,[27] suggesting that changes in excitability in hippocampal theta activity may be related to consolidation processes. Thus, Landfield *et al.*[28] maintain that theta activity in the post learning period may be a correlate of a brain state which is optimal for memory storage. Hypophysectomized rats which show learning and memory deficits[2] have a shorter duration of PS episodes and lack the normally present PS circadian rhythmicity.[29] These deficits can be restored by treatment with various pituitary principles. It is possible, therefore, that vasopressin affects memory consolidation by influencing the quality of RSA of the hippocampus during PS episodes.

Attempts to demonstrate effects of vasopressin analogues in other than "fear" motivated behavior were not very successful. Garrud *et al.*[30] failed to observe an effect of DG-LVP on extinction of a straight runway food running approach response in food deprived rats. It is possible that endogenous release of vasopressin in these food deprived animals might quench the effects of exogenous DG-LVP. In addition, the situation (straight alley) may be too simple to show memory effects of vasopressin analogues since DG-LVP appears to affect sexually motivated approach behavior of male rats in a T-maze (Bohus, in preparation). A higher percentage of male rats which received DG-LVP immediately after each acquisition session, choose the correct arm of the T-maze to search the receptive female goal rat than of saline-treated controls. The effect of DG-LVP again

is of a long-term nature. Thus treated rats keep displaying more correct responses than the controls after discontinuation of the treatment. DG-LVP also delays the disappearance of intromission and ejaculatory behavior of male rats following castration (Bohus, in preparation) when given before or immediately after copulation. Thus, vasopressin not only affects the maintenance of a learned approach behavior but also the genetically determined sexual behavioral repertoire of the male rat.

Vasopressin analogues were found to reverse CO_2-induced amnesia in the rat.[31] In addition, DG-LVP protects against puromycin-induced memory blockade in mice as shown by Lande *et al.*[32] The latter results suggest that vasopressin affects memory through protein synthesis. Interestingly, DG-LVP facilitates the development of resistance to the analgesic action of morphine in mice.[33] Conversely, in the absence of vasopressin, the development of resistance to the analgesic action of morphine as measured in rats on the hot plate is severely disturbed (de Wied and Gispen, in preparation). This disturbance can be restored by treatment with vasopressin analogues. Development of resistance to morphine analgesia may be regarded as a learning process.[34] This view is corroborated by observations showing that protein synthesis inhibitors which impair learning and memory prevent the development of tolerance as well.[35] Thus, similar mechanisms as in learning and memory processes may be involved in the development of tolerance. However, direct studies on macromolecular processes in the brain to determine the biochemical substrate of the consolidating effect of vasopressin analogues are missing. The manipulation of memory processes by these polypeptides, however, offers excellent opportunities for such studies.

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GH-RIF, LH-RH, TRH, MIF-I, SUBSTANCE P, AND ANGIOTENSIN--CENTRAL NERVOUS SYSTEM STUDIES

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THE FIRST CENTRAL NERVOUS SYSTEM (CNS) activity to be reported for hypothalamic releasing factors was found in the Everett DOPA potentiation test which is considered an indicator of central dopaminergic activity. These peptides were MIF-I [(melanocyte-stimulating hormone (MSH) release inhibiting factor (Pro-Leu-Gly-NH_2)], TRH[(thyrotropin (TSH) releasing factor)], LH-RH (luteinizing hormone releasing hormone), and GH-RIF [(growth hormone release inhibiting factor (somatostatin)].[1-5] In addition, MIF-I was found to actively antagonize the symptoms of oxotremorine in an animal model of parkinsonism.[6] Early clinical studies suggested effects of some hypothalamic peptides on the brain of man.[7] In light of these early findings, it seemed advisable to learn more about the effects of the hypothalamic releasing factors and related peptide in other CNS tests.

The Everett DOPA Potentiation Test

Comparative data were obtained for the hypothalamic releasing factors and other peptides in the Everett DOPA potentiation test. The most potent peptide in this test, to date, was found to be MIF-I.[2,8] This tripeptide exhibited significant activity in the dose range of 0.1 to 0.2 mg/kg. Another tripeptide, TRH, was also found to be quite active in the DOPA test in a dose range of 0.2 to 0.4 mg/kg.[3]

By contrast, both GH-RIF as well as LH-RH were considerably less active (1 to 2 mg/kg).[4,5] There appears to be approximately ten-fold difference in potency between MIF-I and TRH as compared to GH-RIF and LH-RH. These potency differences in the Everett DOPA potentiation test among these hypothalamic peptides do not parallel their endocrine activity on the pituitary. Further evidence of CNS effects of the hypothalamic factors was demonstrated in hypophysectomized animals. Thus, the activity of MIF-I, TRH, LH-RH, and GH-RIF in hypophysectomized mice in the DOPA test demonstrated a direct effect on the brain independent of any endocrine effects of the pituitary gland (Table I). These studies were the first demonstration of a nonendocrine effect of the hypothalamic releasing factors--MIF-I, TRH, GH-RIF, and LH-RH. At the same time, the pituitary hormones, MSH as well as TSH (and also T_3 and T_4) have been shown to exhibit activity in the DOPA test. Therefore, the possibility remains that in the intact animal receiving TRH, for example, increased secretion of hormones like TSH (and T_3, T_4) could further sustain the central effects of the releasing factors.

Table I

DOPA Potentiation Test
Hypophysectomized Mice

MIF-I		*TRH*		*LH-RH*		*GH-RIF*		*Angiotensin I*	
Dose (D) (mg/kg, ip)	*Response(R) (1 hr)*	*D*	*R*	*D*	*R*	*D*	*R*	*D*	*R*
0.1	3	0.05	1	0.5	2	0.1	1	0.01	2
0.2	3	0.1	2	1	2	1	2	0.1	2
0.4	3	0.2	3	2	3	10	2	1	1
1	3	0.4	3	4	3				
2	3	0.8	3	8	3				
4	3	1.6	3						
8	3								
16	3								

Substance P		*MSH*	
D	*R*	*D*	*R*
0.1	1	0.01	1
1	1	0.1	2
10	2	1	2

In additional studies in mice, it was found that the activity of TRH, MIF-I and LH-RH is not lost in the absence of a wide range of endocrine and even nonendocrine organs.[9,10]

The dopaminergic function of at least one hypothalamic peptide was further confirmed by use of α-methyl-p-tyrosine and Fla-63. With both compounds, TRH was still active in the DOPA test.

Other peptides tested in the DOPA test include MIF-II, substance P, MSH, and tocinoic acid as well as TSH.[8] All of these substances exhibited significant activity. Angiotensin I was inactive in a dose range of 0.1 to 1 mg/kg.

Serotonin Potentiation Test

The hypothalamic releasing factors MIF-I, GH-RIF, and LH-RH were found to be inactive in the mouse serotonin potentiation test in a dose range of 0.01 to 1.0 mg/kg. In contrast, TRH and LH-RH were active in a dose range of 0.2 to 8.0 mg/kg (Table II).

Other peptides tested included substance P, MSH, and angiotensin I. All of these were found to be inactive. The intriguing CNS actions of MSH in the rat and man have been summarized elsewhere.[11,12]

Table II

Serotonin Potentiation Test

MIF-I		*TRH*		*LH-RH*		*GH-RIF*		*Angiotensin I*	
Dose (mg/kg,ip)	*Response (1 hr)*	*D*	*R*	*D*	*R*	*D*	*R*		
0.5	0	0.1	1	1	0	0.01	0	0.1	0
1	0	0.2	1	2	1	0.1	0	1.0	0
2	0	0.4	2	4	2	1	0		
4	0	0.8	3	8	2				
8	0								

Substance P	
D	*R*
0.01	0
0.1	0
1	0

Oxotremorine Antagonism Test

This animal model of parkinsonism was found to be quite sensitive to the protective actions of MIF-I.[6,13] In a dose range of 1 to 16 mg/kg, MIF-I reduced the parkinsonian-like symptoms induced by oxotremorine. Furthermore, MIF-I was found to potentiate the effects of DOPA in reducing oxotremorine symptoms. Finally, MIF-I was active in reducing symptoms of oxotremorine in hypophysectomized mice in a further demonstration of the nonendocrine effects of MIF-I. In contrast to MIF-I, the other hypothalamic releasing factors, TRH, GH-RIF, and LH-RH, were found to be inactive against oxotremorine.

Discussion

These studies have shown that the hypothalamic releasing factors (MIF-I, TRH, GH-RIF and LH-RH) potentiate the behavioral effects of DOPA both in normal intact as well as hypophysectomized mice. Thus, it was demonstrated for the first time that the hypothalamic releasing factors have a direct action on the brain independent of peripheral endocrine effects. Furthermore, these studies suggest the possibility that hypothalamic and perhaps other peptides may have clinical effects on "mood" and some evidence exists to support this concept.[7] Of the hypothalamic releasing factors, only TRH was effective in the serotonin potentiation test, a finding which suggests that these peptides may be found to have different effects on the CNS of man.

Possibly the most interesting finding with MIF-I was its unusual activity in antagonizing the parkinsonian-like effects of oxotremorine. Preliminary clinical results indicate that MIF-I may have significant antiparkinsonian activity.[14-16] This remarkable activity of MIF-I clearly separates it from the other releasing factors, which are inactive against oxotremorine.

In summary, we have shown that the hypothalamic releasing factors (MIF-I, TRH, GH-RIF, and LH-RH) have central activities independent of their endocrine effects which suggest the possibility of clinical utility in mood and/or neurological disorders.

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NEUROPEPTIDES AND PROTEINS

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IT IS ALREADY MORE THAN 14 years since we postulated that the hypothalamus produces not only releasing hormones regulating the function of the adenohypophysis, but also peptide hormones which regulate cardiac activity and coronary circulation.[1,2] These peptide hormones act without the hypophysis. They have an organotropic activity.

We have isolated three coronarodilatory peptidyl neurohormones from the hypothalamo-neurohypophyseal system, which we refer to as "K," "C" and "G" substances.[3] In amounts of 0, 1-0, 5 μg/kg they induce a significant increase of about 200-300% in the volume of venous blood leaving the heart. This effect lasts for 2-3 hr or more, and has been shown to be due to the increase in the diameter of small capillaries and functions of the reserve vessels of the heart. A significant improvement in heart blood supply by coronarodilatory neurohormones has also been confirmed by the method of radioactive indication at the experimental ischemia of heart and by measuring of the number and diameters of heart capillaries (Figures 1 and 2).

These figures show changes in coronary circulation, the period of half-life of radioactive iodine from experimentally induced acute ischemia of the myocardium in dogs and histochemical data indicating changes in the capillary network of the heart following administration of neurohormone "C". Clinical results have shown that neurohormone "K" and "C" are very effective substances in the treatment of myocardial infarction and other spasmodic diseases of the heart. The results obtained indicate that the neurohormones are released from brain into the circulation after being bound to albumins and γ-globulins of the serum and transported to the heart,

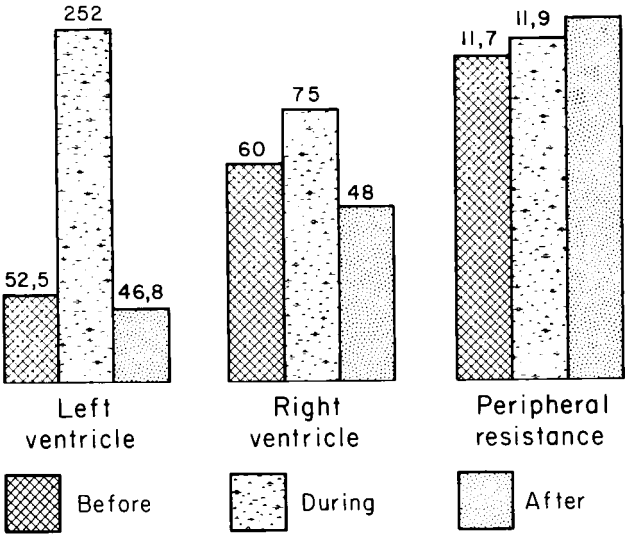

Figure 1: Effect of substance "C" on coronary flow and periferal resistance of dogs (experimental ischemia of myocardium).

	Parts of heart	Diameter of capillaries	Number of capillaries per mm^2
Normal	LEFT VENTRICULAR MUSCLE	4.5 ± 0.05 n=486	772 ± 34 n=4
Normal	INTRAVENTRICULAR SEPTUM	4.0 ± 0.09 n=392	810 ± 42 n=4
30' "C"	LEFT VENTRICULAR MUSCLE	6.17 ± 0.17 n=200 P<0.001	936 ± 32 n=2 P<0.5
30' "C"	INTRAVENTRICULAR SEPTUM	6.35 ± 0.13 n=203 P<0.001	875 ± 45 n=2 P<0.4
60' "C"	LEFT VENTRICULAR MUSCLE	5.3 ± 0.09 n=600 P<0.001	1074 ± 18 n=4 P<0.001
60' "C"	INTRAVENTRICULAR SEPTUM	5.5 ± 0.1 n=448 P<0.001	1100 ± 40 n=4 P<0.005

Figure 2: The effect of neurohormone "C" on the capillaries of some parts of rat heart.

where there are specific receptor proteins for neurohormones "K" and "C". We have recently succeeded in isolating a receptor-neurohormone "C" complex from heart muscle.

It has been established that the hypothalamus produces a coronary constrictor, other than vasopressin, which competes with substances "K" and "C" for the receptor in the heart. However, the N-terminal residue of the coronarodilatory substance has not been determined. It is probable that the terminal amino group is masked by acylation or cyclization. These peptides contain 5-8 amino acid residues with an amino acid composition different from known peptides of brain.

Thus, a family of peptide neurohormones is produced in the hypothalamus which regulate cardiac activity and coronary circulation. We have been able to show that neurohormones "K" and "C" are produced in the magnocellular cells of the hypothalamus where vasopressin and oxytocin are produced. "K" and "C" have been isolated from neurosecretory granules of the hypothalamus and neurohypophysis by the method of LaBella.[4] Figure 3 shows the distribution of "K" and "C" in the sucrose-density gradient. We have been able to show that the sizes of these granules are greater in the hypothalamus than in the neurohypophysis. The presence of substances "K" and "C" in the neurosecretory granules indicates that specific carrier proteins of coronarodilatory neurohormones are found in neurosecretory granules.

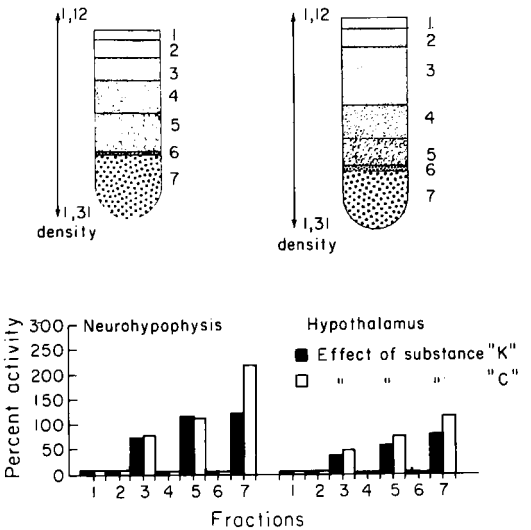

Figure 3: Distribution of biologically active substances ("K" and "C") in the neurosecretory granules of the hypothalamus neurohypophyseal system.

In 1964 I isolated a protein from the hypothalamus possessing a very pronounced coronarodilatory effect.[5] This protein was specifically localized in the hypothalamus, was water soluble, and had a molecular weight of about 50,000. I believe this protein was actually the first specific protein of the hypothalamus isolated.

The first experiments showed that this protein was different from neurophysin in its biological and physicochemical properties; inasmuch as our protein was isolated from the hypothalamus, we decided to isolate also neurophysin and compare the properties of our protein with those of neurophysin. At that time the molecular structure of neurophysin was unknown; its molecular weight was considered to be 30,000 as had been proposed by Van Dyke in 1942,[6] and there were no immunochemical methods for showing neurophysins during the transport of neurosecretory granules. Now we know the molecular structure of neurophysin, which has a molecular weight of 10,000.[7]

We were able to isolate a number of hypothalamic proteins. However, not one of the protein fractions possessed vasoconstrictor or oxytocic activity. This made us propose at that time that the hypothalamus either does not contain neurophysin or that the vasopressin-neurophysin complex in the hypothalamus undergoes enzymatic destruction in the brain by proteolytic enzymes which are exceptionally active in the hypothalamus and which hydrolyze vasopressin. The complex of carrier proteins of coronarodilatory neurohormones isolated from the hypothalamus has, however, high biological activity and markedly increases the volume of the venous blood leaving the heart and the volume capacity of the heart.

During the last years we have been able with Srapionjan to purify the coronarodilatory protein by gel filtration, ion exchange chromatography, and isoelectric focusing. Two protein carriers were obtained for substance "K" and "C" and their amino acid composition was established. Our calculations show that these protein carriers are about 0.003% of the soluble proteins of the hypothalamus. Figure 4 shows an amino acid composition of one of these proteins.

Another important fact is that the protein carriers are probably the sources from which "K" and "C" are formed. After dissociation of the complex of "K" and "C" from their carrier protein, the inert proteins are enzymatically hydrolyzed by trypsin. Two peptides have been isolated from the protein hydrolysate which by their physicochemical and pharmacological properties are very similar to substances "K" and "C". By high voltage electrophoresis and paper

AMINO ACID	AMINO ACID IN NANOMOLES	% OF AMINO ACIDS
ASPARTIC ACID	2,2	5,2
TREONIN	2,4	5,8
SERINE	7,5	17,7
GLUTANIC ACID	1,7	4,9
PROLIN	1,6	3,7
GLICINE	5,5	13,0
ALANINE	2,8	6,6
VALINE	1,5	3,5
ISOLEUCINE	0,9	2,6
LEUCINE	1,1	2,5
TYROSIN	1,6	3,8
PHENYLALANINE	1,2	2,8
HISTIDINE	1,5	3,5
LYSINE	2,0	7,7
ARGININE	0,6	1,4
METHIONINE	TRACES	-
CYSTEINE		-
TRYPTOPHAN	NOT DETERMINED	-

Figure 4: Amino acid composition of the specific protein of cattle hypothalamus.

chromatography we have been able to separate them and to show that they are peptides. The high amounts of serine (17%) and glycine (13%) are worth mentioning.

This protein carrier differs from neurophysin and other proteins of the brain by its physicochemical and biological properties (S-100, 14-3-2, etc.). We were able to obtain data indicating that releasing hormones (unpublished works) as well as the neurohormones "K" and "C" have specific protein carrier in the brain.

Activity of the brain proteases and peptidases, which are probably responsible for the formation of some peptide neurohormones from protein precursors[8,9] is inhibited very strongly by somatostatin, TRF, and LRH at 10^{-9} *M* concentration (Figures 5 and 6) (kindly given to us by D. R. Guillamin).

We have shown that somatostatin inhibited release of the biologically active substances from endocrine pancreas after the excitation of the vagus nerve on the level of pancreas. The question arises about the interrelationships of neurophysin and our proteins in the transport of substances "K" and "C" and vasopressin and oxytocin in the neurosecretory granules. Can substances "K" and "C" bind

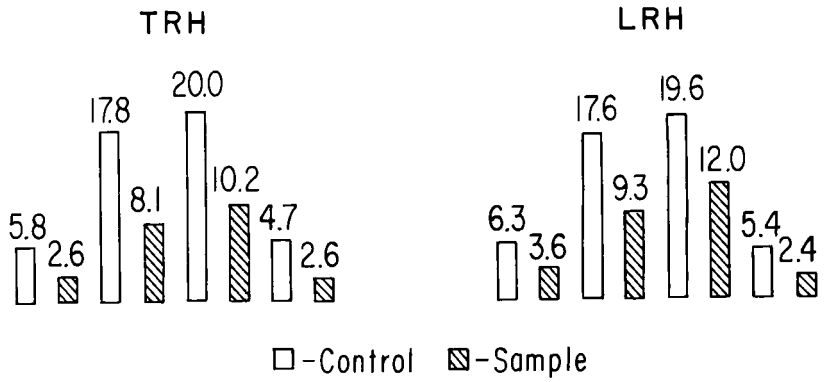

Figure 5: Effect of TRH and LRH on neutral proteinase activity (μmoles/g fresh tissue/hr) of rat brain parts, hypothalamus, neurohypophysis, adenohypophysis and cortex.

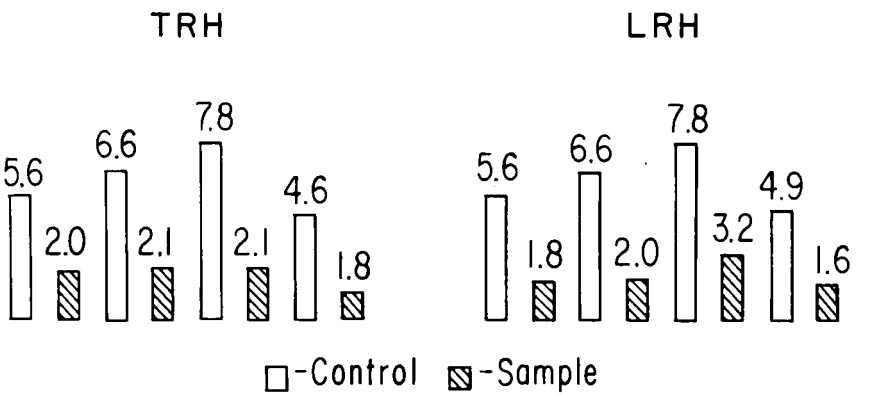

Figure 6: Effect of TRH and LRH on acid proteinase activity (μmoles/g fresh tissue/hr) of rat brain parts, hypothalamus, neurohypophysis, adenohypophysis and cortex.

also with neurophysin? Our experiments have shown that in Tyrode solution (without glucose), on incubation with neurohormone "C" (37°, 60 min) bovine neurophysin II (which was kindly given to us by Dr. Breslow) is bound to a considerable degree. After dialysis against Tyrode solution, neurohormone "C" is still bound to neurophysin II. The complex of neurophysin with neurohormone "C" (250 μg neurophysin) gradually decreases the systemic blood pressure. This effect lasts for a few hours. Vasopressin removes neurohormone "C" from the surface of neurophysin and has a typical

vasopressor effect. These data indicate that neurophysin which is in neurosecretory granules has a high affinity not only to vasopressin and oxytocin but also to neurohormone "C", having an organotrophic effect. The protein carriers may be the parent substances which contain the primary amino acid sequences of the peptide hormones. In the near future, in collaboration with Academician Ovchinnikov, we hope to describe the chemical structures of these unusual substances. At the present time it is established that there are protein and peptide substances in the brain that act as regulatory factors on cardiovascular function and probably on other visceral functions of the visceral organs. We have data showing the clinical usefulness of these substances in myocardial infarction and other spasmatic diseases of the coronary vessels. We have accumulated a large number of observations on the biosynthesis of these specific proteins and peptides of the hypothalamus and about the mechanism of release of these substances in the general circulation.

In conclusion our data indicate that there is a controlling interrelationship and feedback regulation between protein and peptide metabolism of the brain and function of the visceral organs.

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SEXUAL AROUSAL IN MALE ANIMALS: A CENTRAL EFFECT OF ACTH-LIKE PEPTIDES IN MAMMALS

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THE INJECTION OF ACTH OR MSH PEPTIDES into the cerebrospinal fluid (CSF) of mammals induces a dramatic change in behavior, characterized mainly by repeated stretching and yawning movements (stretching-yawning syndrome)[1] and by recurrent episodes of erection and ejaculation.[2]

This report concerns the sexual response to ACTH peptides. We refer to previous publications for a detailed description of the stretching-yawning syndrome and for a discussion of its physiological significance. Here we will give only a brief description of this phenomenon.

Stretching-Yawning Syndrome (SYS)

Ten to twenty minutes after the injection of a few micrograms of ACTH or MSH into the CSF, the animal (dog, cat, rabbit etc.) starts to stretch or yawn in the way it usually does when it awakens from sleep. However, it repeats these movements for many hours at intervals of a few minutes, or even seconds, according to the dose administered. Rabbits and rats exhibit both stretchings and yawnings, cats and dogs mostly stretchings, while in primates yawnings prevail. The onset of this effect is more rapid and the active doses are smaller if the peptides are injected into the third ventricle or into hypothalamic regions closer to the hypophyis.[3]

In cats with chronically implanted electrodes, it has been observed that each stretching or yawning movement induced by ACTH is associated with cortical activation.[4] However, the EEG activation does not appear to be the consequence of the stretching or yawning movement, for these events begin together, and at times cortical arousal precedes the behavioral change. It seems likely that ACTH is responsible for both events. These findings support the idea that stretchings and yawnings have an arousal significance: they might be considered an evolutionary vestige of an adaptive mechanism to aid arousal after sleep or to antagonize sleep when it presses under unsuitable conditions.

Sexual Response to ACTH and MSH

This effect has been observed in our laboratories in rats, rabbits, cats and dogs, and it has been confirmed by Baldwin *et al.* in rabbits[5] and Kinnard (reported by MacLean)[6] in squirrel monkeys.

The sexual response begins 10 to 20 minutes after the injection of ACTH peptides into the lateral ventricles and accompanies the SYS for the first 2 to 5 hours. It is characterized by recurrent episodes of penile erection, each episode often culminating in ejaculation. In adult male rabbits, sexual stimulation may be so intense that, during the first few hours following treatment, the animals may ejaculate up to a dozen times. During an episode of sexual excitement the animal frequently yawns or stretches, although the two phenomena may also occur separately. Moreover, castration abolishes the sexual response to ACTH but not its capacity to induce the SYS.[7]

Interestingly, during the episodes of sexual stimulation, animals do not seek to mount either male or female partners. Thus, ACTH-like peptides, unlike drugs acting on brain monoamines,[8] do not enhance social interaction. These findings indicate that erection and ejaculation or mounting behavior can be influenced differentially. The present study was aimed at clarifying the amino acid sequence necessary to elicit the sexual response and the mechanism by which ACTH peptides exert their central effect.

Amino Acid Sequence Essential for Eliciting the Sexual Response

This study was carried out on adult male rabbits, cats and rats with stainless steel cannulae chronically implanted into the lateral ventricle. We tested the natural or synthetic peptides listed in Table I. The results show that

Table I

Penile Erection and SYS Induced by Peptides Related to ACTH

Peptide	*Dose Intraventricularly (μg or I.U.)*	*Animal Species*	*No. Animals Displaying Penile Erection**	*SYS*
Natural:				
β_p-ACTH	5	rabbit	10/10	10/10
	1	rabbit	6/10	8/10
α_p-MSH	5	rabbit	5/5	5/5
β_p-MSH	5	rabbit	5/5	5/5
β_s-LPH	10	rabbit	5/5	5/5
Synthetic:				
DW-75	1	rabbit	8/8	8/8
Alkali-treated				
$ACTH_{1-24}$	5	rabbit	8/8	8/8
$ACTH_{1-16}$	5	rat	10/10	10/10
	5	cat	5/5	5/5
$ACTH_{1-13}$	5	rat	5/5	5/5
	5	cat	4/4	4/4
Inactive:				
STH	20	rabbit	0/5	0/5
LYS-vasopressin	10 I.U.	rabbit	0/10	0/10
Oxytocin	0.2 I.U.	rabbit	0/10	0/10
Bradykinin	30	rabbit	0/20	0/20
	10	cat	0/4	0/4
LHRH	10	rat	0/10	0/10
TRH	5	rat	0/10	0/10
MIF	5	rat	0/10	0/10
GIF	5	rat	0/10	0/10

Experimental details are reported in Gessa *et al.*[7] DW-75=(D-Ser^1,Nle^4,Val^{25})$ACTH_{1-25}$-NH_2; p=pig; s=sheep.

*At least three episodes of penile erection during the 3-hr observation.

only those peptides possessing ACTH or MSH activities induce erection and the SYS, the effective dose being roughly equal for inducing either effect.

Indeed, these behavioral changes were induced by natural ACTH and MSH peptides, by β-lipotropic hormone and by synthetic peptides related to ACTH and MSH, such as $ACTH_{(1-24)}$, $_{(1-16)}$, $_{(1-13)}$ and (D-Ser^1, Nle^4, Val^{25})$ACTH_{1-25}$-NH_2.

Natural or synthetic ACTH fully retain the capacity to induce erection and the SYS after being boiled in 0.1 *N* NaOH, a procedure which degrades ACTH molecule and destroys its adrenocorticotropic but not its intrinsic MSH activity. However, although all the active peptides possess MSH activity, there is no correlation between their MSH potency and their ability to induce behavioral changes. As Table I shows, the following peptides, unrelated to ACTH or MSH, were found inactive in producing either the SYS or the sexual response: STH, LYS-vasopressine, oxytocine, bradykinin, LH-RH, TRH, MIF and GIF.

From the analysis of the structure of the active peptides, it appears that the "core sequence" essential for producing the behavioral changes is the esapeptide Glu-His-Phe-Arg-Try-Gly. This sequence is present in all the active peptides.

On the Mechanism of the Central Action of ACTH

The consideration that the steroidogenic effect of ACTH is mediated by cyclic AMP formation[9] and by stimulation of protein synthesis[9] prompted us to investigate whether similar mechanisms might be involved in the behavioral effects of centrally injected ACTH. We investigated if this effect was influenced by theophylline, a compound which protects cyclic-AMP by inhibiting cyclic 3',5'-nucleotide phosphodiesterase,[10] or by cycloheximide, an inhibitor of protein synthesis.

As Table II shows, the administration of cycloheximide at a dose as low as 5 mg/kg, completely prevented both the sexual response and the stretching-yawning effect of β-$ACTH_{1-24}$.

On the contrary, as Table III shows, the effects of ACTH were potentiated by the intraventricular injection of theophylline, which by itself was inactive.

These results suggest that the behavioral effects of ACTH require the formation of cyclic-AMP and the synthesis of a new protein: this mechanism might explain the latency to the onset of the behavioral changes after the administration of the peptide.

Table II

Inhibition by Cycloheximide of the Behavioral Effects of Intraventricularly Injected ACTH in Male Rats

Cycloheximide mg/kg i.p.	$ACTH_{1-24}$ *µg/animal*	*No. of Rats Displaying erections*	*stretchings*
0	1	10/10(4.6±1.2)	10/10(12.3±2.6)
5	1	0/10	2/10(1)
10	1	0/10	1/10(3)
20	1	0/10	0/10

Cycloheximide was injected 3 hours prior to $ACTH_{1-24}$. The latter was injected into a lateral ventricle.
In parentheses the no. of erections and stretching acts (mean ±S.E.) observed during the first two hours after the injection of ACTH.

Table III

Potentiation by Theophylline of the Sexual Response to ACTH in Rabbits

Treatment		*µg/Animal Intra-ventricularly*	*No. of Animals Showing Penile Erection*
————	$ACTH_{1-24}$	1	3/8
Aminophylline +	$ACTH_{1-24}$	50 + 1	9/9
Aminophylline	————	50	0/5

Aminophylline was given 30 min prior to ACTH.

Physiological Significance

An important question with regard to the physiological significance of our findings is whether peptides capable of producing the above behavioral changes are normally present in the mammalian brain. We isolated from the rat hypothalamus an ACTH-like peptide that produced both the SYS and the sexual response when injected into the lateral ventricle of male rabbits. Table IV shows that the amount of this material is markedly increased in rats both hypophysectomized

Table IV

Penile Erection and the SYS Induced in Rabbits by ACTH-like Peptides Isolated from the Hypothalamus of Intact and Hypophysectomized-Adrenalectomized Male Rats

Source of Active Peptide	*Dose Injected Into Lateral Ventricle. Material Contained In:*	*Rabbits with Erection/Treated Rabbits*	*Rabbits with SYS/Treated Rabbits*
$ACTH_{1-24}$	1 μg	5/5	5/5
Rat pituitary	1 hypophysis	6/7	7/7
Hypothalamus from intact rats	4 hypothalami	3/6	3/6
Hypothalamus from hypophysectomized-adrenalectomized rats	1 hypothalamus	2/4	3/4

Extraction and isolation procedures for the peptide material and other technical details are reported in Bertolini *et al.*[11]

and adrenalectomized. These results indicate that this peptide is synthetized in the hypothalamus and its concentration increases under conditions known to increase the concentration of corticotropin-releasing-factor.

In conclusion, the physiological significance of the central response to ACTH-peptides is supported by the presence of active peptides in the hypothalamus, by the smallness of the active doses, by the fact that the behavioral changes are induced in all animal species tested, and that they are an exaggeration of physiological responses. Among the problems to be clarified is whether the concentration of the active peptides in the hypothalamus varies under physiological conditions, particularly in relation to sexual behavior.

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PURIFICATION OF THE SLEEP-PROMOTING SUBSTANCES FROM SLEEP-DEPRIVED RAT BRAIN

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THE PURPOSE OF THIS STUDY IS THE extraction and purification of a specific substance(s) to promote sleep. A key question in our experiment is which compound(s) directly causes specific centers of the central nervous system to promote its function. While intracisternal injection of certain substances such as acetylcholine, 5HT and GABA may promote sleep, the mechanisms are not known. Conceivably, the chemical transmitters act directly at the sleep centers, making particular neurons more accessible to generating sleep signals. Compared to what we know about the sleep centers and the general functions of chemical transmitters, our knowledge of the specific substances to promote sleep is limited. We can find three candidates for such substances. The first substance ("Sleep Factor Delta")[1] is a peptide isolated by Monnier and his collaborators. The second (The Sleep-Promoting Factor S),[2] which is also a peptide, has been purified by Pappenheimer and his group. On the other hand Drucker-Colin[3] and his associates reported the specific proteins in the midbrain perfusate of naturally sleeping cat, of which concentration increased at a state of rapid eye movement sleep. In previous report[4] we showed the possible existence of the sleep-promoting substances in the brain stem of sleep-deprived rats.

Male rats were deprived of their sleep by electrical shock for 24 hours, using our special sleep-deprivation apparatus.[5] Their brain stems were separated in ice-cold

saline and homogenized with 5 volumes of water in a Waring blender. The homogenate was dialyzed for 5 hours against water. The outer solution was lyophilized and stored at -74° until use. For control experiment, the material was prepared from not sleep-deprived rats. The lyophilized material was purified on SP Sephadex column by linear gradient from water to 0.25 *M* ammonium formate. The active fractions obtained by SP Sephadex column chromatography were purified on Sephadex G-10 by 0.05 *M* ammonium formate. The active fractions obtained by Sephadex G-10 column chromatography were used for pronase treatment.

For bioassay we used two methods. One is the continuous monitoring of slow waves of electroencephalogram (EEG) and of the locomotor activities of male rats before, during and after intraventricular infusion of the samples. The other method is the continuous monitoring of the spontaneous discharge of the abdominal ganglion of crayfish before and after application of the samples. The effect was estimated from the reduction rate of discharges.

Figure 1 shows the effect of each fraction obtained from SP Sephadex column chromatography. The ordinate shows the reduction rates of discharges which indicate the rate of pulse numbers for 5 minutes before and after application of the samples. One of the active compounds was contained in the fractions 23-25 and the other in fractions 26-28. GABA was located in fractions 28-29; 5HT, 5HTP and melatonin were located in fractions behind fraction 38.

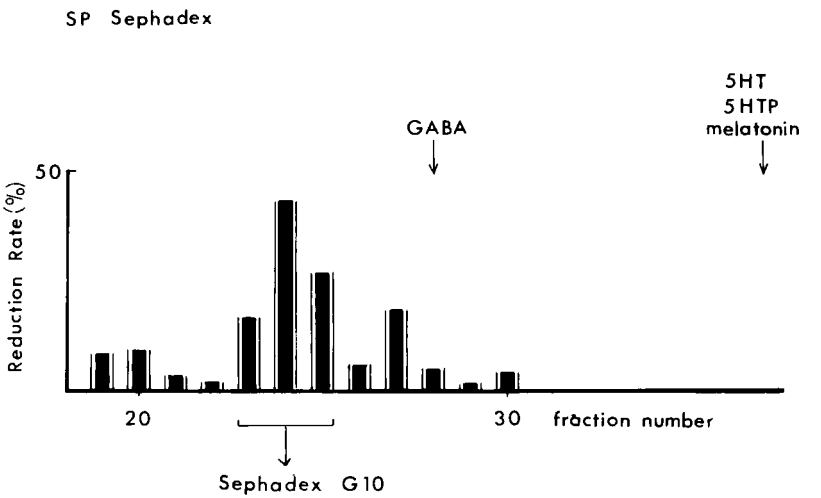

Figure 1: The effect of each fraction obtained by SP Sephadex column chromatography on the spontaneous discharges of the abdominal ganglion of crayfish.

The combined material of fractions 23-25 was further purified on Sephadex G-10. Figure 2 shows the reduction rates of each fraction from Sephadex G-10. The column was eluted by 0.05 *M* ammonium formate. There were three active fractions, *i.e.*, fractions 17-19, fractions 23-25, and fractions 30-32, of which activity inhibited the spontaneous discharges. By contrast, fractions 26-29 exhibited the effect of enhancement. Gly-gly-gly-glycine (mol wt. 246) was used for the marker of molecular weight.

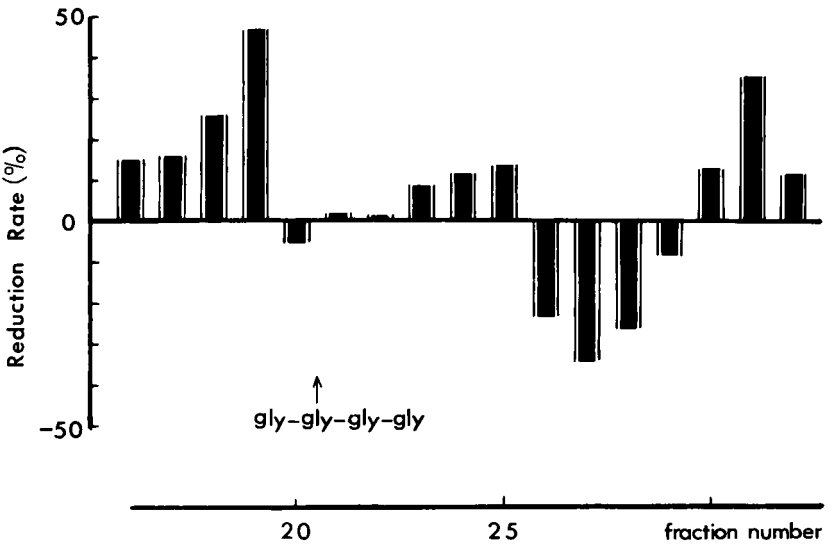

Figure 2: The effect of each fraction obtained by Sephadex G-10 column chromatography on the spontaneous discharges of the abdominal ganglion of crayfish.

The effect of fraction 19 is shown in Figure 3. The profile presents the integrated discharges per 3 seconds. The discharge rate reduced after application of fraction 19.

Figure 4 shows the effect of the fraction 19 on the nocturnal locomotor activities of a male rat before, during and after the administration. Furthermore, the slow waves in EEG increased during this period. In our preliminary experiment, fraction 19 lost its activity after pronase treatment.

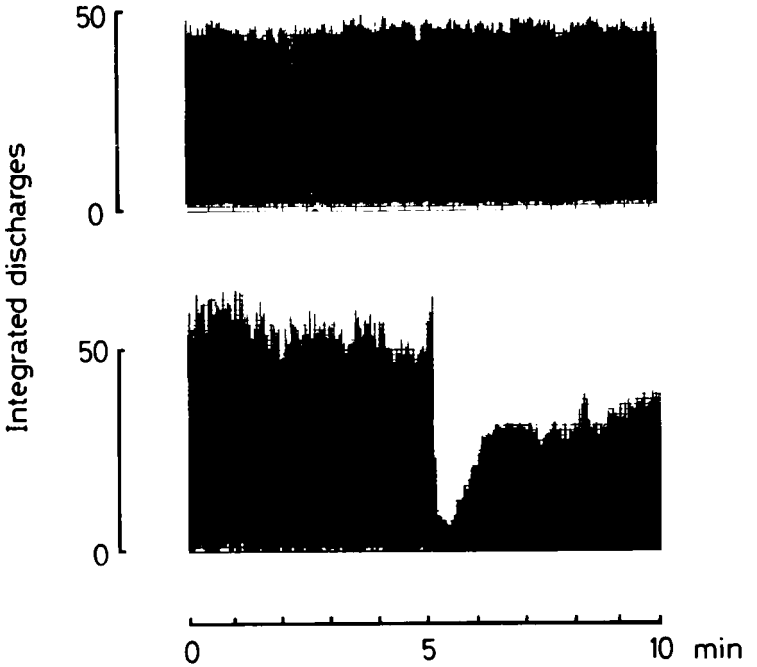

Figure 3: The effect of fraction 19 obtained by Sephadex G-10 column chromatography on the integrated discharges of the abdominal ganglion of crayfish.

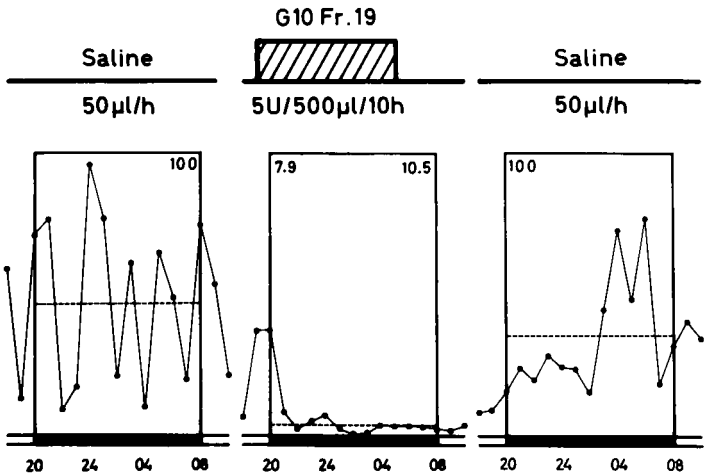

Figure 4: The effect of fraction 19 obtained by Sephadex G-10 column chromatography on the nocturnal locomotor activities of a male rat.

From these results it is suggested that fraction 19 contains the active compounds which modulate the neuronal mechanism of sleep-promotion. The active compounds in fraction 19 might have the similar molecular weight to those of the sleep-promoting Factor S and "Sleep Factor Delta."

Further purification of the active substances is now in progress.

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LEARNING-INDUCED BRAIN PEPTIDES

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THE ATTEMPTS MADE IN THE LAST fifteen years to detect chemical events taking place in the brain during acquisition and retention of information have used three main approaches. Results obtained by the first two, the search for chemical correlates of learning and memory[1] and the study of the effect of metabolic inhibitors on these processes,[2] have suggested that the main event was an increase in RNA and protein synthesis. By their very nature, however, these approaches could not define the precise molecular species involved or specify the significance of the chemical changes associated with learning.

The third approach, the traditional method of bioassays, is uniquely suited to detect small amounts of biologically active material of unknown chemical composition in complex mixtures. In spite of the conspicuous success of the method in laying the foundations of the doctrine of hormones, vitamins, and neurotransmitters, its application to the problem of learning and memory has been regarded with suspicion.[3,4] The present report deals mainly with attempts at demonstrating by means of chemical methods, the presence in the brain of peptides induced by learning that have previously been isolated under the guidance of bioassays.

The first of these substances, named scotophobin (SP) was extracted from the brain of rats whose dark preference had been converted into dark avoidance.[5] Table I shows the originally identified pentadecapeptide and some of its naturally-occurring and synthetic derivatives that have been found to possess more or less specific behavioral activities. By densitometry of its dansyl derivative, we were able to follow the formation of SP in the brain during dark avoidance training, and study its regional distribution.[8]

Table I

Behaviorally Active Scotophobin Derivatives

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	H-Ser-	Asp-	Asn-	Asn-	Gln-	Gln-	Gly-	Lys-	Ser-	Ala-	Gln-	Gln-	Gly-	Gly-	Tyr-NH_2
B*				H-Ser-	Glu-										
C**				H-Gln-											
D***	H-Ser-				Glu-						Glu-				
E***								H-Lys-			Glu-				

* Naturally-occurring in dark avoidance-trained brain.

** By-product of the solid-phase synthesis of A; shows same activity as B.

*** Synthetic derivatives found to induce nonspecific facilitation of passive avoidance.

A second peptide, named ameletin (Am), was extracted from the brain of rats habituated to, *i.e.*, failing to be startled by a sound stimulus.[9] Using the loss of startle responses as an assay, we purified the active material. Because of the small amount of pure substance obtained, analytical work was done by the ultramicromethod described by one of us,[10] using Dns and ^{3}H-labeled Dns reagents. The following sequence was obtained: <Glu-Ala-Gly-Tyr-Ser-Lys. The hexapeptide was synthesized[11] and a chemical procedure was devised for estimating Am in the brain. This was similar to the method used for SP[8] but included further purification of the Dns derivative by free-flow electrophoresis.

This method was used for studying the formation of Am during habituation. Table II shows the results obtained in brains of rats submitted to daily sessions of habituation to the repeated sounds of an electric bell at 5 sec intervals for 1 hr and killed 24 hr after the last session. The level of Am in the brain reached its peak after 5 days of habituation and decreased gradually afterwards, in spite of continued training.

Preliminary estimates of the regional distribution of the peptide are shown in Table III. Five rats were habituated for five days and their brains were divided into the four parts shown. Unlike SP, most of which was found in the cortex, Am is about equally distributed throughout the brain, almost half of it outside the cortical areas. In one experiment the specificity of the sound stimulus was tested. Rats were habituated to the gong-like sound of a metal hammer hitting a brass plate, instead of the electric bell. No Am was found in their brains.

Two other peptides at present under investigation have been extracted from brains of goldfish trained for color discrimination. One group (BG) learned to avoid the blue light and take refuge in the green; another group (GB) was trained to the opposite behavior.[12] Extraction and purification of these peptides was done by the procedure used for SP.[5] When purification reached the stage of a single Dns spot in several solvent systems, analysis by the Dns method, after acid and enzymic hydrolysis, gave the following amino acids: Ala, Glu, Gly_2, Ile, Leu, Lys_2, Phe, Pro, Ser, Tyr, Val.

The N-terminal group, revealed after alkali treatment, was <Glu. Digestion with CP A and B resulted in the successive release of Lys, Ser, Gly, Tyr, Lys, and Leu and was stopped, presumably by Pro. Aminopeptidase M (after unblocking of the N-terminus) released Ala, Glu, Gly, Ile and Val and traces of Phe and Pro. The sequence could, however,

Table II

Estimation of Ameletin in Brains of Rats Habituated to a Sound Stimulus (Electric Bell)

Days in Training	*ng/g±S.D.*		*ng/brain±S.D.*	
0	0		0	
3	134	25.5	209	28
5	284		426	
7	229	29	388	40
10	174		296	
15	199		279	
20	132		198	

Five brains in each group. Brains were pooled except in groups 3 and 7 in which estimations were done in individual brains.

Table III

Regional Distribution of Ameletin in Brain of Habituated Rats

	ng/g	*ng/brain*	*%*
Fronto-parietal cortex	300	96	22.9
Temporo-occipital cortex	362	127	30.4
Subcortical areas	68	58	13.9
Brain stem and cerebellum	381	137	32.8

Brains collected after five days of habituation. Determinations were made in pooled portions collected from five brains.

not to be ascertained, except that Phe was preceding Pro. Further analysis of this N-terminal fragment by dipeptidyl amino peptidase, with determination of the N-terminal amino acid of each of the dipeptides obtained and comparison of these with known synthetic dipeptides, completed the structure so that, pending further verification, the following tentative

sequence can be proposed: <Glu-Ile-Gly-Ala-Val-Phe-Pro-Leu-Lys-Tyr-Gly-Ser-Lys.

The peptide extracted from the brain of GB rats has been purified and is currently being analyzed. It has approximately the same molecular size as BG. These compounds were given the name chromodiopsin (for color discrimination) followed by the initials of the color avoided and the color preferred.

The results obtained up to now suggest that acquisition of a learned behavior is associated with the formation of a peptide in the brain. The specificity of this association and its significance for the coding of neural information are being investigated in this and other laboratories.

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STRUCTURAL REQUIREMENTS FOR THE BIOLOGICAL ACTIVITY OF NEUROTENSIN, A NEW VASOACTIVE PEPTIDE

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RECENTLY WE REPORTED THE ISOLATION of a new hypotensive peptide from acid-acetone extracts of bovine hypothalami.[1] The amino acid sequence of this peptide, neurotensin (NT) has been determined to be <Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH.[2] Synthesis of this tridecapeptide by the Merrifield solid phase procedure yielded material chemically and biologically indistinguishable from the isolated native peptide.[3] NT is a potent pharmacological agent having a biological resemblance to mammalian bradykinin, *i.e.*, it produces hypotension in the anethetized rat, increases vascular permeability, induces a pain sensation, and affects the contractibility of various nonvascular smooth muscles. However, NT also possesses biological properties which may not be shared with the kinin family, *i.e.*, intravenous injection of NT into anesthetized rats brings about an increased hematocrit and a cyanosis,[1] an increase in ACTH secretion that is inhibited by pretreatment with morphine,[4] an increase in LH and FSH secretion without an effect on GH or TSH secretion,[5] and a rapid hyperglycemia.[6] This report describes some of the biological effects of several partial sequences of NT and discusses the implications of the structure-activity relationships obtained.

The partial sequences of NT were synthesized according to the Merrifield solid phase procedure,[7] and the products were purified by chromatography on Sephadexes and preparative high-voltage paper electrophoresis.[1] In the figures these partial sequences are referred to as tetra, penta, hexa, etc. according to their length. The NT-(2-13)-dodecapeptide was synthesized as well as generated from NT by

treatment with the specific enzyme, pyrrolidonecarboxylyl peptidase. The cleaved peptide was purified by high-voltage paper electrophoresis at pH 6.5 (mobility relative to lysine, 0.43). The NT-(1-10)-decapeptide was obtained by treatment of synthetic NT with carboxypeptidase A.

Results and Discussion

Whereas NH_2-terminal partial sequences of NT as large as the NT-(1-10)-decapeptide were found to be ineffectual, COOH-terminal partial sequences of 5 or more amino acids in length induced hypotension (Figure 1), hyperglycemia (Figure 2), increased cutaneous vascular permeability (Figure 3), and contracted the isolated guinea pig ileum (Figure 4). In addition for each peptide tested the threshold dose for the induction of cyanosis,[1] as well as the ED_{50} for the intensity of the cyanosis, graded 5 min after injection, was similar to that for the hyperglycemic effect. The parallel log dose-response relationships obtained for the partial sequences can be interpreted as reflecting reduced affinities for receptors but similar intrinsic activities compared to NT. Thus, the smallest peptide with full intrinsic activity is the pentapeptide, H-Arg-Pro-Tyr-Ile-Leu-OH.

The estimated potencies of the peptides tested relative to NT for the biological responses examined are summarized in Figure 5 and illustrate the essential nature of the COOH-terminal region of NT for each of these biological activities. Whereas removal of the COOH-terminal three amino acids from

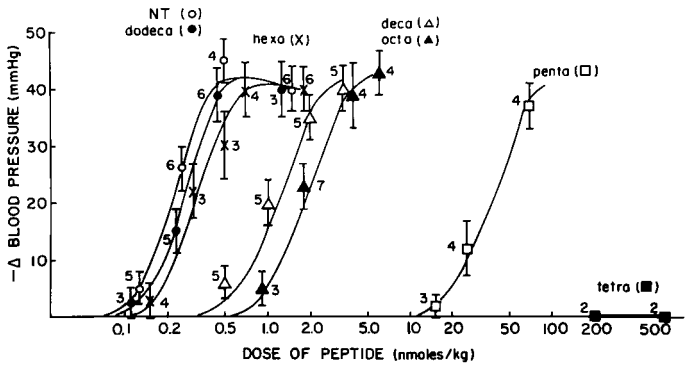

Figure 1: Hypotensive effect of NT and its COOH-terminal partial sequences. Plotted is the decrement in systemic blood pressure measured *ca* 30 seconds after tail vein injection of test samples into rats weighing 250-300 g as previously described.[1] Since NT displays acute tachyphylaxis each determination was done in a separate animal.

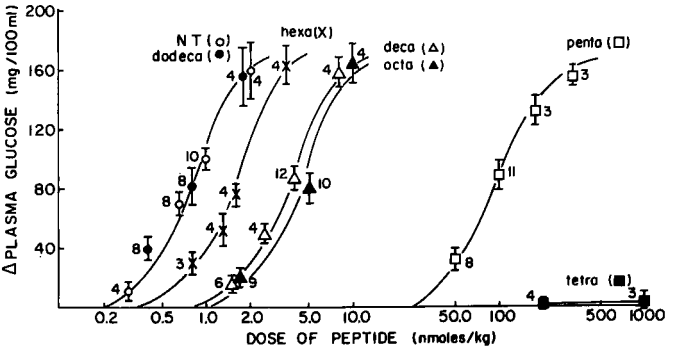

Figure 2: Hyperglycemic effect of NT and its COOH-terminal partial sequences. Plotted is the increment in plasma glucose levels measured 15 min after tail vein injection of test samples into rats weighing 250-300 g as previously described.[3]

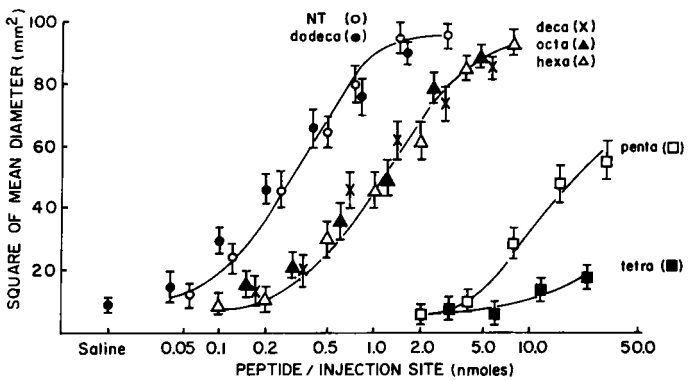

Figure 3: Increased vascular permeability produced by NT and its COOH-terminal partial sequences. Data was obtained by intradermal injection of peptides dissolved in 0.1 ml 0.85% saline at multiple sites on the shaved backs of adult guinea pigs previously injected with a 1% solution of Evan's blue dye (1.5 ml/kg). Plotted is the square (mean±S.E., n=3) of the average of the minimum and maximum diameters of each spot measured 15 min after injection.

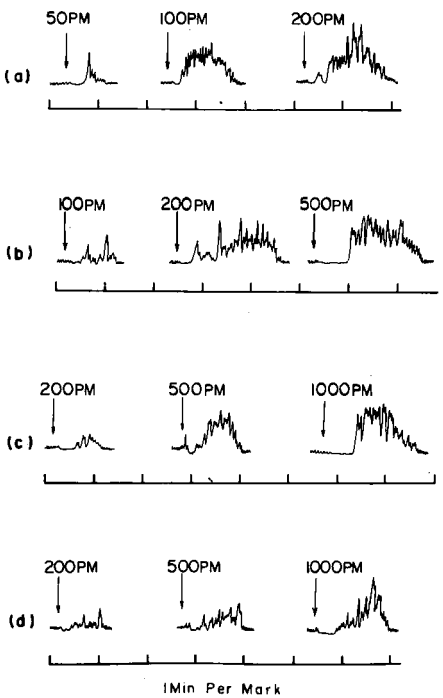

Figure 4: Contractile effect of NT and its COOH-terminal partial sequences on isolated guinea pig ileum. Freshly dissected sections of ileum were suspended in a 30-ml bath of Tyrode's solution at 37° and aerated with O_2-CO_2 (95:5).[1] The final concentrations of each peptide are given at the arrows: a, NT; b, NT-(2-13)-dodecapeptide; c, NT-(4-13)-decapeptide; d, NT-(6-13)-octapeptide.

PEPTIDE	ACTIVITY IN TEST SYSTEM (% of NT)			
	A	B	C	D
<Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH	100	100	100	100
<Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-OH	≤0.2	≤0.2	ND	ND
H-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH	80	100	100	50
H-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH	20	25	25	25
H-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH	10	20	25	20
H-Arg-Arg-Pro-Tyr-Ile-Leu-OH	60	55	25	ND
H-Arg-Pro-Tyr-Ile-Leu-OH	0.5	1.0	1.5	ND
H-Pro-Tyr-Ile-Leu-OH	≤0.1	≤0.1	≤0.3	ND
H-Tyr-Ile-Leu-OH	ND	≤0.1	ND	ND
H-Ile-Leu-OH	ND	≤0.1	ND	ND

ND- NOT DETERMINED

Figure 5: Relative biological potencies of NT and some of its partial sequences. Given is the $ED_{50}NT/ED_{50}X$ times 100% for each peptide, X for the biological activities tested; A, hypotensive effect; B, hyperglycemic effect; C, vascular permeability; D, ileal contractility.

NT destroys activity (<0.2%) as does amidation of the COOH-terminus,[8] nine amino acids must be omitted from the NH_2-terminus of NT before a biologically inactive product is obtained. The dramatic increase in apparent receptor affinity attendant upon addition of a second arginine residue to the NH_2-terminus of H-Arg-Pro-Tyr-Ile-Leu-OH argues for an important role for this residue in binding. One unexpected result was that the hexapeptide was found to be considerably more potent than the octa- and decapeptides in inducing hypotension and hyperglycemia (Figures 1 and 2). This might be attributed to an enhanced binding because of the increased positive charge (+3 instead of +2) in the neighborhood of the arginyl residues. With addition of the next four amino acids to obtain the NT-(4-13)-decapeptide biological activity does not increase. However, a doubling of activity accompanies the addition of the tyrosyl and leucyl residues to obtain the NT-(2-13)-dodecapeptide, suggesting a role for these residues in enhancing binding. A hypothetical model for NT-receptor interaction is depicted in Figure 6. Strong, probably ionic interactions of the receptor with the negatively charged COOH-terminus and the positively charged arginyl side chains are pictured to contribute strongly to the binding energy. A large part of the intrinsic activity of the peptide is likely to derive from interactions of the side chains of the COOH-terminal four amino acids with a hydrophobic site on the receptor. The

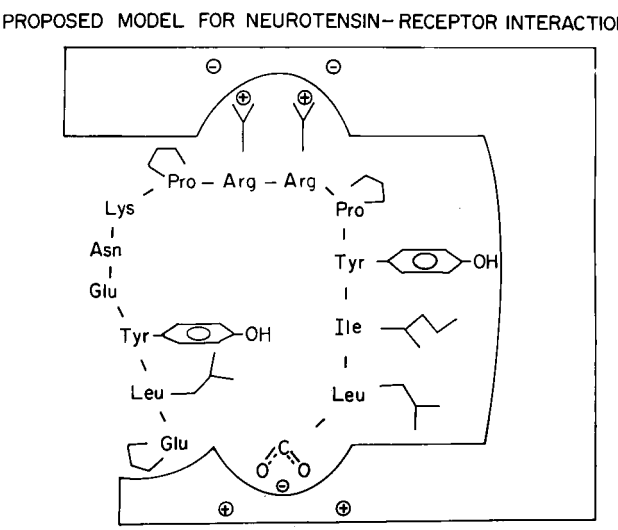

Figure 6: Proposed model for a NT-receptor interaction.

NH_2-terminal portion of NT might possibly serve to favor the proper conformation for the rest of the molecule, Leu^2 and/or Tyr^3 being especially important in this regard. The fact that the relative activities of these peptides are very similar in the various test systems investigated suggests that, if there are indeed separate receptors for each of these effects, they have rather similar requirements for the binding and action of NT.

It is interesting that the peptide Xenopsin, <Glu-Gly-Lys-Arg-Pro-Trp-Ile-Leu-OH recently isolated from the skin of the frog, Xenopus laevis,[9] bears a striking resemblance to the COOH-terminal region of NT while lacking a corresponding NH_2-terminal portion. Xenopsin induces hypotension, cyanosis, and hyperglycemia in rats with *ca* 20% potency relative to NT which is similar to that of the NT-(6-13)-octapeptide. Assuming that the <Glu and the Gly residues do not contribute strongly to the binding, these results show that the substitution of a Lys for Arg^3 and a Trp for Tyr^6 does not dramatically affect the biological activity of NT-(6-13)-octapeptide. This apparent phylogenetic relationship and the data presented here argue strongly that the major determinants of the biological activity of NT reside primarily in its COOH-terminal region.

Acknowledgments

We wish to thank Dr. Robert Fellows, Departments of Physiology and Medicine, Duke University Medical Center, Durham, North Carolina for his gift of pyrrolidonecarboxylyl peptidase and John Dryer for his excellent technical assistance.

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SECTION VI

NEUROHYPOPHYSEAL HORMONES AND NEUROPHYSIN PROTEINS

DEDICATED TO
DR. JOSEF RUDINGER

REMEMBERING JOSEF RUDINGER

Joseph S. Fruton. Yale University, New Haven, Connecticut.

PROFESSOR WALTER HAS ASKED ME to open today's proceedings with some words about our departed friend Josef Rudinger, who died on April 30th at the tragically early age of 50.

At the time of his death, Dr. Rudinger was Professor at the Eidgenössische Technische Hochschule in Zürich, where he was a member of the Institute for Molecular Biology and Biophysics. Until 1968, he had been associated with the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Science in Prague. It was at Prague that he did his outstanding work in peptide chemistry. Among his many important achievements perhaps the best known are his studies on synthetic analogues of oxytocin, and it is appropriate therefore that we should pay tribute to his memory at the session dealing with neurohypophyseal hormones.

Professor Rudinger's work on structure-function relationships of peptide hormones was only a part of his notable contributions to the advancement of the science and art of peptide chemistry. I need only recall his outstanding studies on the use of the tosyl group in the protection of amino groups, on the azide method of coupling, and on the synthesis of peptides containing residues of glutamine or diamino acids. These were all pioneering efforts that have influenced the development of peptide synthesis during the past twenty years. At this Fourth American Peptide Symposium it is well to remember also that the first European Peptide Symposium was held in 1958 in Prague and that it had been organized by Professor Rudinger. What began rather modestly (there were 27 participants at the

1958 meeting) soon developed into the principal international organization devoted to the promotion of peptide chemistry, and Professor Rudinger's contributions to its success were evident at each meeting. Any of you who have attended any of the thirteen European Peptide Symposia held thus far will recall how much he enriched and enlivened the proceedings with incisive comments, always offered with courtesy and good humor. We were fortunate in having him as a participant in the Third American Peptide Symposium three years ago, and you will remember the pleasure we all derived from his company at the scientific sessions and at the social gatherings. At these symposia, we saw him not only as a great scientist, but also as a citizen of the world, for his charm, his integrity, his easy command of languages, all helped to build lasting friendships among peptide chemists from countries with widely different political regimes.

For many of us, the loss of Josef Rudinger deprives us of a cherished friend. For all of us it means that a great chemist and scientific statesman has been halted prematurely in a career that could have done so much more for the advancement of our area of science and for the promotion of international good will.

MEMBRANE EFFECTS OF NEUROHYPOPHYSEAL HORMONES AND RELATED STUDIES WITH PARATHYROID HORMONE

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and

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NEUROHYPOPHYSEAL HORMONES (NHHs) act on the contractility of uterine, oviductal and vascular smooth muscle and the myoepithelial cells of the mammary gland. NHHs, and/or their derivatives, influence memory, affect and behavior by action on the central nervous system and metabolism by action on the liver and adipose tissue. However the principal and unequivocally physiologic role of these peptides, particularly of the vasopressins, is the facilitation of water conservation by an action on the permeability of NHH-sensitive epithelia in the vertebrate kidney and on functionally-related epithelia in amphibian bladder and skin. Almost certainly the primary event in all of the above noted NHH actions is the selective attachment of the hormone to the plasma membranes of the target cell. Therefore, in a fundamental sense, all of these actions qualify for consideration in any general discussion of membrane effects of neurohypophyseal hormones. However, in this report, we are limiting our considerstion only to those membrane effects -- on the collecting duct of

the mammalian kidney and on the toad urinary bladder -- that are related to the antidiuretic (hydroosmotic) action of these peptides.

Specifically we are reviewing recent and ongoing studies of (1) the phenomenon of "receptor reserve" in NHH action on membrane permeability in the toad bladder, (2) the localization of NHH-stimulated adenylate cyclase and cyclic AMP-dependent protein kinase in the plasma membrane envelope of the collecting duct epithelial cells of the mammalian kidney (and related observations pertinent to parathyroid hormone action on the mammalian proximal nephron), (3) the possible function of microtubules (and microfilaments) at a distal step in the hydroosmotic action of NHHs, (4) the separation of, and NHH action on, the mitochondria rich and granular cells of the toad bladder, and (5) factors other than hormone degradation that modulate NHH action.

Receptor Reserve

While studying the inhibitory effect of the prostaglandin, PGE_1, on the NHH-induced increase in permeability of the toad bladder, we found that although PGE_1 proved to be a non-competitive inhibitor for both oxytocin and the analog [2-O-methyltyrosine]-oxytocin (2-O-Me-OT), it was still possible for oxytocin (but not 2-O-Me-OT) to elicit a maximal hydroosmotic response from the bladder in the presence of high concentrations of PGE_1. On the basis of this observation we suggested that oxytocin (but not 2-O-Me-OT) at "receptor saturating" concentrations generates a stimulus in excess of that required to elicit a maximal response and, accordingly, that oxytocin (and other neurohypophyseal hormones with high intrinsic activities) is (are) capable of evoking a maximal hydroosmotic response with only partial occupation of the number of receptors available -- a phenomenon referred to as "receptor reserve".[1]

This concept was supported by studies in the toad bladder of the intrinsic activity of a series of neurohypophyseal hormonal peptides evaluated by measuring a response at an early step (adenylate cyclase activation) and at the final step (hydroosmosis) in the reaction sequence which constitutes the action of NHH on membrane permeability.[2,3] The relative stimulatory potencies of a set of agonists (natural NH hormones and analogs) were tested on an adenylate cyclase membrane preparation and also in

the intact bladder. We found that the intrinsic activities of these peptides, *i.e.* their ability to evoke a maximal response, differed greatly when measurements of adenylate cyclase activation in the broken cell system and of the hydroosmotic response in the intact system were compared. Specifically, each of the peptides at saturating concentrations elicited a different maximal response in the cyclase system whereas most of these same peptides elicited the same maximal response in the intact system. Thus, when the intrinsic activity of the peptide which elicited the greatest maximal response in the cyclase system -- arginine vasopressin (AVP) in our initial study[2] -- was defined as 100%, the other peptides were seen to have exhibited variably lower intrinsic activities in the cyclase system. On the other hand, most of the peptides studied were found to have exhibited maximal (100%) intrinsic activity in terms of the final hydroosmotic effect in the intact system. In fact, it was only when intrinsic activity, as measured in the cyclase system, fell below 30% (of the intrinsic activity of AVP) that we began to see any decrease whatever in intrinsic activity as measured in the intact system. In other words, it was necessary for the maximal response capacity at the early cyclase step to fall by more than 70% before we were able to detect any decrease in the maximal response capacity at the final step in hormone action. Accordingly most of the neurohypophyseal peptides that we have studied to date can stimulate adenylate cyclase to a much greater level of activity than is required to elicit a maximal response in the final effector system. These findings, therefore, supported our proposal that the bladder has a "receptor reserve" with respect to NHH-induced changes in membrane permeability. More generally these findings indicated the need to include the parameter of receptor reserve in the application of current receptor theory to the analysis of the mechanism of action of any hormone (or other agent) when (a) the target tissue response involves a chain of sequential events and (b) the experimental data have been derived from observations on an intermediate or late event in the chain.

We do not yet know whether the receptor reserve phenomenon can be attributed to an excess of actual receptor-cyclase units, whether it is a consequence of the broad range over which adenylate cyclase can be activated, whether it depends on a mechanism for altering the efficiency of the process which couples hormone-receptor interaction

to cyclase activation or on a mechanism affecting phosphodiesterase activity, or on any combination of these and other factors. Although it has been shown that exposure of the toad bladder to progressively increasing concentrations of NHH leads not only to excessive cyclase stimulation but also to an accumulation of cyclic AMP in the tissue which exceeds the requirement for maximal stimulation of transepithelial Na^+ transport and water flow,[4] additional studies are still needed to gain further insight into the nature of the reserve phenomenon. We continue, therefore, to emphasize that there are no mechanistic implications in our use of the term "receptor reserve" which we employ only descriptively to indicate that early steps in hormone action -- "at" or "close to" the initial hormone-receptor interaction-- often manifest much more activity than can be translated into the final physiological effect. Thus the responsive system is provided with a margin of safety in the event (a) of defective function developing at an "early" step in hormone action, or (b) of a decline in hormone production, release or transport to target cells.

Localization of Hormone-Sensitive Adenylate Cyclase and Cyclic AMP-Dependent Protein Kinase in Renal Cortical and Medullary Epithelial Cell Membranes

To discern the nature of the molecular events underlying the hormonal regulation of a transepithelial transport process it is necessary to separate the plasma membrane of the polarized epithelial cell into its functionally different luminal and contraluminal components. Such separations were attempted with epithelial cells derived from the renal cortex where brush border microvilli serve as a morphological marker not only for the proximal convoluted tubule as a whole but also for the luminal (apical) component of the plasma membrane envelope of the proximal tubular epithelial cells and Na^+-K^+-ATPase serves as a marker for the contraluminal (basal-lateral) plasma membrane.[5-7] All brush border fractions isolated by density gradient centrifugation have been found to be contaminated with Na^+-K^+-ATPase-containing contraluminal membrane. However with the application of preparative free-flow electrophoresis such contamination was reduced to extremely low levels and it was possible to obtain well defined luminal and contraluminal membrane fractions from epithelial cells of the renal cortex.[7] Because it had been established

that parathyroid hormone(PTH)-stimulated adenylate cyclase was largely confined to the renal cortex[8-12], we decided to take advantage of the methodology that had been developed for membrane separation in the renal cortex and focus our efforts on the suborganellar localization of PTH-cyclase before we turned to the renal medulla where NHH-stimulated cyclase in preferentially localized. Accordingly we prepared partially purified plasma membranes from renal cortical cells by differential centrifugation in an isotonic sucrose medium and then resolved these membranes into luminal (brush border microvilli) and contraluminal (basal-lateral membrane) fractions in a Hannig FF4 free-flow electrophoresis apparatus.[13,14] The fractions were assayed for the activities of alkaline phosphatase (marker enzyme for luminal membranes), Na^+-K^+-ATPase (marker enzyme for contraluminal membranes), cyclic AMP-dependent protein kinase, cyclic AMP-dependent phosphoprotein phosphatase and adenylate cyclase in the absence of hormones and in the presence of PTH, ADH, epinephrine and calcitonin. It can be seen in Figure 1 that the adenylate cyclase of these membrane preparations responds almost exclusively to PTH -- and also that this PTH-stimulated cyclase activity is largely confined to the contraluminal membrane fraction. It should also be noted that the very low cyclase response of all fractions to ADH shows that the membranes in these fractions are derived primarily from proximal tubular cells rather than from cells of the distal tubule or cortical collecting duct.

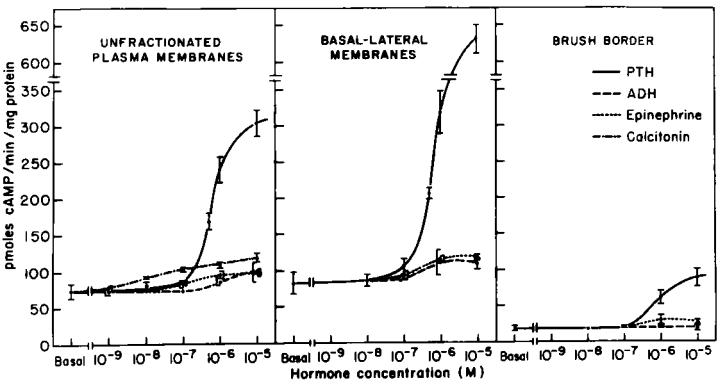

Figure 1: Effects of hormones on adenylate cyclase activity in unfractionated plasma membranes, basal-lateral membranes and brush border membranes.[13] Each point represents the mean ± S.E. of triplicate determinations using five separate preparations.

In Figure 2 it can be seen that the protein kinase activity (determined by measuring the transfer of the terminal phosphate of ATP to membrane protein) and alkaline

Figure 2

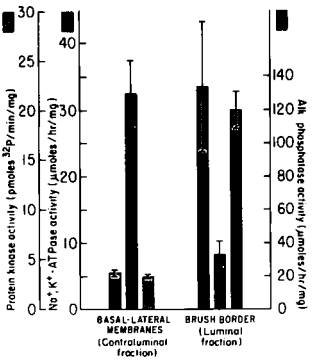

Figure 3

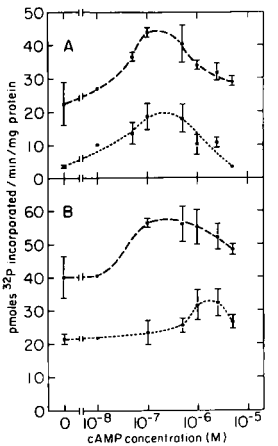

Figure 2: Specific activities of intrinsic protein kinase, Na^+-K^+-activated ATPase and alkaline phosphatase in basal-lateral membranes and brush border microvilli.[14] Results represent the mean ± S.E. of triplicate determinations in each of three separate preparations.

Figure 3: A. Effect of cyclic AMP on intrinsic protein kinase activity of brush border microvilli (•— — — —•) and basal-lateral membranes (•----------•).[14]
B. Effect of cyclic AMP on the phosphorylation of brush border microvilli (•— — — —•) and basal-lateral membranes (•----------•) in the presence of cytosolic protein kinase.[14]
Results represent the mean ± S. E. of triplicate determinations using three separate preparations.

phosphatase activity, unlike Na^+-K^+-ATPase activity, are very much greater in the luminal than in the contraluminal membrane fraction. The cyclic AMP dependence of the protein kinase associated with these fractions is shown in Figure 3A.

The *relative* cyclic AMP dependence of the enzyme (*i.e.*, the percent increase above basal activity) is greater in the contraluminal than in the luminal fraction. However, at all concentrations of cyclic AMP, the absolute level of phosphorylation of the luminal fraction greatly exceeded that of the contraluminal fraction. Figure 3B illustrates the effect of cyclic AMP on the phosphorylation of luminal and contraluminal membranes when proximal tubular cytosolic protein kinase is added to the system. It can be seen that the inclusion of this soluble kinase in the system boosted the baseline phosphorylation of both membrane fractions substantially -- but the cyclic AMP responsiveness of the contraluminal membrane was reduced -- and the apparent maximal phosphorylation of the luminal membrane was increased by only 25% above the level of self-phosphorylation. In other words, the phosphorylation due to the intrinsic kinase of the luminal membrane was bettered by only 25% when cytosolic kinase was added to the system. On the basis of this finding we have concluded that the luminal membrane contains the predominant cyclic AMP-dependent self phosphorylating system of the PTH-sensitive proximal tubular cell and that this system consists of a membrane-bound kinase and its membrane-bound substrate.

It is of interest that *in vivo* physiological responses correlate with our *in vitro* enzyme localizations, as indicated in Figure 4. The lower half of Figure 4 shows the contraluminal localization of PTH-stimulated cyclase and the luminal localization of cyclic AMP-dependent kinase -- revealed by the electrophoretic distribution of these enzymes. The upper portion of Figure 4 shows the results of micropuncture experiments carried out by Karl Baumann and co-workers[15] who used the shrinking droplet method (to measure the inhibitory effect of PTH on the reabsorption of isotonic fluid from the proximal tubule lumen) in combination with peritubular capillary perfusion so that PTH or dibutyryl-cyclic AMP could be applied at either the contraluminal or the luminal side of the proximal tubule. It can be seen that peritubular, *i.e.* contraluminal, application of PTH evokes a much greater inhibition of isotonic volume flux than does intratubular, *i.e.* luminal, application. Conversely, in the case of cyclic AMP, intratubular application evokes a greater inhibition than does peritubular application. Thus these *in vivo* responses support the conclusion, drawn from our *in vitro* studies, that PTH-stimulated adenylate cyclase is preferentially, if not exclusively, distributed in the plasma membrane of

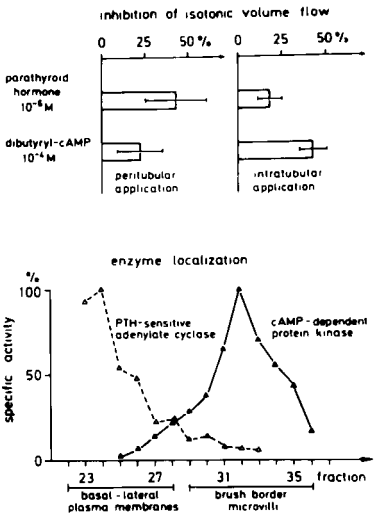

Figure 4: See text for explanation.

the contraluminal pole of the hormone sensitive cell, whereas nucleotide-dependent kinase is preferentially distributed in the luminal pole of the cell. Furthermore, these findings indicate that, although PTH has access to the luminal as well as the contraluminal cell surface *in vivo*, its action is initiated at the latter locus.

In principle, the assymetric phosphorylation of the tubular cell membrane might directly underlie all of the effects of PTH on renal transport processes -- or such an alteration might account for the regulation of only one transport system, for example, the system responsible for phosphate excretion -- in which case the other PTH effects may be due to action of the hormone at another locus in the cell. For example, it is possible that a PTH-induced increase in cytosolic calcium concentration may inhibit the Na^+-K^+-ATPase and the resulting decrease in sodium reabsorption may, in turn, inhibit the sodium-linked transport of calcium and glucose. However, as far as our localization studies are concerned, we would like at this time only to suggest that the mechanism of action of PTH on the proximal tubule cell involves an asymmetrical distribution of enzymes in its plasma membrane.

With our PTH studies serving as guidelines we turned our attention back to the neurohypophyseal hormones and their renal target tissue, specifically the papillary

collecting duct cell. In the collecting duct, unlike the proximal tubule, there is little morphologic evidence of polarity and, therefore, we had a lower expectation for successful resolution of luminal and contraluminal membranes on free-flow electrophoresis. However, to our surprise, we were able to resolve the collecting duct cell plasma membrane even more effectively than the plasma membrane of the cells of the renal cortex.[16] In these studies, the luminal and contraluminal localization of HCO_3^--ATPase and Ca^{++}-ATPase, respectively, had particular advantage in the identification of the luminal and contraluminal plasma membranes of the collecting duct cells -- because the classical markers, alkaline phosphatase and Na^+-K^+-ATPase, are present at very low concentrations.

Figure 5 shows the separation of HCO_3^--ATPase and Ca^{++}-ATPase after free-flow electrophoresis of plasma membranes isolated from cells of the renal papillary collecting duct. It should be noted that these markers are not

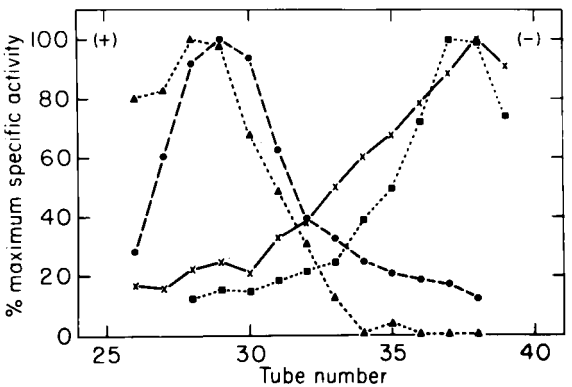

Figure 5: Separation of papillary plasma membranes by free flow electrophoresis.[16] The distribution of HCO_3-ATPase (▲·····▲), intrinsic protein kinase in the presence of μM cAMP (•———•), adenylate cyclase in the presence of μM arginine vasopressin (×———×), and Ca-ATPase (■·····■) is given as a percent of the highest specific activity found for each enzyme. Tubes 25 to 30 were combined to obtain fraction I (luminal membranes); tubes 35 to 40 were combined to obtain fraction II (contraluminal membranes).

soluble enzymes which migrate per se but rather it is the membrane fragments to which these enzymes are bound that are migrating differentially in the electric field.

As in the proximal tubule study, the fraction with the highest specific activities of our marker enzymes were collected separately and evaluated for hormone-sensitive adenylate cyclase activity and cyclic AMP-dependent protein kinase activity.

Figure 6 shows adenylate cyclase activity in the presence and absence of the antidiuretic hormone, arginine vasopressin (AVP). It can be seen that the cyclase activity of the luminal membrane has a low basal activity and

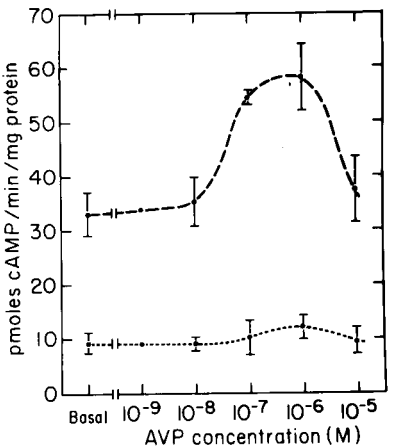

Figure 6: Effect of arginine vasopressin (AVP) on the adenylate cyclase activity of contraluminal membranes (---) and luminal membranes (•••).[16]

shows virtually no response to hormone whereas the cyclase activity of the contraluminal membrane has a four-fold greater basal activity and an unequivocal response to ADH. Thus NHH-stimulated adenylate cyclase appears to be localized almost exclusively in the contraluminal membrane of the ADH-sensitive collecting duct epithelial cell.

In contrast to the contraluminal localization of NHH-sensitive adenylate cyclase, the cyclic AMP-dependent protein kinase activity of the collecting duct epithelial cell is confined to the luminal membrane (Figure 7). It is

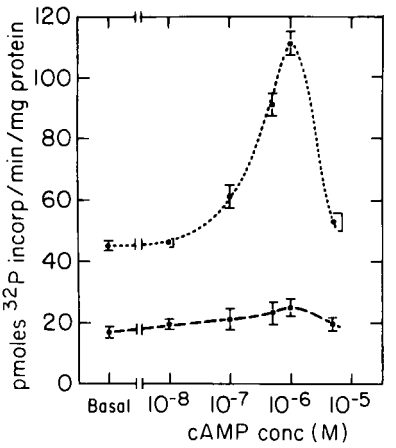

Figure 7: Effect of cAMP on intrinsic protein kinase activity of luminal membranes (•••) and contraluminal membranes (---).[16]

apparent, therefore, that in papillary collecting duct cells only the luminal membrane contains a cyclic AMP-dependent self-phosphorylating system consisting of a membrane-bound protein kinase and its membrane-bound substrate.

In the course of these studies on plasma membrane-bound protein kinase, we also investigated a soluble protein kinase which we extracted from the cytosolic fraction of papillary epithelial cells. We found that this kinase did not substantially increase the cyclic AMP-dependent phosphorylation of the luminal membrane. The marked difference in membrane phosphorylation effected by the membrane-bound kinase, in contrast to the cytosolic kinase, suggests that enzyme-substrate proximity--rather than enzyme-substrate specificity--is the critical feature of cAMP-mediated phosphorylation of the luminal membrane of the NHH-sensitive collecting duct epithelial cell.

We are tempted to speculate that the NHH-induced increase in luminal membrane permeability results from a cyclic AMP-mediated increase in the level of phosphorylation because we can identify an intrinsic cyclic-AMP dependent protein kinase in the luminal membrane but we have not as yet found cyclic AMP-dependent phosphoprotein phosphatase at this locus. However others [17-21] have reported activation by cyclic AMP of a membrane-bound phosphoprotein phosphatase acting on a specific protein (protein D) in

a membrane fraction from toad bladder. The occurrence of a similar phenomenon in renal membranes cannot be excluded at this time because it is likely that dephosphorylation of some specific phosphoprotein(s) may be masked by a predominating concurrent phosphorylation of other membrane proteins. If such masking occurs it is possible that a cyclic AMP-dependent phosphorylation is required to activate the specific phosphoprotein phosphatase for which protein D serves as substrate.

Based on the enzyme localizations reported above, we are pursuing the following working hypothesis for the mechanism of action of PTH and ADH on their respective proximal and distal target cells in the mammalian kidney: (a) Hormone-receptor interaction and activation of adenylate cyclase in the contraluminal membrane; (b) Generation of cAMP and its diffusion to the luminal membrane; (c) Activation of luminal membrane-bound protein kinase and possibly phosphoprotein phosphatase; and (d) Change in the state of phosphorylation of the luminal membrane which leads through one or more subsequent events to an increase in the tubular rejection of phosphate in the case of PTH--and to an increase in permeability to water in the case of ADH.

Irrespective of how the elements of this working hypothesis withstand the test of time, it should be noted that the method of free-flow electrophoresis provides the basis for the development of vesicle systems that reveal the individual functions of the contraluminal and luminal poles of transporting epithelial cells. More generally, the approach employed in this study for the resolution of the plasma membrane into basal and apical components should find application in a variety of other efforts to elucidate the biochemical basis of the action of hormones, drugs and disease states on water and solute transport processes.

The Possible Function of Microtubules (and Microfilaments) in the Hydroosmotic Action of Neurohypophyseal Hormones

Colchicine (C), vinblastine (V) and podophyllotoxin (P), antimitotic compounds which bind to microtubule subunit protein (tubulin) and prevent the assembly of microtubules -- and cytocholasin B (CB) which disrupts microfilaments -- have been shown to inhibit the hydroosmotic but not the natriferic action of NHH and cyclic AMP on toad bladder.[22] Also, C, V, and Ca^{2+} have been shown to inhibit NHH-induced antidiuresis in the rat[23] and to prevent the polymerization of tubulin isolated from bovine renal medulla.[24] However lumicolchicine, an analog of colchicine that lacks antimitotic

activity and does not bind to tubulin, has no inhibitory action on the NHH- or cyclic AMP-induced hydroosmotic response of the amphibian bladder or on the antidiuretic response of the mammalian kidney. Moreover the reversibility, concentration-dependence and time-dependence is similar for inhibition by C, V and P of NHH-induced water movement in the toad bladder and of the interactions of C, V and P with microtubular protein *in vitro* and microtubular systems *in vivo*.[22]

Based on the foregoing findings it has been suggested[22-24] that microtubules -- and possibly microfilaments -- participate in the hydroosmotic action of NHHs at a step after the generation of cyclic AMP, but that these organelles are not involved in the natriferic action of NHHs. In addition, because NHHs and cyclic AMP stimulate exocytosis in the toad bladder epithelium[25] and because in other systems the mechanism of exocytosis involves microtubular protein, and possibly microfilaments, it is reasonable to suppose that these organelles may participate in the hydroosmotic action of NHHs through their involvement in the mechanism for release of secretion granules from the bladder epithelial cells. This suggestion[22,25] is based on the idea that the addition to the apical cell surface of secretory material or the incorporation of the secretion granule membrane into the luminal (apical) membrane of the effector cells may be responsible for the permeability change.

The following alternative or additional possibilities have been suggested: (a) microtubules, and possible microfilaments, may play a role in the hydroosmotic action of NHHs via an effect of cyclic AMP on a directional cytoplasmic streaming process;[22] (b) changes in the structure (*e.g.* by cyclic AMP-dependent phosphorylation) of plasma membrane-associated tubulin or juxtaplasmalemmal components of microtubules, and possible microfilaments, might alter the permeability properties of the plasma membrane;[23] (c) cyclic AMP might affect the state of polymerization and thereby the permeability of a submembrane layer of microtubular protein;[23] or (d) there may be no interaction between cyclic AMP and microtubular protein or microfilaments, the role of the microtubule and/or microfilament system(s) being merely to provide a structural framework for a functionally effective fixation and orientation of cyclic AMP-dependent enzymes and their substrates and modulators. Thus, by disrupting this framework, the cyclic AMP-protein kinase-phosphoprotein phosphatase-etc. system can be rendered inoperative in the hormonal target cell.[23]

Separation of Mitochondria Rich and Granular Cells of the Toad Bladder; Interaction of NHH and Aldosterone with the Separated Cells

By layering the disaggregated toad bladder mucosal cells over discontinuous gradients of Ficoll, it was possible to obtain highly enriched populations of the two major cell types, mitochondria-rich (MR) and granular (G).[26]

These separated cells retain their morphologic identity and exhibit brisk consumption of oxygen.[27] When these separated mucosal cells were treated with oxytocin, the MR cell but *not* the G cell exhibited an increase in cyclic AMP content. This finding suggests that the initial steps in NHH action on the toad bladder occur in the MR cell and that cyclic AMP generated in this cell might be transferred to adjacent G cells.

Aldosterone, the natural mineralocorticoid of the toad, enhances the natriferic and hydroosmotic responses of the toad bladder to vasopressin[28-30] as a consequence of the larger increment of intracellular nucleotide induced by a given dose of NHH in the steroid-treated tissue.[31] This synergism of NHH and steroid hormones suggests that their loci of action are in close proximity. We therefore examined the binding of aldosterone by the two major cell types and found that ^{3}H-aldosterone was displaceably bound by the MR but *not* by the G cell, indicating that the initial action of mineralocorticoid as well as NHH occurs in the MR cell[32] although both cell types contain specific receptors for corticosterone, the natural glucocorticoid of the toad.

It was proposed that aldosterone induced the synthesis of specific protein(s) that mediate(s) sodium transport, *e.g.*, vasopressin receptors, ATPase subunits, or sodium permease. Using a double-label isotope method, we examined the effects of aldosterone upon the synthesis of protein in the tissue and found at least two aldosterone-induced proteins in the soluble fraction of the MR cell.[33] We also examined the membrane fractions of the separated cells and isolated an aldosterone-induced lipoprotein from the plasma membranes of MR cells.[32] This protein, which is not found in the granular cell, is currently being examined for binding sites for NHH, amiloride and ouabain. The presence of a membrane particle unique to the MR cell has recently been observed by Wade (personal communication).

Although the findings outlined above indicate that the early steps in NHH and mineralocorticoid action take place in the MR cell, it is likely that the G cell, or both cell types, are involved in the final effects (increased osmotic water movement, increased sodium transport) because (a) the area of the flask-shaped MR cell exposed to the urine is probably too small to accomodate the observed fluxes, (b) there is direct evidence for water movement into the G cell during NHH-stimulated hydroosmotic flux[34] and (c) recent freeze-fracture studies of G cell membranes have revealed a clustering of membrane particles correlated with the physiologic response of the tissue.[35]

Factors other than Hormone Degradation that Modulate NHH Action

Divalent Ions

Either depletion or high concentrations of calcium reduces the response to NHH of adenylate cyclase membrane preparations but neither condition alters the binding of ^{3}H-NHH to these preparations.[36,37] There is some disagreement concerning the relative effects of deficiency and excess of calcium on hormone binding and on basal vs. hormone-stimulated cyclase activity; however it seems generally accepted that both low and high calcium concentrations decrease the NHH-induced activation of adenylate cyclase following receptor occupation. In the intact toad bladder calcium (20 mM) blocks the hydroosmotic but not the natriferic response to NHH; however this concentration of calcium does not block either the hydroosmotic or the natriferic responses to cyclic AMP.[38,39] These findings not only served to localize the inhibitory action of calcium at the receptor-cyclase level but also led to the concept that natriferic and hydroosmotic responses are mediated by different cyclic AMP pools within the hormone-sensitive cell. A similar dissociation of effects is seen when the toad bladder is treated with PGE_1.[40]

Manganese, like calcium inhibits the response of NHH-sensitive adenylate cyclase to NHH.[41] Magnesium increases basal enzyme activity to a greater degree than NHH-stimulated activity; thus there is a decrease in the relative stimulation of the enzyme by NHH.[42]

Monovalent Ions

The reactivity of NHH-sensitive adenylate cyclase is increased by sodium or potassium concentrations below 100 mM and inhibited by higher concentrations;[43,44] lithium inhibits both NHH-sensitive cyclase[44] and vasopressin-induced antidiuresis.[45] Of particular interest is a series of recent observations implicating some aspect of the sodium transport process, the sodium transport pool or the concentration of intracellular sodium as a feedback regulator of the cyclic AMP content and natriferic response of the hormone-stimulated toad bladder.[46-48] These observations are analogous to the "dilution desensitization" reported by Eggena[49] and to earlier studies[50-54] of autoinhibition of the NHH-induced hydroosmotic response. In these earlier studies it was found that the vasopressin-mediated flux across the toad bladder not only consititutes a final effect of the hormone but also participates in the overall set of processes which limit the duration and decrease the magnitude of the target organ's response.[52,53] In preliminary studies of the mechanism of this self-induced resistance to NHH in the toad bladder, we have found that the modulating (inhibitory) signal exerts its effect at a late step in the hormone-initiated reaction sequence, namely, after the cyclic AMP-dependent alteration in the state of phosphorylation of a possible preeffector or effector element in the luminal (apical) diffusion barrier(s). Similar studies are now in progress to localize the site of modulation of a number of other natural and pharmacological factors[30,52] that influence the reactivity of the toad bladder to NHH.

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INTERACTIONS OF NEUROHYPOPHYSEAL HORMONAL PEPTIDES WITH ISOLATED RAT FAT CELLS

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THE CONVERSION OF GLUCOSE to CO_2 in adipose tissue and lipocytes is under the control of a number of hormonal effectors including adrenalin, glucagon and insulin.[1] The amplification of the basal level of carbohydrates oxidation by those effectors, so called "insulin-like" action, can also be elicited by oxytocin, [Arg^8] or [Lys^8] vasopressin and related nonapeptide analogs.[2] We have investigated in details this metabolic action using isolated rat fat cells and "ghosts" prepared from those cells. We show that this property of the nonapeptide hormones can apparently be correlated with the existence of well-defined oxytocin receptors localized on the plasmatic membrane. These receptors seem to be independent from the previously described insulin receptors[3] and from the adenylate cyclase activities of these cells. This system provides a good example of an hormone action which is not mediated through a modification of the basal level of both cyclicAMP and cyclicGMP.

Methods

Suspension of rat lipocytes were prepared according to the method of Rodbell modified according to Gliemann.[4] The cell concentration was evaluated by triglyceride content as measured by the Lambert and Neish method.[5] Ghosts of the rat fat cells were obtained according to Avruch and Wallach.[6] The conversion of 1-^{14}C glucose to $^{14}CO_2$ by the

lipocytes preparation was determined using the technique described in [4]. Tritiated oxytocin (14 to 30 Ci/m mole) was used in the binding studies. This radioactive hormone had biological and chemical properties undistinguishable from the unlabelled peptide used as reference.[7] The standard binding assays were performed according to the filtration technique of Cuatrecasas.[3] The adenylate cyclase activity was evaluated by the conversion of α^{32}-P ATP into labeled cyclicAMP separated by means of the Ramachandran technique.[8] CyclicAMP and cyclicGMP concentrations in the cells were measured using the protein binding assays.[9,10]

Characterization of the Oxytocin Receptors

Oxytocin Activates the Basal Glucose to CO_2 Oxidation by the Fat Cells

For concentrations ranging from 0.1 n*M* to 0.1 μ*M*, oxytocin was efficient in enhancing the basal level of glucose metabolism of these cells (Figure 1). The half

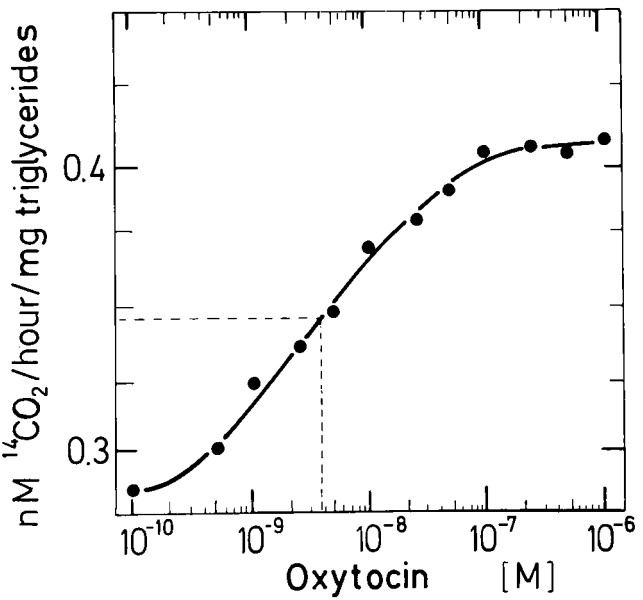

Figure 1: Ability of oxytocin to enhance the rate of glucose oxidation by isolated rat fat cells.

maximum response was attained with oxytocin concentrations close to 5 n*M*, and a total increase of 60% of the basal level of respiration was produced by 1 μ*M* oxytocin.

Oxytocin Binds to the Rat Fat Cells

Under conditions where the cells appear to be sensitive to hormones the binding of [^{3}H]oxytocin was evaluated. This appear to be a biphasic process and two linear binding isotherms are clearly apparent when the data are plotted according to Scatchard (Figure 2). They indicate the

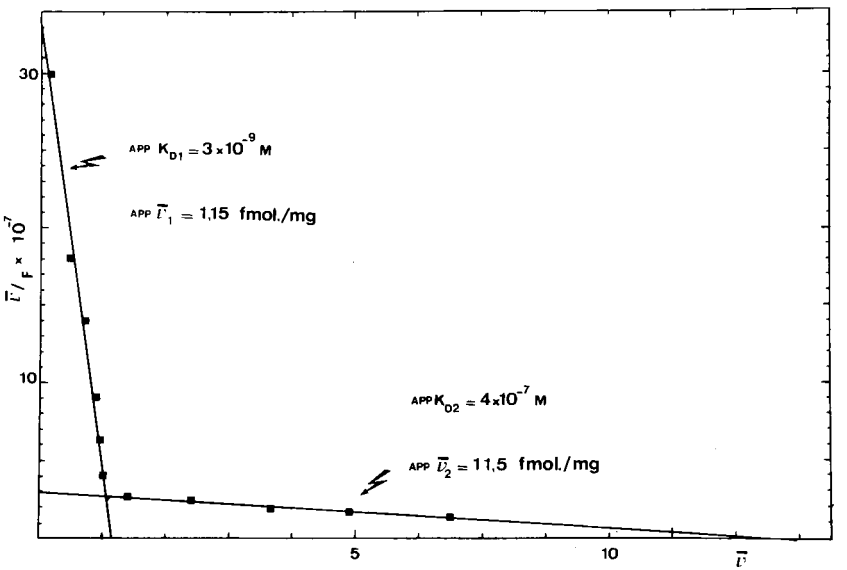

Figure 2: Specific binding of [^{3}H]-oxytocin (14 Ci/mmole) to isolated rat fat cells: results plotted according to Scatchard. For each concentration of [^{3}H]-oxytocin studied, control incubations were performed in the presence of a 10^3 fold excess of unlabelled oxytocin. The non-specific binding (not represented here) has been substracted from the total binding. $\bar{\nu}$ = f. mol. of oxytocin bound/mg of triglycerides. F is the free oxytocin concentration.

existence of two classes of binding sites with different properties: the first one with a high affinity (3 n*M*) and a low capacity (~3.10^4 sites per cell); the second category which could not be saturated under the conditions used, exhibits a rather low affinity (0.4 μ*M*) but a much higher capacity, It should be noted that the high affinity binding sites are half saturated by oxytocin concentrations comparable with those needed to produce half the maximal biological response.

Binding of Oxytocin to the Fat Cells is a Time-Dependent Process

At 30° equilibrium was reached in 8 minutes (Figure 3). When the ligand radioactivity was displaced by a twenty fold dilution of the medium, the dissociation of the hormone-receptor complex appeared as a first order kinetic process (k_1 = 2.3 x 10^8 $min^{-1}.M^{-1}$; k_{-1} = 0.47 min^{-1}). These observations clearly indicate that the binding of oxytocin to the isolated rat lipocytes can simply be described as a bimolecular reaction of the hormone with its receptor. Moreover, it suggests that the activation of the cellular glucose metabolism elicited by this effector is apparently directly connected with a discrete number of oxytocin binding sites. That these receptors might be localized on the plasmatic membrane was confirmed by binding measurements carried out on the ghosts. A similar biphasic process was observed. However, a loss of both apparent affinity and total capacity resulted from cell disruption.[7]

Oxytocin Binding Sites are Specific and Independent from the Insulin Receptors of the Fat Cells

Since insulin is one of the major effectors of the carbohydrate metabolism in the fat cells, it was interesting to check for the possibility that oxytocin might simply act through the insulin receptors *via* degenerated interactions with these structures. Competition studies run in the presence of insulin, ACTH and glucagon clearly demonstrated that oxytocin binding remained unaffected by a large range of concentrations of those hormones (Figure 4). It is concluded that the oxytocin receptors here characterized are spatially independent from the other hormone receptors of the fat cells. The fact that [Lys^8] vasopressin was relatively weak in competing with oxytocin

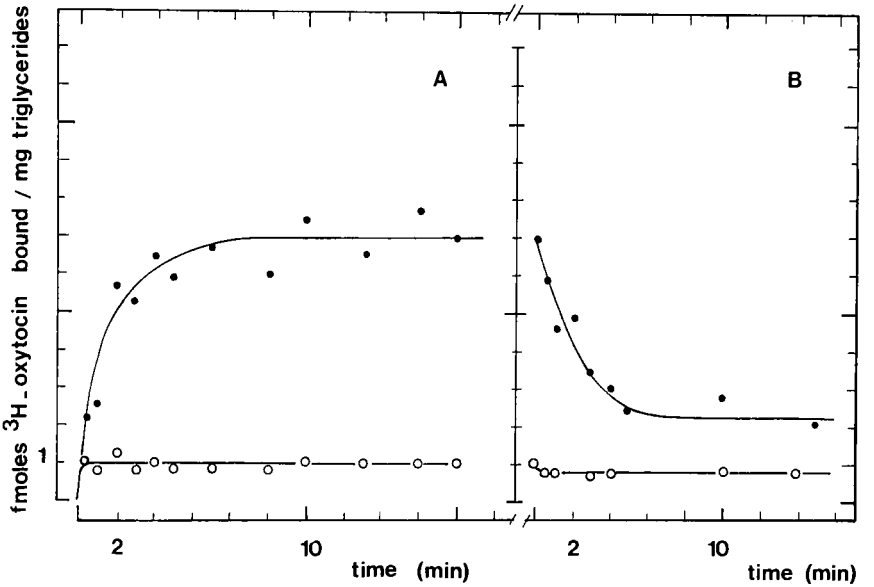

Figure 3: Time-course of the association and dissociation of [^{3}H]- oxytocin (12.5 n*M*) with isolated fat cells receptors. A) Total binding (●——●——●), as non-specific binding (o——o) (in the presence of native oxytocin 10 µ*M*) were measured as a function of time at 30° (Figure 3A: association curve). B) To test the reversibility of the binding, the cells were first incubated at 30° during 20 min in the presence of 12.5 n*M* [^{3}H]-oxytocin. The hormone concentration was then lowered 20 times by dilution. Residual total binding (●——●——●) and non-specific binding (o——o) were measured as a function of time after dilution (Figure 3B: dissociation curve). (◉) and (◎) represent independent measurements of the bound radioactivity after 20 min incubation at 30° with [^{3}H]-oxytocin 0.625 n*M* (◉) and in similar conditions with a 10^3 fold excess of cold hormone (◎).

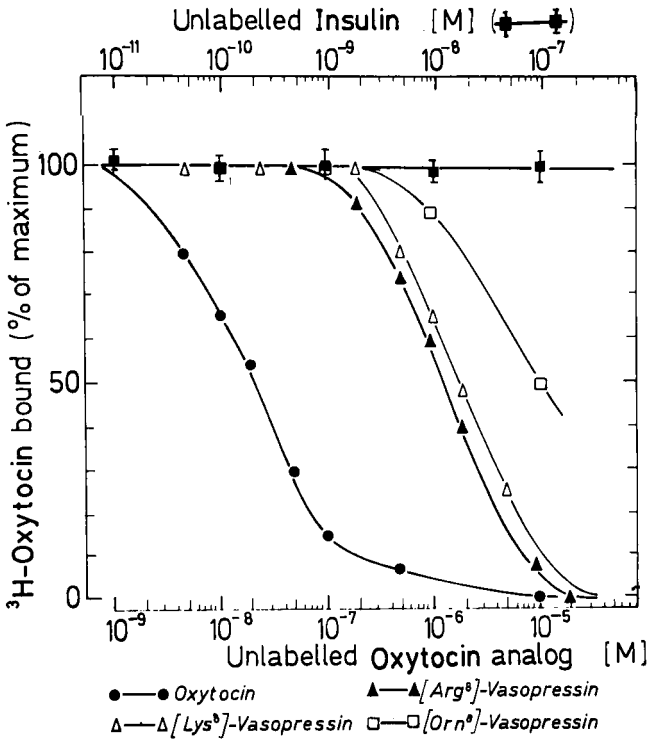

Figure 4: Effect of insulin (●—●) and some oxytocin analogs on [^{3}H]-oxytocin binding to isolated rat fat cells. Binding of [^{3}H]-oxytocin alone was 1 fmole per mg of triglyceride and taken as 100% binding.

for the binding sites (Figure 4) reinforces the idea that these receptors exhibit a rather strict specificity for oxytocin.

Oxytocin Produces No Measurable Effects on the Basal Activity of the Ghosts Adenylate Cyclase, Nor Does It Cause Variation of the Cellular Levels of Both CyclicAMP and CyclicGMP

The basal or adrenaline-stimulated, adenylate cyclase activity of freshly prepared "ghosts" remained unaffected by both oxytocin and insulin (from 10^{-9} to 10^{-4} *M*). CyclicAMP level in the isolated cells was found constant both in the presence or in the absence of insulin and oxytocin. Insulin raised the basal level of cyclicGMP,

in accord with a previous report of Cuatrecasas,[11] while oxytocin did not.

It is concluded that the action of oxytocin on the rat fat cells metabolism does not imply direct interactions with these nucleotide cyclases. This is an example of membrane bound hormone receptors which are not connected with those enzyme systems.

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MAGNESIUM-NEUROHYPOPHYSEAL HORMONE INTERACTIONS IN CONTRACTION OF ARTERIAL SMOOTH MUSCLE

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EVIDENCE HAS ACCUMULATED WHICH suggests that magnesium (Mg) ions potentiate neurohypophyseal hormones (NHHs) and analogues on different types of smooth muscles, including that of blood vessels (see references 1 and 2). It has been suggested that Mg potentiates the activity of NHHs on blood vessels by primarily affecting the affinity (or binding) of the hormones to their receptors rather than intrinsic activity.[2] Previous studies on blood vessels, however, did not employ either: 1) complete dose-response curves (DRC), 2) several different concentrations of external Mg ($[Mg^{++}]_o$), 3) blood vessels from rats (the species which is primarily used to assay and standardize NHH), or 4) more than two to three NHHs, effects of different amino acid moieties in the NHH would have to be investigated. The present *in vitro* experiments utilizing nine synthetic NHHs and analogues, a range of $[Mg^{++}]_o$ (0,0.2,1.2 and 6.0 m*M*), complete DRC, and rat aortic strips, were therefore undertaken to acquire this information. In addition, since previous studies indicated that $[Mg^{++}]_o$ can affect binding and transport of calcium (Ca) in vascular muscle,[3,4] selected tissues were analyzed for total Mg and Ca content.

The aortae were obtained from male rats, cut helically into vascular strips, 1.3-1.5 mm in width by 25 mm in length, and set up under isometric recording conditions as described previously.[5,6] Only male rats were employed since sex and sex hormones are known to affect the reactivity of blood vessels to NHH.[7,8] All aortic strips were

initially equilibrated for 2 hr in muscle chambers containing Krebs-Ringer bicarbonate (KRB) solution with 1.2 m*M* Mg^{++}.[1,5] After the incubation period, certain vascular strips were exposed either to MG^{++} -free KRB solutions or to KRB solutions containing various concentrations of Mg^{++} for a period of 10 min. Complete, cumulative DRC were then obtained for one of nine different synthetic NHHs and analogues.[5] In other experiments, aortae were analyzed for total Ca and Mg by atomic absorption spectroscopy.[3]

If the presence of $[Mg^{++}]_o$ potentiates NHHs and analogues solely by enhancing the binding of these peptides to their respective receptors,[2] then one might expect to see a parallel displacement of the concentration-effect curves for the NHH to the left of those obtained in Mg^{++} -free solutions with no change in maximum contractile response. If, however, $[Mg^{++}]_o$ potentiates these peptides on certain vascular muscles by acting at receptors and/or some other cellular site,[3,4,9] then one should not see a leftward parallel shift of the DRC. A comparison of the data obtained in different concentrations of $[Mg^{++}]_o$ (*i.e.*, 0.2, 1.2,6.0m*M*) (Figures 1-3), with those obtained in Mg^{++} -free solution (Figure 4), indicate that although different concentrations of $[Mg^{++}]_o$ strongly potentiate the contractile actions of vasopressin and analogues on rat aorta, the DRC are not shifted in a distinctly parallel manner to the left of those obtained in Mg^{++} -free solution. Instead, these results indicate that the slopes of the DRC are steepened and the maximum contractile responses are differentially affected by the concentration of $[Mg^{++}]_o$; 1.2 m*M*$[Mg^{++}]_o$ appears to yield the greatest shift of the DRC to the left. In most cases, the exceptions being [Lys^8] -, [Phe^2, Lys^8]-, 1-Deamino-[Arg^8]-, and 1-Deamino-[Phe^2, Arg^8]-vasopressin, 1.2 m*M*$[Mg^{++}]_o$ also results in the greatest degree of contractile response.

The data shown for oxytocin ([Ile^3, Leu^8]-vasopressin) and [Ile^3, Orn^8]-vasopressin (Figure 2 vs. Figure 4) indicate that although the maximum contractile response to these peptides are increased almost six-fold and five-fold, respectively, by 1.2m*M*$[Mg^{++}]_o$, the ED_{50}'s are not significantly shifted to the left of those observed in Mg^{++}-free solution as is the case for the other peptides. In fact, $[Mg^{++}]_o$ shifts the ED_{50}'s rightward for these two peptides. Such data thus strongly contradict the concept that $[Mg^{++}]_o$ solely potentiates activities of NHHs and analogues on vascular muscle by enhancing hormone-receptor affinity.[2]

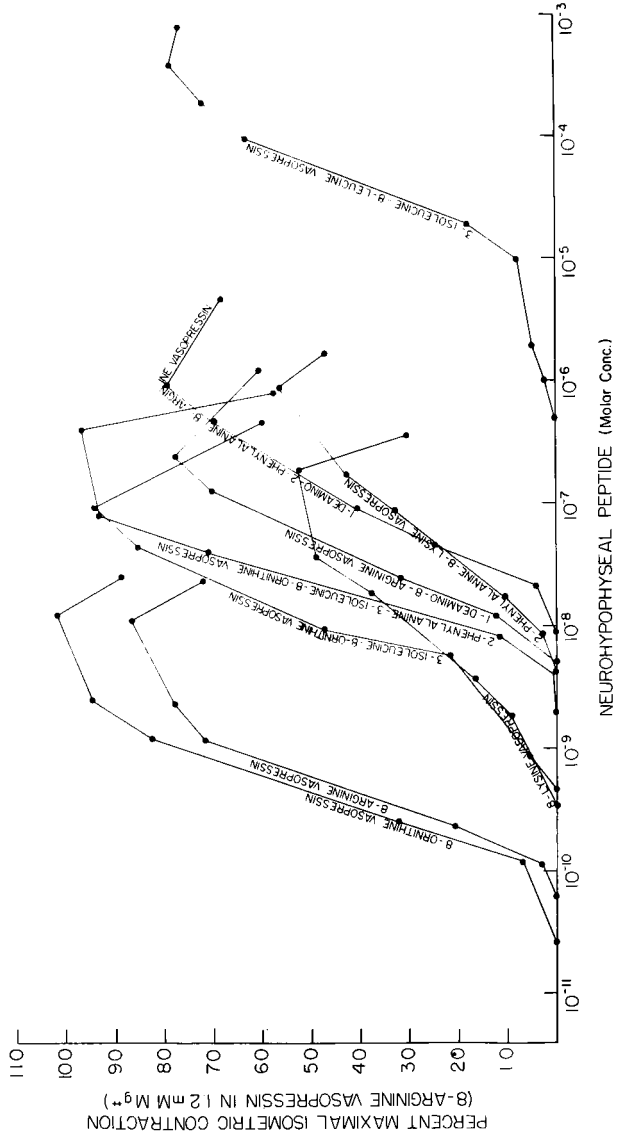

Figure 1: Comparative contractile actions of NHHs and analogues on isolated rat aortas suspended in KRB solution containing 0.2m*M*$[Mg^{++}]_o$. *N* = 6 - 20 different strips for each dose-response curve. Cumulative log dose-isometric response curves.

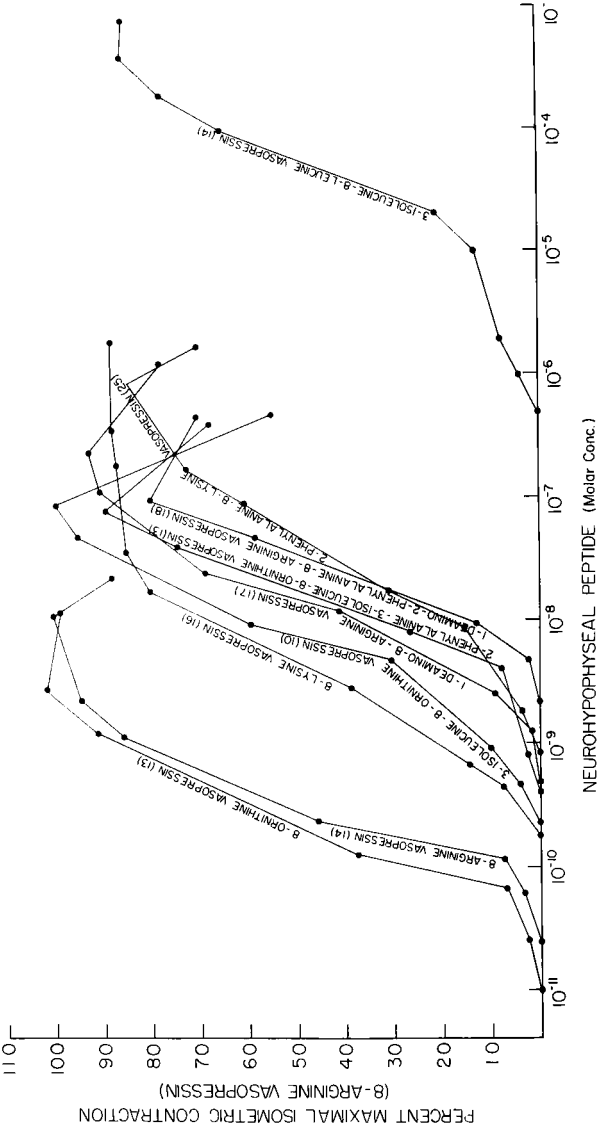

Figure 2: Comparative contractile actions of NHHs and analogues on isolated rat aortas suspended in KRB solution containing 1.2m*M* $[Mg^{++}]_o$. Numbers in parentheses denote the number of different male rats utilized.

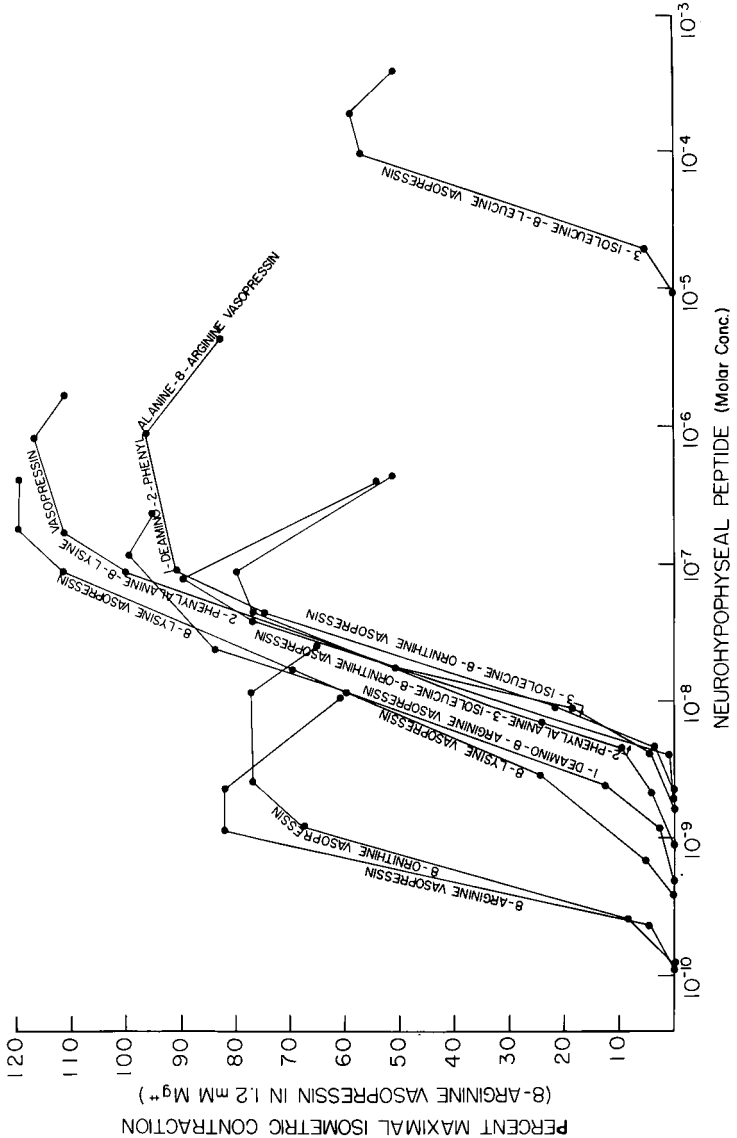

Figure 3: Comparative contractile actions of NHHs and analogues on isolated rat aortas suspended in KRB solution containing 6.0m*M*$[Mg^{++}]_o$. *N* = 6 - 25 for each dose-response curve.

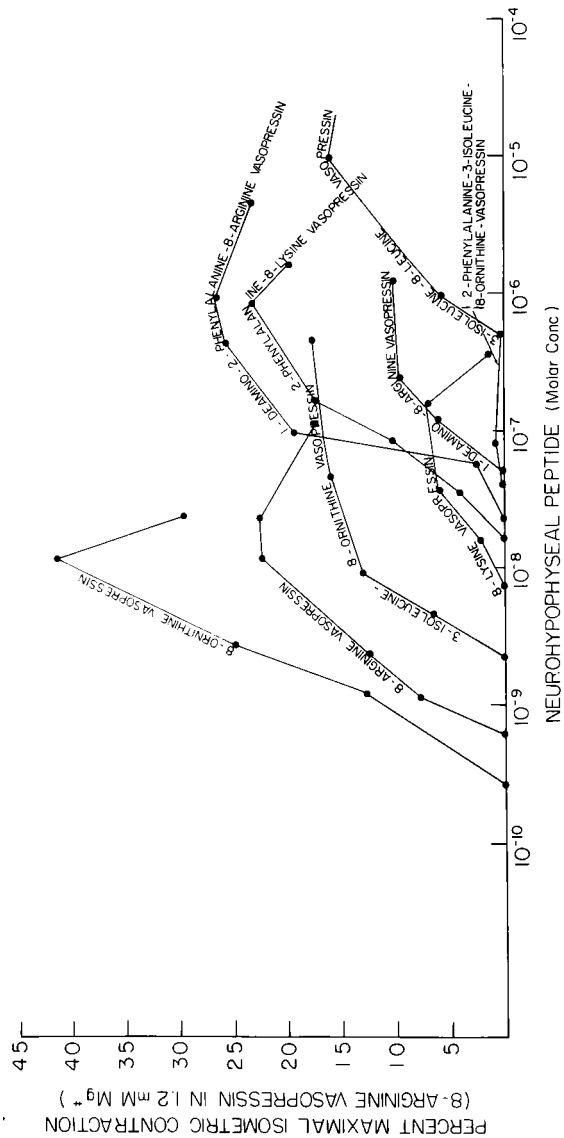

Figure 4: Comparative contractile actions of NHHs and analogues on isolated rat aortas suspended in Mg^{++}-free KRB solution. N = 10 - 30 for each dose-response curve.

The data for [Phe^2, Ile^3, Orn^8]-vaspressin indicate that major amino acid changes in the native mammalian NHH can bring about an extreme degree of potentiation of maximal response in the presence of $[Mg^{++}]_o$ (Figure 2 vs. Figure 4); this analogue is potentiated approximately 130-fold in 1.2m*M* $[Mg^{++}]_o$ when compared to the almost zero response seen in Mg^{++}-free KRB (Figure 4). In this context, the data in Figures 1 - 3, when compared to Figure 4 (Mg^{++}-free KRB), serve to illustrate that alteration of the vasopressin molecule in positions 1,2,3, and/or 8 not only alters the positions of the DRC relative to those observed in zero Mg^{++}, but indicates that these positions (amino acid moieties, etc.) interact differentially with $[Mg^{++}]_o$ to produce contraction. It can readily be seen that the presence of a physiologic concentration of $[Mg^{++}]_o$ (*i.e.*, 1.2m*M*) (Figure 2) approximately equalizes the maximal contractile response to all nine NHH whereas in the absence of $[Mg^{++}]_o$, the values range from as little as 1% to 40% (Figure 4). In addition, these data indicate that although the presence of $[Mg^{++}]_o$ significantly enhances the maximal contractile responses of all NHH peptides studied ($P < 0.001$), the relative increase in the contractile response is somehow dependent on the chemical structure of the amino acid substituents in the NHH analogues. The presence of an amino group in position 1 decreases the relative degree of potentiation in maximal contractile response obtained in the presence of 1.2m*M* $[Mg^{++}]_o$ (Figure 2 vs. Figure 4) from approximately 10-fold as in Deamino-AVP to 5-fold as in AVP. Removal of the phenolic hydroxyl in position 2 also decreases the relative degree of potentiation in maximal contractile response; for example, 1-Deamino-[Phe^2, Arg^8]-vasopressin and [Phe^2, Lys^8] vasopressin versus Deamino-AVP and LVP (Figures 2 and 4).

The findings with [Ile^3, Orn^8]-vasopressin indicate that the ED_{50} with $[Mg^{++}]_o$ is not appreciably altered when compared to that in the absence of Mg; these data suggest that interaction of Mg with an aromatic group (or phenylalanine) might be critical for NHH potency in arterial muscle. A decrease in basicity in position 8 relative to AVP can result in increases in the relative degree of potentiation of maximal response when $[Mg^{++}]_o$ is present, for example compare, especially, LVP and oxytocin to AVP (Figures 1 - 3 vs. Figure 4). Our results also tend to suggest that the length of the side chain in this position may be quite important in Mg potentiation; [Orn^8]-vasopressin is potentiated more by 1.2m*M* Mg than is AVP.

A previous preliminary report suggested to us that the greater the rat pressor potency of the NHH, the greater may be the shift of the cumulative DRC to the left in the presence of $[Mg^{++}]_o$.[9] A plot of the data shown in Figures 2 and 4, *i.e.*, the ratios of the ED_{50}'s of the NHH in 1.2m*M* $[Mg^{++}]_o$ to those in the absence of $[Mg^{++}]_o$, versus rat pressor potency, clearly indicates that the degree of potentiation of a neurohypophyseal peptide on rat aortic smooth muscle by $[Mg^{++}]_o$ is a direct function of the rat pressor potency of the NHH, unlike that observed on uterine smooth muscle.[10] In other terms, the more potent the NHH or analogue is in raising rat blood pressure, the more dependent it is on extracellular Mg for inducing contraction on rat aorta. At the very least, these present observations indicate that the receptor which subserves contraction for NHH on mammalian blood vessels is probably different from the one in uterine smooth muscle for vasopressin and oxytocin.

Turning our attention to total tissue Ca and Mg contents, we noted that exposure of equilibrated rat aortic strips to Mg^{++}-free KRB solution for 60 min not only lowers the total tissue Mg by approximately 50% (3.82 ± 0.15m*M*/kg wet wt. vs. 2.02 ± 0.19, $N = 6$) but concomitantly results in a 25% increase in total tissue Ca content (8.03 ± 0.36 vs. 10.21 ± 0.47, $N = 6$). Qualitatively, similar results have been noted for rabbit aorta.[3] Such data suggest that, at least in aortic smooth muscle, Mg can affect binding and/or movement of Ca,[3,4] thus supporting the idea that $[Mg^{++}]_o$ may enhance maximal contractile responses in vascular muscles by displacing Ca from intracellular sites.[3,4,9]

In conclusion, the present experiments demonstrate that: 1) $[Mg^{++}]_o$ potentiates the contractile actions of a variety of NHHs and analogues on arterial smooth muscle (ASM). 2) $[Mg^{++}]_o$ can alter the ED_{50}'s, intrinsic activities, structure-activity relationships, as well as steepness of the DRC of these molecules on ASM. 3) The amino acid moieties in positions 1,2,3, and 8 of the vasopressin molecule interact, differentially, with $[Mg^{++}]_o$ to produce contraction of ASM. 4) The length of the side chain, and basicity, in position 8 of the vasopressin molecule may be important in Mg potentiation in ASM. 5) Interaction of Mg with an aromatic group in position 3 may be critical for potency of vasopressin hormones on mammalian ASM. And 6) $[Mg^{++}]_o$ not only affects Mg content of ASM but Ca as well. Collectively, it is difficult, if not impossible to interpret such data as supporting the concept that $[Mg^{++}]_o$ solely potentiates activites of NHH analogues on vascular muscle by enhancing

hormone-receptor affinity. Rather, these data, when taken together with other sutdies on ASM,[3,4,9] suggest that Mg probably acts at sites other than just receptor in the production of NHH-activated contractions of ASM.[3,4,9] In addition, the present findings indicate, as suggested previously,[9] that the Mg-dependence of these peptides on at least one ASM-rat aorta is a *direct* function of the rat pressor potency of the molecules.

Acknowledgments

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A SIMPLE, RAPID, AND QUANTITATIVE *IN VITRO* MILK-EJECTING ASSAY FOR NEUROHYPOPHYSEAL HORMONES AND ANALOGS

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THE MILK-EJECTING ACTIVITIES OF THE NEUROHYPOPHYSEAL hormones and related analogs have been the least seldom documented of the common biological activities used to measure the potencies of these peptides. This is primarily because the *in vivo* methods generally used[1,2] are inherently quite difficult, while the *in vitro* methods which have been presented[1,3,4] are rather subjective in nature and not readily amenable to quantitative evaluation.

We present here a simple, rapid, and quantitative *in vitro* method for studying the milk-ejecting activities of neurohypophyseal hormones and related analogs. The method uses mammary glands from lactating albino mice, and is sensitive and specific for neurohypophyseal hormones and analogs and can be used to study both agonist and antagonist activities.

Methods and Materials

Lactating albino mice from which pups have been removed 18-24 hr previously are used. The animals are sacrificed by cervical dislocation and a longitudinal slit is made in the ventral integument. The skin is then pulled open laterally and the mammary glands are removed

intact and placed in a Krebs Ringer bicarbonate (KRB) solution (g/l; NaCl, 6.85; KCl, 0.35; $NaHCO_3$, 2.10; $CaCl_2 \cdot 2H_2O$, 0.28; $MgSO_4 \cdot 7H_2O$, 0.29; $Na_2HPO_4 \cdot H_2O$, 0.16; glucose, 2.0; Trizma·HCl, 6.85; Trizma base, 0.80). The glands are sliced into small fragments (approximately 4x4x4 mm) by a razor blade, returned to fresh KRB, and placed on a rotator for about 5 to 10 min with 2-3 changes of the incubation medium. The KRB is poured off and the pieces of gland placed on a moistened filter paper in a Petri dish. Samples of the tissue fragments of 100 ± 10 mg are rapidly weighed out and transferred into one ml of KRB in a 15 ml plastic beaker. Each mouse usually yields enough tissue to prepare 36 beakers. After all samples have been prepared, the KRB is again removed and 5 ml of control or experimental solution is added to each of the beakers containing the mammary tissue. The beakers are then shaken (84 cycles/min) in a Dubnoff metabolic incubator under 95% O_2-5% CO_2 for 30 minutes at 26° (pH 7.5). The solution from each of the beakers is then poured into spectrophotometer tubes and the optical density at 630 nm is determined on a Spectronic 20.

Oxytocin, lysine vasopressin, arginine vasopressin, vasotocin, mesotocin, and related analogs were prepared in our laboratory in highly purified form. Samples of vasotocin were also generously provided by Drs. Maurice Manning and Roderich Walter. Crystalline [1-β-mercaptopropionic acid]oxytocin (deamino-oxytocin) was prepared as previously described[5] and found to be identical to a sample generously provided by Dr. V du Vigneaud. The USP Posterior Pituitary Reference Standard solution was a gift from Dr. Walter Chan and had an activity of 2.29 U/ml of oxytocic and 2.16 U/ml of pressor activity. The peptide hormones and analogs were lyophilized in lactose (2 mg/g, for storage and simplicity of weighing) and were weighed out and dissolved just prior to use. In this form the peptide hormones had activities indistinguishable from the pure lyophilized powders (Figure 1).

Results and Discussion

Using the *in vitro* milk-ejecting assay outlined above, highly reproducible dose-response curves are obtained. This is illustrated in Figure 1 for oxytocin and shows the results obtained using three separate preparations of the hormone. The figure also illustrates the use of the hormone lyophilized in lactose wherein the activity of the lactose-hormone mixture (2) was indistinguishable from that of the

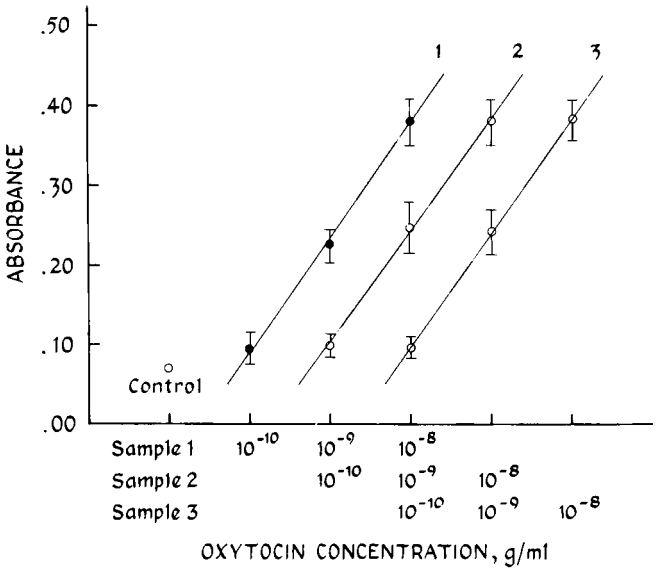

Figure 1: In vitro milk-ejecting response of mouse mammary gland tissue to three different samples of oxytocin. Samples 1 and 2 were synthetically different samples. Samples 2 and 3 were from the same preparation but sample 3 had been lyophilized (as was sample 1) with lactose (0.2% oxytocin). The three preparations were each tested at the same time under identical conditions and within the same tissue preparations. The means and the 95% confidence limits are indicated. Each point represents the mean of 9 - 11 replicates from a total of three mice.

hormone itself (3). We routinely measure optical density at 630 nm, though measurements at other wavelengths (*e.g.* 540 nm) may also be used. We have also found that the response is proportional to the weight of tissue used, and that similar results are obtained for either 15 or 30 minute incubations. The *in vitro* method reported here is sensitive to about 0.01 mU of USP standard and to about 5×10^{-11} g/ml of crystalline deamino-oxytocin (Figure 2). Against the USP standard, crystalline deamino-oxytocin was found to have a potency of about 340 U/mg (Table I). However, the slope of the curves for deamino-oxytocin and the USP standard are often not parallel over the dose-response range, probably because of the presence of

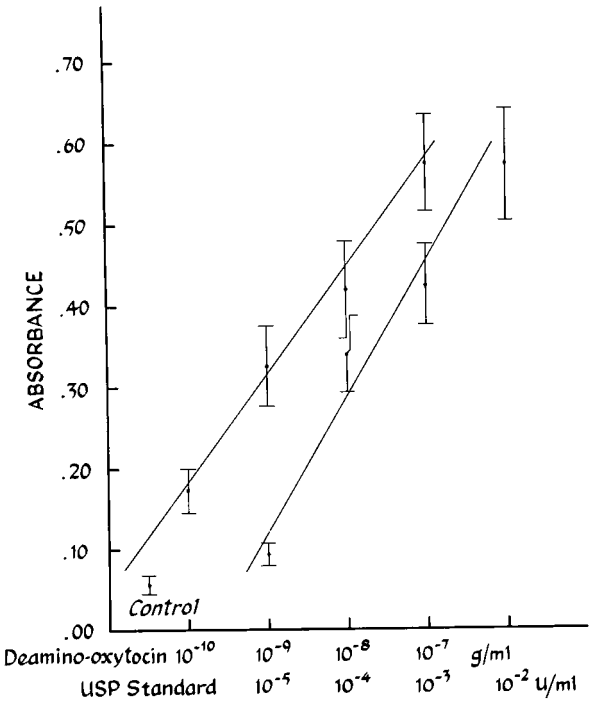

Figure 2: Milk-ejecting response of mammary gland tissue to crystalline deamino-oxytocin (g/ml) and USP standard (U/ml). Values represent the means and 95% confidence limits. Each point represents the mean of 46 - 53 replicates obtained from a total of 15 mice.

vasopressin in the latter. Both lysine vasopressin and arginine vasopressin usually have different slopes than deamino-oxytocin over the dose-response range. On the other hand oxytocin, mesotocin, glumitocin and vasotocin have dose-response curves parallel to that of deamino-oxytocin. Thus we use crystalline deamino-oxytocin as our standard and define its activity as 500 U/mg. The potency of oxytocin and several other neurohypophyseal hormones and analogs is given in Table I, and the values are compared with activities previously reported in the literature. The bioassay is highly specific for neurohypophyseal hormones since α-MSH, prolactin, luteinizing hormone, human chorionic gonadotropin, insulin, glucagon

Table I

Milk-Ejecting Activities

of Neurohypophyseal Hormones and Analogs

Compound	*U/mg Activity This Method*	*U/mg Activity Other Methods*[a]
Deamino-oxytocin	500[b] (340[c])	541 ± 13[d], 400 ± 8[e]
Oxytocin	428 ± 25	410 ± 16[f], 450 ± 30[g,h], 430[i], 360[j]
Arginine Vasopressin	116 ± 18	30 - 120[k], 64 ± 8[l], 68[i]
Lysine Vasopressin	29 - 53	60 ± 10[h,l], 31 ± 2[m], 45[g]
Arginine Vasotocin	321 ± 22	270[i], 210[l]
Mesotocin	471 ± 70	328 ± 21[n,o]
Glumitocin	81 ± 6	53[p]
[1-Penicillamine]-oxytocin	1.6 ± 0.2	nil[q]
Tocinamide	1.2 ± 0.1	0.5[r]
Tocinoic Acid	0.34 ± 0.01	---

[a]Rabbit Mammary Gland (*in vitro*).
[b]See text.
[c]Vs. USP Standard.
[d]Ferrier, B. M., D. Jarvis and V. du Vigneaud. J. Biol. Chem., 240, 4264 (1965).
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and several other peptide hormones had no milk-ejecting activities nor did these substances either enhance or inhibit the activity of deamino-oxytocin. Biogenic amines such as histamine, serotonin, epinephrine, norepinephrine, and melatonin were also without milk-ejecting activity. Acetylcholine (10^{-3} to 10^{-6} *M*) did have milk-ejecting activity, and this activity could be selectively blocked by atropine (2×10^{-4} *M*). Atropine, however, did not block the activity of deamino-oxytocin (Figure 3), demonstrating the different receptor specificities for these two substances. The tripeptide side chain of oxytocin (Pro-Leu-Gly-NH_2) was without milk-ejecting activity, but both tocinoic acid and tocinamide (the ring moiety of oxytocin) have milk-ejecting activities (Table I). Of particular note is the observation that [1-L-penicillamine] oxytocin, which is generally without agonist activity[6] acts as a partial agonist in this assay (Table I). The latter analog can also act as an antagonist in this system. Further structure-activity studies of neurohypophyseal peptides are in progress and will be reported elsewhere.

Summary

A simple quantitative *in vitro* assay method for milk-ejecting activity using mammary gland pieces from lactating albino mice is presented. The assay can be "set up" in a few minutes and 30-40 data points can be determined simultaneously in a one to two hour period. The method is sensitive and can readily detect neurohypophyseal hormones such as oxytocin at concentrations of 0.1 - 1 nanograms/ml. Dose-response curves for neurohypophyseal hormones and analogs can be readily obtained.

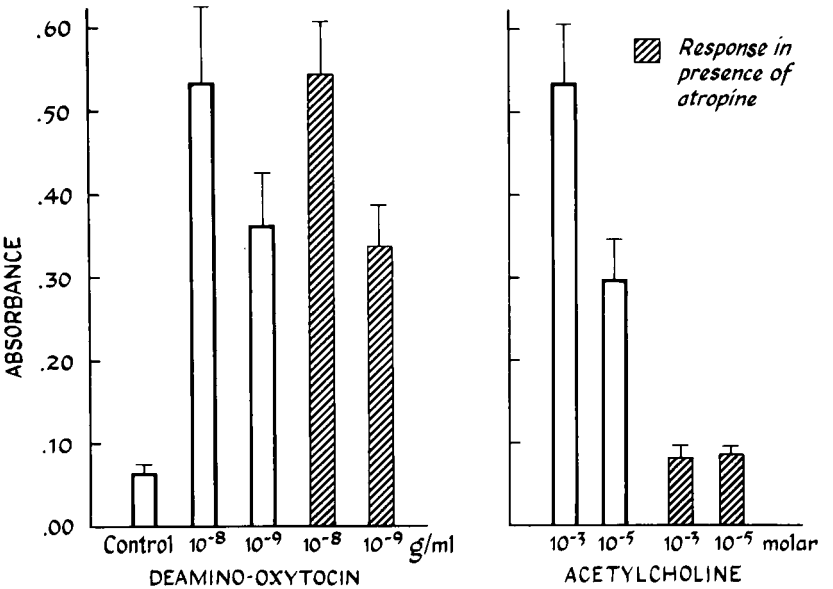

Figure 3: Demonstration of the specificity of atropine blockade of acetylcholine-induced milk ejection. Mammary gland tissue was incubated in deamino-oxytocin (10^{-8} and 10^{-9} g/ml) and acetycholine (10^{-3} and 10^{-5} *M*) and in the same concentrations of these hormones but also in the presence of atropine (2×10^{-4} *M*). Tissues were allowed to preincubate in the cholinergic antagonist approximately 10 minutes before they were transferred to the media containing the hormones. Values represent the means and 95% confidence limits. Each mean value obtained from 16 samples of mammary tissue, 4 replicates from each of 4 mice.

Acknowledgments

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THE DESIGN OF NEUROHYPOPHYSIAL PEPTIDES POSSESSING SELECTIVELY ENHANCED AND INHIBITORY PROPERTIES

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Introduction

THE ORIGINAL SYNTHESIS OF OXYTOCIN by du Vigneaud and collaborators[1] made possible the study of the structure-activity relationships of this and related molecules. These studies were carried out in a number of laboratories both in the U.S. and in Europe. The advent of the Merrifield solid-phase method[2] provided a further impetus to these studies by allowing the synthesis of a larger number of peptides in a much shorter period of time. Over 300 analogs of oxytocin and vasopressin have been synthesized and pharmacologically evaluated in an attempt to delineate those structural and topochemical features which determine the characteristic biological activities. The highlights of these studies have been summarized.[3] The thrust of the early studies was chiefly in making individual changes and noting the resultant effects on biological activities. Predicting the effects of these changes was not possible. However, now that a relatively large number of synthetic analogs have been examined, it is possible to discern patterns and correlations between structural changes and consequent changes in biological activities.

Thus in many instances one may predict the effects of structural changes, individually or in combination, on biological activities. We are thus approaching one of the long-standing goals of those who study structure-activity relationship of the neurohypophysial peptides; the design of peptides exhibiting selectively enhanced or inhibitory properties.

Compounds possessing specific oxytocic, antidiuretic or pressor properties would be of potential clinical value and might also aid in the study of receptors and of the mode of action of these peptides. Specific inhibitors of these responses could be of value for clinical use and also for studying peptide receptor interactions.

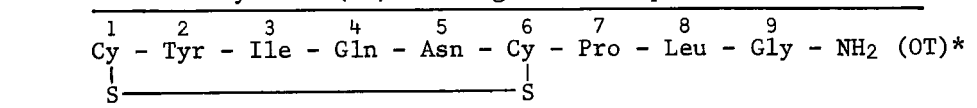
The purpose of this presentation is to outline an approach towards designing peptides with selectively enhanced and inhibitory properties based on an analysis of pharmacological data from our own and from other laboratories. Although the approach appears to have possible limitations for the design of specific oxytocic agents it has considerable potential for the design of specific antidiuretic and pressor agents and for specific inhibitors of the oxytocic and pressor responses.

Selectivity: Definitions

Oxytocin and vasopressin exhibit overlapping spectra of biological properties (Table I) with the former possessing relatively enhanced rat uterus (RU) and milk ejecting

Table I

Some Pharmacological Activities (Units/mg)
of Oxytocin (OT) and Arginine Vasopressin (AVP)[3]



Peptide	*RU*	*ME*	*P*	*A*
Oxytocin	520	475	4	4
Arginine Vasopressin	14	70	370	320

* In AVP, positions 3 and 8 are occupied by Phe and Arg.

(ME) potencies relative to vasopressin which in turn possesses relatively enhanced pressor (P) and antidiuretic (A) potencies relative to oxytocin.

Analysis of the data from over 300 synthetic analogs of oxytocin and vasopressin provides the following pattern of selectivities; 1) Intra-Oxytocin Like: RU/ME 2) Inter-Oxytocin-Like-Vasopressin-Like: (a) RU/P (b) RU/A 3) Intra-Vasopressin-Like: (a) A/P (b) P/A

RU/ME Selectivity

Only a few known analogs exhibit slightly enhanced RU/ME selectivity primarily due to increased RU activity rather than diminished milk-ejecting activity.

These are carba-1-oxytocin[4], 4-threonine-oxytocin[5] and deamino-oxytocin[6] with RU/ME values respectively of 4, 1.7 and 1.5. Attempts at further enhancing specificity by combining in one molecule the structural changes which individually enhance selectivity indicates that these effects are not additive. For example, [1-deamino-4-threonine]oxytocin[7] has an RU/ME ratio of only 0.4.

RU/P Selectivity

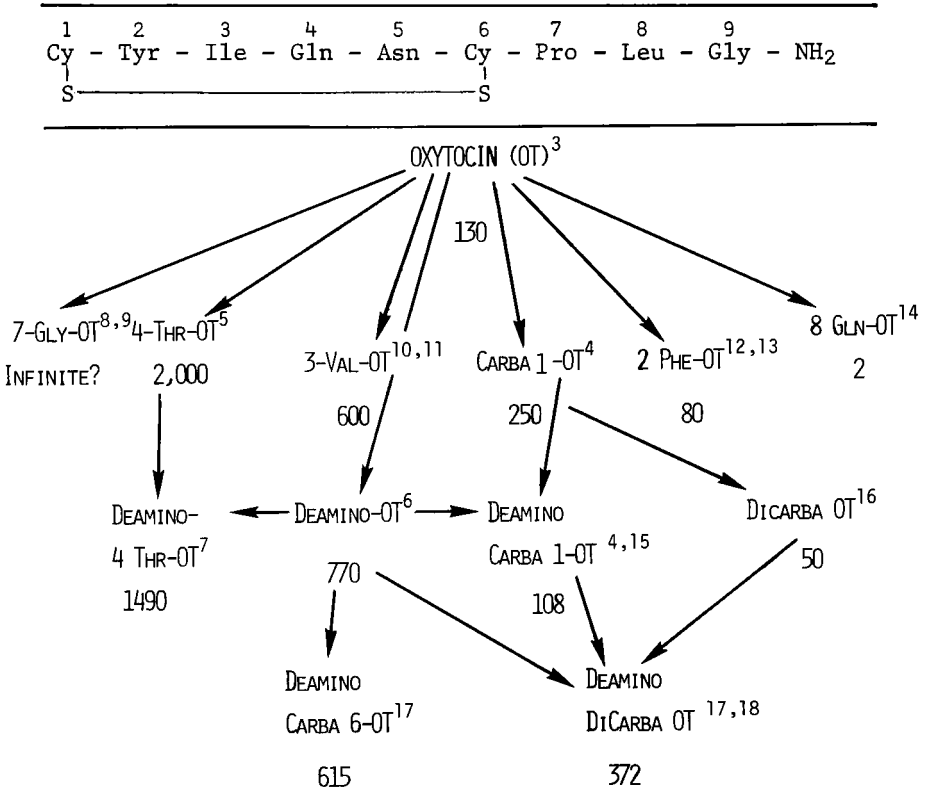
A number of different individual modifications in oxytocin can lead to enhanced RU/P selectivity relative to oxytocin. These involve substitutions in positions 1, 3, 4 and 7. Curiously diminished RU/P selectivity relative to oxytocin results from changes in positions 2 and 8. The enhanced selectivity arises in most instances by the virtual elimination of pressor activity. These effects appear not to be additive (Table II) but not enough analogs have been studied to give a definitive answer on this.

RU/A Selectivity

Oxytocin has an RU/A value of 130. As shown in Table III changes in positions 4 and 7 lead to dramatic enhancements by diminishing antidiuretic activity and, in the case of threonine substitution in position 4, by also enhancing oxytocic potency.[5] Substitutions at positions 1, 2, 3 and 8 lead to diminished ratios relative to oxytocin. These findings suggest that in designing highly specific oxytocic agents lacking both antidiuretic and pressor activities one stands a much better chance for success by concentrating on analogs with changes in

Table II

Structural Changes in Oxytocin Which Enhance or Diminish RU/P Selectivity Relative to Oxytocin



positions 7 and 4 than on those modified at positions 1, 2, 3 and 8. Additivity has not been determined. In this regard the synthesis and pharmacological evaluation of [4-threonine-7-glycine]-oxytocin should provide an answer. Should the effects indeed be additive this peptide would be a highly active and specific oxytocic agent and, of more intriguing interest, might point the way to even more highly potent and selective oxytocic agents of potential clinical value.

Table III

Oxytocin Peptides Exhibiting Enhanced and Diminished RU/A Selectivity Relative to Oxytocin

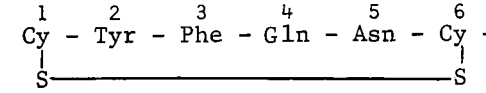
Peptide	*RU*	*RU A*
7-Gly-OT[8]	330	33,000
4-Thr-OT[5]	920	600
Oxytocin[3]	520	130
Carba 1-OT[4]	734	79
Deamino carba 1-OT[15,4]	1899	68
2-Phe-OT[12,31]	32	60
Deamino-OT[6]	803	40
8-Gln-OT[14]	58	10
Deamino carba 6-OT[17]	929	8

Antidiuretic/Pressor Selectivity

The structural changes which enhance A/P selectivity in AVP analogs are shown in Table 4. These were established by studies in a number of different laboratories[19-24] and

Table IV

Structural Changes Which Enhance A/P Selectivity of AVP

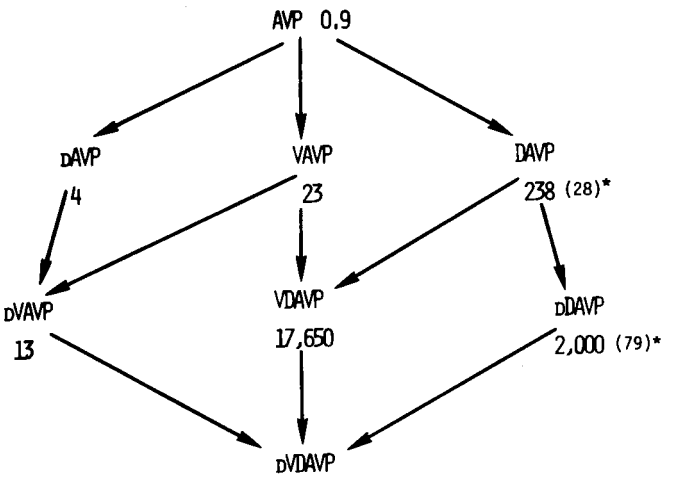


1. D-Arg Substitution[20]
2. Enhancement of lipophilicity at position 4[22,23]
3. Deamination at position 1[19]
4. Combinations of all three[27-29]
5. Phe substitution at position 2[21]
6. DiCarba substitution ?[24]

were also shown to be additive in combinations of 2[19,22, 25,26]. Further studies in our laboratories demonstrated that these effects are additive in a combination of three changes.[27-29] Thus dVDAVP possesses an infinite A/P ratio (Table V). We have also established the relative

Table V

Structural Changes of AVP Showing Additive Nature of Effects on A/P Ratios[27-29]



Legend: AVP, Arg Vasopressin; dAVP, 1-Deamino-AVP; VAVP, 4-Val-AVP; DAVP, 8-*D*-Arg-Vasopressin; dVAVP, 1-Deamino-4-Val-AVP; VDAVP, 4-Valine-8-*D*-Arg-Vasopressin; dDAVP, 1-Deamino-8-*D*-Arg-Vasopressin; dVDAVP, [1-Deamino-4-Val-8-*D*-Arg]-Vasopressin.

*A/P values originally reported by Zaoral *et al.*[20,25]

contributions of these three changes *i.e.* deamination at position 1, enhancement of lipophilicity at position 4 and *D*-Arg substitution at position 8 towards enhancing A/P selectivity.[28,29] Whether more than three changes can be incorporated into one molecule with additive effects on A/P selectivity could be ascertained by combining in one molecule four of the structural features listed in Table

IV. [1-Deamino-3-phenylalanine-4-valine-8-*D*-arginine]-Vasopressin is an obvious choice in this regard for comparison with dVDAVP.

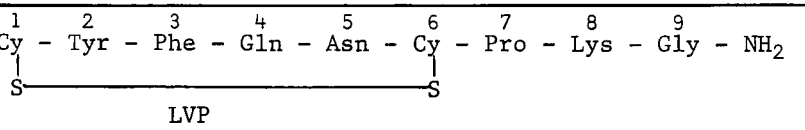
The precise estimation of Carba-1, Carba-6, or DiCarba substitutions towards enhancing A/P selectivity is not yet possible due to the lack of appropriate analogs of AVP. Thus Carba-1-AVP, [Deamino-carba-1]-AVP, Carba-6-AVP or DiCarba AVP have not yet been reported. [Deamino-Carba-6]-AVP has an A/P ratio of only 7.0.[30] [Deamino]-AVP as an A/P ratio of 4.[19] Thus Carba-6 substitution contributes relatively little towards further enhancing A/P selectivity. [1-Deamino-Carba-6-8-*D*-arginine]-Vasopressin has an A/P value of ~1700.[30,31] By comparison [1-deamino-8-*D*-arginine]-Vasopressin (dDAVP) has an A/P value of 2,000.[28,29] Thus in this instance substitution of Carba 6 in dDAVP did not appear to increase A/P selectivity. In the absence of hard data the effect of Carba-1 substitution may be deduced in the following some-what round about fashion. Substitution of dicarba *i.e.* Carba-1 + Carba-6 in [1-deamino]-AVP brought about a 10-fold reduction in pressor activity and a corresponding increase in A/P selectivity from 4 (dAVP)[19] to 34[24] ([1-deamino-diCarba]-AVP). On the basis of this and the above-mentioned very limited data it might be fair to assume that Carba-1 substitution leads to enhanced A/P selectivity and that it does in fact make a much greater contribution than Carba-6 substitution. Thus [Deamino-Carba-1-8-*D*-Arginine]-Vasopressin should make a very attractive model for studying this assumption. The effect of 4-valine substitution in this last compound on A/P selectivity would help to further delineate how many structural changes can be incorporated in one molecule with consequent enhancements of A/P specificity.

P/A Selectivity

Relatively few analogs exhibiting this type of selectivity have been synthesized. For a summary see[32]. Structural changes of lysine vasopressin (LVP) which lead to P/A selectivity based on rat pressor assays are shown in Table VI. These are additive (Table VII). Effects on regional blood flow of peptides exhibiting P/A selectivity (based on rat pressor data) have been shown to manifest both tissue and species variability.[33] Thus the design of peptides with enhanced P/A selectivity based on routine rat pressor assay data are subject to this limitation.

Table VI

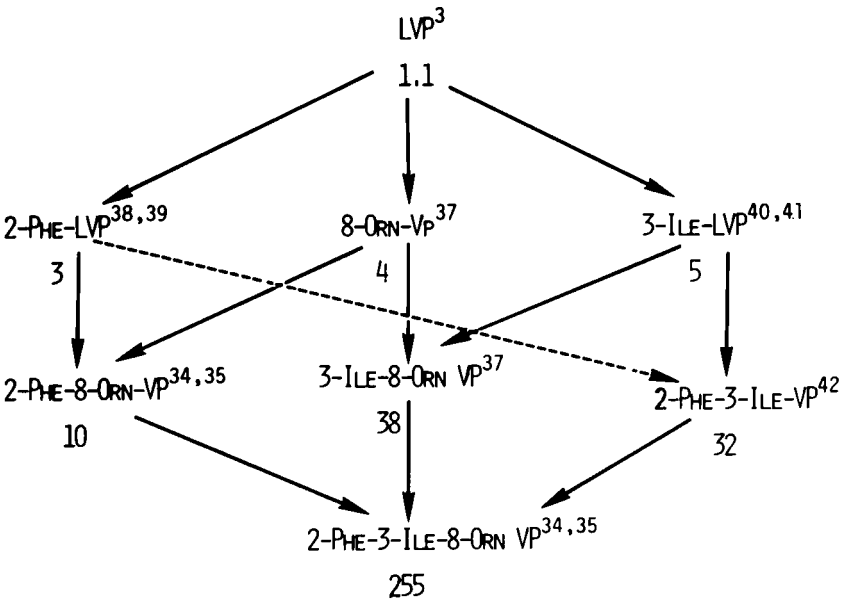
Structural Changes Which Enhance P/A Selectivity of Lysine Vasopressin (LVP)



1. Orn substitution at position 8[37]
2. Phe substitution at position 2[38,39]
3. Ile substitution at position 3[40,41]
4. Combinations of 1-3[34,35]
5. Asn in position 4[36]

Table VII

Structural Changes in LVP Which Enhance P/A Selectivity in an Additive Fashion



Nonetheless, as an approximation, the rat pressor values offer a most promising first line of approach. So far the peptide exhibiting the highest P/A value is 2-phenylalanine-8-ornithine vasotocin[34,35] (P/A, 255, Table VII). This value might be further enhanced by one of the following approaches or by a combination of the two. (a) Since 4-Asn-LVP has a P/A ratio of 2,[36] the substitution of asparagine in place of glutamine in [2-Phe-8-Orn]-VT might lead to a further enhancement of the P/A ratio. (b) From studies to date phenylalanine at position 2 coupled with ornithine at position 8 appear to be the optimal substitutions in these positions for enhancing P/A selectivity. Position 3 substitutions have not been explored to the same degree. Thus substitutions of other aliphatic amino acids at position 3 in [2-Phe-8-Orn]-VT might lead to enhancement of P/A selectivity.

Inhibitors

The design of effective specific antagonists of the oxytocic, antidiuretic and pressor responses of oxytocin and vasopressin has received much attention over the years since the original synthesis of oxytocin. Joe Rudinger provided an excellent summary of most of the thinking and accomplishments in this area up to 1970.[32] Very little progress has been made towards realizing an effective inhibitor of the antidiuretic response. However very substantial progress has been made towards obtaining effective inhibitors of the oxytocic response to oxytocin and the rat pressor responses to LVP and AVP (Table VIII).

Curiously both sets of inhibitors are based on the discovery that [1-deamino-penicillamine]-oxytocin is a potent inhibitor of oxytocin.[43,44] More potent inhibitors of the oxytocic response were obtained by successively replacing the dimethyl grouping with a diethyl grouping[45] and more recently with a pentamethylene group[46] (Table VIII). Substitution of the diethyl grouping in LVP produced a compound which antagonized the oxytocic and pressor responses to oxytocin and LVP respectively.[48] Substitution of threonine in the 4 position of [1-deamino-penicillamine]-oxytocin has given what appears to be the most potent antagonist of the oxytocic activity of oxytocin known to date. Our preliminary findings indicate that this material has a pA_2 value of approximately 7.7.[47] We had shown earlier that the highly specific antidiuretic peptides

Table VIII

Inhibitory Properties of Some Analogs of Oxytocin, LVP and AVP

Analog	*Anti Oxytocic* PA_2[a]	*Anti Pressor* PA_2[a]
[1-β-Mpa (β-Me_2)]-OT[43,44]	6.94	6.27
[1-β-Mpa (β-Et_2)]-OT[45]	7.24	6.24
[1-β-Mpa (β-$(CH_2)_5$)]-OT[46]	7.43	weak
[1-β-Mpa-(β-Me_2)-4-Thr]-OT[47]	~7.7	?
[1-β-Mpa-(β-Me_2)]-LVP[48]	6.84	7.15
dVDAVP[28,49]	Agonist	~6.8
1-Penicillamine-dVDAVP[49]	~7.3	~7.5

Legend: [a]PA_2 [H. O. Schild, Brit. J. Pharmacol. 2:189 (1947)] represent the negative log to the base 10 of the average molar concentration (M) of the antagonist which will reduce the response of the uterus of the rat to 2X units of agonist to the response to X units of the agonist.

[1-deamino-4-valine-8-*D*-arginine]-Vasopressin (dVDAVP) is a potent inhibitor of the rat pressor action of AVP.[28] More recent studies have shown that it possesses a pA_2 value of ~6.8.[47] Substitution of the penicillamine grouping in dVDAVP produced an even more potent antagonist of the pressor response. Thus 1-penicillamine dVDAVP possesses a rat pressor PA_2 value of ~7.5.[49] This material has also been shown to inhibit the oxytocic action of oxytocin.

In attempting to further enhance the antagonistic properties of [1-deamino-penicillamine-4-threonine]-oxytocin the substitution of the diethyl grouping or the pentamethylene group for the dimethyl grouping would now seem worthwhile.

For the design of an anti-antidiuretic peptide there are as yet no clear pathways to follow. One possible approach might be to take a highly selective pressor agent and incorporate into this molecule the deamino-penicillamine, deamino-diethyl or deamino-pentamethylene groupings. By analogy with the effect of the penicillamine substitution in dVDAVP one might hope that one of the resulting compounds would be an anti-antidiuretic agent.

Conclusion

Table IX attempts to summarize the additive and non-additive natures of the different kinds of selectivities outlined in this presentation. One severe limitation of

Table IX

Selectivity: Summary

1.	Intra Oxytocin-Like: $\frac{RU}{ME}$ Non-Additive
2.	Inter-Oxytocin-Like/Vasopressin-Like
	(a) RU/P Non-Additive
	(b) RU/A Additivity has not been tested
3.	Intra-Vasopressin-Like:
	(a) A/P Additive
	(b) P/A Additive

this kind of analysis should be mentioned before concluding. This relates to the fact that this kind of approach relies very heavily on data from widely differing sources. Selectivity ratios for given peptides either synthesized in different laboratories or pharmacologically evaluated in different laboratories or both can vary widely. We have encountered many instances of this in the present literature survey. Nonetheless, with this limitation in mind we hope the foregoing shown that this approach does possess real merit for the design of neurohypophysial peptides possessing desired biological properties.

Acknowledgments

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IODO AND TRITIO DERIVATIVES OF NEUROHYPOPHYSEAL HORMONES

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IN CONNECTION WITH A STUDY of neurohypophyseal hormone (NHH) receptors in uterus and kidney, we required radioactively labeled oxytocin (OT) and lysine vasopressin (LVP) of high specific activity. Because carrier free ^{35}S-Cys is not available commercially, only carrier free ^{125}I and ^{3}H were considered as possible labels of an NHH. For this reason, we prepared monoiodotyrosine and diiodotyrosine derivatives of the hormones and defined their biological properties in the rat oxytocic assay[1] and in an adenylate cyclase assay[2] which employs the NHH-responsive cyclase of bovine renal medullary membranes. This strategy was required since these iodo derivatives are analogs of the parent hormone and their biological action must be ascertained prior to preparing them labeled with ^{125}I. Furthermore, an iodinated NHH analog can serve as an intermediate for the preparation of the ^{3}H-NHH.[3]

Oxytocin was iodinated in methanol solution containing NH_4OH, by the addition of one meq of 3.9% I_2 in $CHCl_3$ for the preparation of monoiodo (MI) derivatives, and 2 meq for the preparation of diodo (DI) derivatives. Purification of MIOT and DIOT was accomplished by gel filtration on Sephadex G-25 with 1% AcOH. OT appears first in the effluent, followed by MIOT (as reported by Thompson[4] for the preparation of ^{125}I-monoiodotyrosine^{2}-OT) and DIOT which appear later because they are adsorbed. A second and third gel filtration of MIOT insured high purity and constant biological activity. The lyophilized products, MIOT and DIOT, were obtained in 30% yield each, based on

starting OT. Alternatively, MIOT and DIOT were purified by countercurrent distribution or by partition chromatography on Sephadex G-25 employing the solvent system (A) n-BuOH:PrOH:benzene:1% AcOH containing 0.5% Py, 12:1:1:16. In either case, a final purification step was accomplished by gel filtration on Sephadex G-25. Elemental analysis data was consistent with the monoacetate tetrahydrate for MIOT and for a monoacetate trihydrate for DIOT. The optical rotations were $[\alpha]_D^{24}$-17° for MIOT and -7.9° for DIOT (c 0.5, 1 *N* AcOH). The uv spectra showed λ_{max} (H_2O) 282 nm (ε = 2,360) for MIOT and 286 nm (ε = 2,200) for DIOT. On thin layer chromatography (tlc) MIOT and DIOT were homogeneous with R_f^A = 0.56 and 0.76 respectively, whereas OT has R_f = 0.28. On thin layer electrophoresis (tle) (0.1 *M* pyridine-AcOH, pH 5.6; 400 volts for 4 hrs), the following mobilities were observed: OT = 3.9 cm, MIOT = 3.5 cm and DIOT = 2.5 cm, all peptides moving as one component. MIOT and DIOT were each hydrolyzed with a mixture of leucine amino peptidase and papain and the hydrolysates were shown by tlc (solvent A) to contain monoiodotyrosine and diiodotyrosine respectively.

LVP was iodinated by an identical procedure and the resulting MILVP and DILVP were isolated and purified by gel filtration on Sephadex G-25. The elemental analyses were consistent for a diacetate in the case of MILVP and diacetate dihydrate for DILVP. The optical rotations were $[\alpha]_D^{24}$-22.4° for MILVP and -15.8° for DILVP (c 0.5, 1 *N* AcOH). The uv spectra showed λ_{max}(H_2O) 282 nm (ε = 2,660) and 286 nm (ε = 2,530) respectively for MILVP and DILVP. On tle (0.1 *M* pyridine-AcOH, pH 5.6, 400 volts for 2.5 hrs), the electrophoretic mobilities were LVP = 9.7 cm, MILVP = 8.4 cm and DILVP = 6.1 cm, showing homogeneity of all products.

For biological testing, MIOT had to be freshly prepared. In the rat oxytocic assay MIOT was inactive as an agonist, but was a competitive inhibitor of OT (apparent $K_i = 10^{-7}$ *M*) thus corroborating recent independent findings by Marbach and Rudinger.[5] Additionally, MIOT showed no stimulatory activity in the NHH-specific renal cyclase, but was a competitive inhibitor of OT (apparent $K_i = 10^{-4}$ *M*) when both peptides were added simultaneously. However, when the renal cyclase preparation was preincubated with MIOT for ten minutes before adding OT, non-competitive inhibition of OT (at 10^{-6} and 10^{-5}*M*) was observed with no effect on basal or fluoride-stimulated cyclase activity. After several months, frozen solutions of MIOT showed

OT-like activity in both assays, suggesting that the analog is not completely stable. DIOT was neither an agonist nor an antagonist in both the oxytocic assay and the adenylate cyclase assay. MILVP ($10^{-5}M$) was a partial agonist in the renal cyclase assay. However, MILVP appears to be unstable and after purification appears to slowly release traces of LVP. Upon preincubation of MILVP with renal medullary membranes for 10 min, followed by addition of LVP, the former was found to be a stronger non-competitive inhibitor (at 10^{-6} and 10^{-5} *M*) than MIOT. In the cyclase assay, DILVP is neither an agonist nor an antagonist.

MIOT (10^{-4} to 10^{-3} *M*) modifies the parathyroid hormone-sensitive bovine renal cortex cyclase system and the glucagon-responsive rat liver cyclase system, indicating lack of specificity.

Thus, MIOT and MILVP lack sufficient affinity and/or specificity to warrant the preparation of ^{125}I-OT or LVP to measure specific OT of LVP binding in uterine or renal medullary systems. However, iodo compounds were useful for preparation of tritiated hormones.

In model reactions, DIOT (or DILVP) was dissolved in MeOH-EtOAc (1:2), and hydrogenated in the presence of 5% Pd-$CaCO_3$ for 40 min (20 min for DILVP), which are modified conditions from those employed originally by Agishi[3] and subsequently by Morgat *et al.*[6] and Pradelles *et al.*[7] Gel filtration on Sephadex G-25 showed the formation of an impurity which travelled faster than OT. The impurity was almost the exclusive product when hydrogenation was conducted for 4 hrs, and was tentatively characterized as 1,6-diala-OT (1,6-diala-LVP), since upon amino acid analysis ala (2 mole) and not cys was found. To facilitate purification, we prepared a Neurophysin-Sepharose solid support which was used for affinity chromatography of the products of hydrogenation of DIOT and DILVP. Such method of purification alone or in combination with gel filtration or partition chromatography on Sephadex G-25 gave the pure samples of ^{3}H-OT and ^{3}H-LVP.

Homogeneity of the tritiated hormones was verified on tlc employing solvent A for ^{3}H-OT and n-BuOH:EtOH: pyridine:0.5% AcOH (5:1:1:8) for ^{3}H-LVP. The uv spectra of both products gave the expected uv $\lambda_{max}(H_2O)$ = 275 nm. The labeled hormones showed the characteristic dose response curves in the oxytocic and adenylate cyclase assays, which were used in conjunction with the total number of counts recovered in the samples, Folin-Lowry color yields and

uv spectra were used to determine specific activities of 25, 31 and 25 Ci for three different preparations of ^{3}H-OT and 26 Ci/nmole for a preparation of LVP.

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THE RETENTION OF ANTIOXYTOCIC ACTIVITY BY THE RING MOIETIES OF [1-β-MERCAPTO-β,β-DIETHYLPROPIONIC ACID]-OXYTOCIN AND [1-β-MERCAPTO-β,β-PENTAMETHYLENEPROPIONIC ACID]OXYTOCIN

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THE QUESTION OF WHETHER biological activities associated with oxytocin are intrinsic to the distinctive 20 member disulfide ring moiety was examined by Ressler[1] through the synthesis and study of tocinamide [1].

$$\overbrace{CyS^1 - Tyr^2 - Ile^3 - Gln^4 - Asn^5 - CyS^6} - NH \qquad [1]$$

In this compound the Pro-Leu-Gly-NH_2 side chain of oxytocin is missing but the amide character of the C-terminus is maintained. From this and later work it was determined that tocinamide[2-4] and the corresponding analog of deamino-oxytocin, deaminotocinamide,[2,5] possess 0.6 and 6.2%, respectively, of the oxytocic activity of oxytocin. Tocinoic acid[6] and deaminotocinoic[6] acid, where the C-terminus is a carboxylic acid rather than a carboxamide group, exhibit approximately 1/10th the oxytocic potency of the corresponding amide analogs. These results indicate that, while the tripeptide amide side chain of oxytocin is necessary for the expression of the full biological activity of oxytocin, the disulfide ring structure itself possesses some degree of intrinsic receptor affinity. This led us to consider the question of whether the disulfide ring moieties of some inhibitors of oxytocin might retain receptor affinity and inhibitory potency.

Studies[7-12] of the effect of alkyl substituents on the hemi-cystine residue in position 1 of oxytocin and deamino-oxytocin indicated that β,β-dialkyl substitution[10] yielded potent inhibitors of the oxytocic, avian vasodepressor, and rat pressor activities of oxytocin and vasopressin. A series of deamino analogs was synthesized which exhibited antioxytocic potencies increasing in the order [1-β-mercapto-β,β-dimethylpropionic acid]oxytocin[7] < [1-β-mercapto-β,β-diethylpropionic acid]oxytocin ([β-Mpa(β-Et_2)[1]]-oxytocin) [11] < [1-β-mercapto-β,β-pentamethylenepropionic acid]oxytocin ([β-Mpa(β-$(CH_2)_5$)[1]]oxytocin).[12] Accordingly, we have incorporated the two most effective inhibitory modifications into analogs of tocinamide and tocinoic acid.

The protected polypeptide precursors (*e.g.* β-Mpa (β-Et_2)(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-OBzl) were synthesized by classical stepwise solution methods. Each tocinamide and tocinoic acid analog pair was generated in the same flask after subjecting the appropriate protected hexapeptide Bzl ester to a preliminary 4 hr reflux period in liquid NH_3 so that some of the protected polypeptide ester would be converted to the amide. The remaining Bzl protecting groups were removed by reduction with Na and the mixture of disulfhydryl hexapeptide amide and acid was cyclized by oxidation with ICH_2CH_2I.[13] The product mixture was separated into an amide peak and an acid peak by partition chromatography[14] on Sephadex G-25 in the solvent system 1-butanol/pyridine/H_2O (6:1:7). The acid peak was strongly retarded relative to the amide peak in this basic system. Further purification of the amide and acid peaks by gel filtration[15] on Sephadex G-25 in 0.2 *N* HOAc or on Sephadex LH-20 in DMF served to remove dimeric material. All four analogs were found to be devoid of oxytocic, avian vasodepressor and rat pressor activities when tested against U.S.P. posterior pituitary reference standard but produced inhibition of the oxytocic and avian vasodepressor responses to synthetic oxytocin.[11] The $\overline{M}$ values represent the average molar concentration of inhibitor needed to reduce the appropriate biological response for a dose of 2X units of oxytocin to that normally seen for X units.[16]

Table I

Inhibitory Potencies

Analog		antioxytocic $X10^7\overline{M}$	antioxytocic pA_2	antiavian vasodepressor $X10^8\overline{M}$	antiavian vasodepressor pA_2
[β-Mpa(β-Et_2)^1]	tocinoic acid	3.3 (15) σ = 1.4	6.48	18 (11) σ = 10	6.75
	toxinamide	2.1 (28) σ = 0.6	6.68	4 (8) σ = 2	7.42
	oxytocin[11]	0.6 (9) σ = 0.1	7.24	0.8 (8) σ = 0.2	8.11
[β-Mpa(β-$(CH_2)_5$)^1]	tocinoic acid	4.3 (16) σ = 0.8	6.37	35 (8) σ = 13	6.46
	tocinamide	1.8 (41) σ = 0.5	6.74	5 (11) σ = 2	7.28
	oxytocin[12]	0.4 (33) σ = 0.1	7.43	0.5 (22) σ = 0.2	8.30

$pA_2 = -\log \overline{M}$.[16] The number of determinations is given in parentheses and σ is the standard deviation.

The tocinamide analogs exhibit similar potencies in each of these two assay systems and in each pair of compounds the tocinamide analog is more potent than the tocinoic acid analog. It is apparent from these data that the compounds reported here, representing just the 20 member disulfide ring fragments of two potent inhibitors of oxytocin, have retained to a very significant extent the antioxytocic and antiavian vasodepressor properties of the intact oxytocin analogs. It is interesting to note that both tocinamide analogs are effective inhibitory agents in both assay systems while tocinamide and deaminotocinamide have agonist activity in the oxytocic assay but not in the avian vasodepressor assay.

The compounds reported here were also tested for inhibitory activity against synthetic lysine vasopressin in the rat antipressor assay.[12] Both [β-Mpa(β-Et_2)^1]tocinamide and [β-Mpa(β-Et_2)^1]tocinoic acid exhibited weak and variable rat antipressor effects while [β-Mpa(β-$(CH_2)_5$)^1] tocinamide and [β-Mpa(β-$(CH_2)_5$)1] tocinoic acid showed no inhibition. These results parallel those found previously for the parent oxytocin analogs in this assay system, where [β-Mpa(β-Et_2)^1]oxytocin proved to be a moderately strong rat antipressor compound ($\overline{M}$ = 5.7 x 10^{-7}; pA_2 = 6.2)[12] while the antipressor effect of [β-Mpa(β-$(CH_2)_5$)^1] oxytocin was weak ($\overline{M}$ < 12 x 10^{-7}).[12]

Acknowledgment

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DEGRADATION BY HUMAN PLACENTAL TISSUE EXTRACTS OF OXYTOCIN RADIOACTIVELY LABELLED IN POSITIONS 2 AND 3

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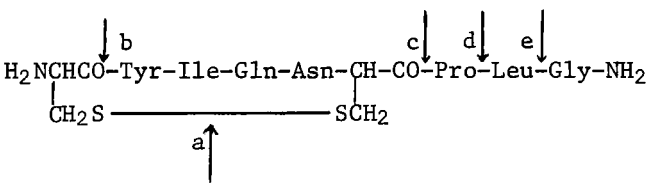
THE STUDY OF ENZYMATIC INACTIVATION of polypeptide hormones at either their target organs or by other biological systems is of prime importance in contributing to the knowledge of the mechanism of hormone action. In this context much attention has been given to the role of enzymes in the biological inactivation of the neurohypophysial hormones, oxytocin and vasopressin (for reviews see refs. [1] and [2]).

In the scheme below the known sites of enzymatic cleavage of oxytocin are shown:-



(a) Cleavage of the disulphide bond by action of thiol-protein-disulphide oxidoreductases is known to occur in human placental tissue homogenates[3-6] and possibly in the myometrium[7] and uterus.[8]

(b) Attack of the amide bond between the N-terminal cystine residue and adjacent tyrosine. This mechanism of inactivation is best exemplified by pregnancy cystine aminopeptidase.[1]

(c) Liberation of radioactivity labelled Pro-Leu-Gly-NH_2, albeit in small quantities, has recently been demonstrated after incubation of ^{14}C-labelled oxytocin with rat and rabbit hypothalamic fractions.[9]

(d) A novel enzyme capable of cleaving the prolyl-leucine bond of oxytocin (and the prolyl-arginine bond of vasopressin) has been partially purified from human uterine tissue[10] and also identified in rat brain tissue [11] and kidney extracts.[2]

(e) Hydrolytic cleavage of the leucyl-glycinamide bond with a subsequent release of glycinamide is a mechanism associated with kidney,[12] uterus[13] and brain extracts.[11]

In this paper we have reinvestigated the sites of cleavage of radioactively labelled oxytocin by human placental extracts.

Methods and Materials

Placentas were obtained by normal vaginal delivery and the villi and connective tissue scraped from the chorionic plate and washed free of blood with cold (4°) saline (0.9% w/v). The tissue was homogenized in an equal volume of phosphate buffer (0.01*M*, pH 7.4) using an Ultra Turrex T45 homogenizer (Janke and Kunkel & Co., Germany) after which the particulate fraction was separated by centrifugation at 1400g for 10 min. The supernatant was dialyzed for 18 hr in Visking 18/32 cellophene membranes against excess phosphate buffer (0.01*M*, pH 7.4) at 4°. Term pregnancy serum was similarly dialyzed. Serum was also treated with anti-oxytocinase serum as previously described.[14]

A solution (0.5ml) of either [9-glycinamide-1-^{14}C]-oxytocin (^{14}C-oxytocin, specific radioactivity 29.6 mCi/m*M*) or [2-tyrosine-3-^{3}H]-oxytocin ((^{3}H-tyrosine) oxytocin; specific radioactivity 1.8 Ci/m*M*) was incubated at 37° with either dialyzed pregnancy serum (50µl) or dialyzed placental homogenates. Samples (30µl) were removed at varying time intervals using a capillary tube and plunged into boiling water for 15 min and precipitated material subsequently removed by centrifugation (15000g for 10 min). $ZnCl_2$ ($10^{-4}M$) was also added to some of the incubation mixtures.

The supernatant (20µl) was applied in 1.5 cm bands to pieces (20 x 40 cm) of Whatman No. 1 chromatography paper, 10 cm from one end. Separation of peptides and amino acids was carried out by electrophoresis at pH 1.9 [formic acid 2.5% v/v) and acetic acid (8.7% v/v)] at 1500V (17-19 ma) for 40 min at 10° using the apparatus described by Troughton *et al.*[15]

After electrophoresis, the dried paper was cut into longitudinal strips (2.5 cm wide) corresponding to the direction of migration of the individual samples. Areas of radioactivity were detected using an Actigraph III paper scanner (Nuclear Chicago, U.S.A.) and their positions related to authentic samples of oxytocin, tyrosine, glycine, glycinamide and leucylglycinamide. These latter standards were separated on the same sheet of paper as the test samples and were located with ninhydrin. In some cases the portions of each paper strip corresponding to the positions of the standards were cut out and the amino acids eluted with water. The eluate was collected in scintillation vials, freeze-dried and after addition of Bray's solution was counted for radioactivity.

Chymotryptic activity of placental homogenates was measured by the method described by Hummel[16] using N-benzoyl-*L*-tyrosine ethyl ester as substrate. One unit of activity is that amount of enzyme that will hydrolyse one micromole of substrate/min at 25° at pH 7.8.

Results

The effect of incubating pregnancy serum and serum treated with anti-oxytocinase serum with [2-tyrosine-3-^{3}H]-oxytocin is shown in Figure 1a. Oxytocin was degraded at approximately the same rate as the release of labelled tyrosine. After treatment with anti-oxytocinase only trace amounts of radioactive tyrosine were liberated. Experiments with placental extracts (Figure 1b) showed a similar release of tyrosine and disappearance of oxytocin. Zinc ions ($10^{-4}M$) which does not inhibit oxytocinase (C.W. Small & W. B. Watkins, unpublished results) reduced the rate of release of tyrosine as did treatment with anti-oxytocinase. No liberated tyrosine was detected when zinc was added to the anti-oxytocinase treated placental homogenates *i.e.* the effects of zinc and antioxytocinase on placental degradation of oxytocin were additive (Figure 2a).

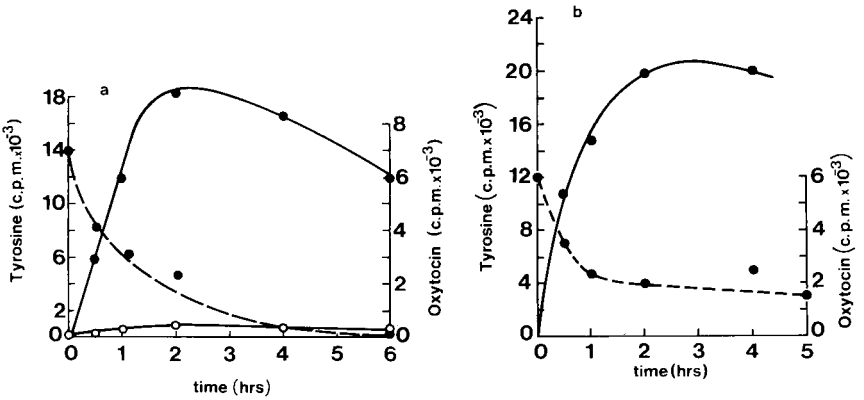


Figure 1: (a) Degradation of [^{3}H-tyrosine]-oxytocin by pregnancy serum (●——●) and pregnancy serum treated with anti-oxytocinase serum (o——o). ^{3}H-tyrosine released (●——●, o——o) and tritiated oxytocin present in incubation medium at various time intervals (●– –●). (b) Degradation of [^{3}H-tyrosine]-oxytocin (●– –●) and release of [^{3}H-tyrosine] (●——●) on incubation of labelled peptide with placental homogenates.

When a placental extract was incubated with [9-glycinamide-1-^{14}C]-oxytocin, the radioactively labelled oxytocin decreased rapidly over a period of 2 hrs (Figure 2b). The reaction product that predominated was glycinamide which reached a maximum amount after approximately 4 hr, and was accompanied with the concomitant appearance of glycine. This conversion was completed after a period of 24 hr. Leucylglycinamide was also detected in the reaction mixture during the 30 min to 4 hr incubation period with the maximum release of the dipeptide occurring after 1 hr. Figure 3 shows that the rate of release of glycinamide and tyrosine from oxytocin are similar.

Some of the properties of the glycinamide releasing system are given in Table I. The enzyme is relatively stable at 20° for 1 hr but is almost completely inhibited by Zn^{++} [10^{-4} *M*] and 2-nitro-4-carboxyphenyl-N,N-diphenyl carbamate (NCDC) and partially inhibited by EDTA. In contrast to the oxytocinase activity which residues primarily in the microsomal fraction of a placental homogenate, the enzyme (s) responsible for the release of ^{14}C-glycinamide are present in the soluble phase.

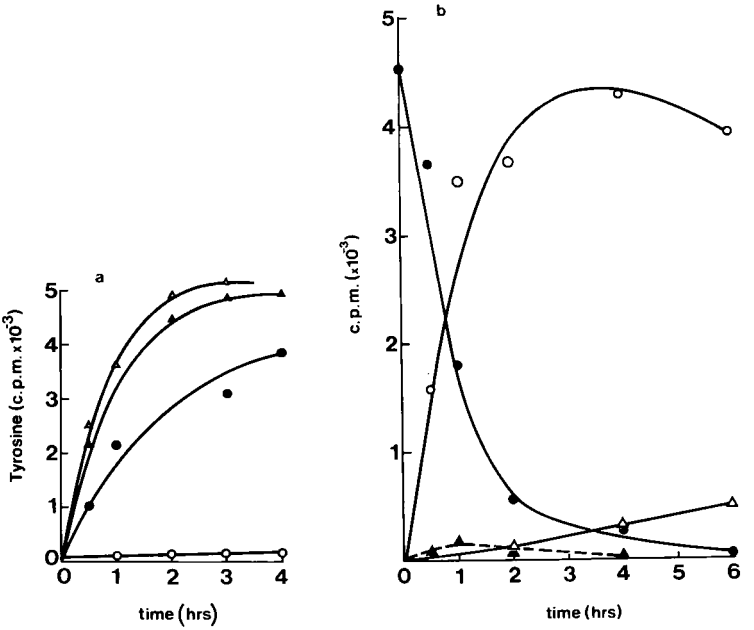


Figure 2: (a) Effect of treated placental homogenates on the release of ^{3}H-tyrosine from [^{3}H-tyrosine]-oxytocin. Untreated homogenate (Δ——Δ); homogenate plus $Zn^{2+}(10^{-4}M)$ (▲——▲); treated with anti-oxytocinase serum (●——●); treated with anti-oxytocinase serum plus $Zn^{2+}(10^{-4}M)$ (o——o). (b) Appearance of ^{14}C-labelled compounds from [^{14}C-glycinamide]-oxytocin at various times after incubation with placental extracts. Residual labelled oxytocin (●——●); glycinamide (o——o), glycine (Δ——Δ), leucylglycinamide (▲——▲).

The content of chymotrypsin in various placental homogenates in compared in Table II with other tissues known to contain chymotrypsin activity. That NCDC inactivates the placental enzyme capable of hydrolysising N-benxoyl-*L*-tyrosine ethyl ester is consistent with its identity as a chymotrypsin-like enzyme. Only minimal chymotryptic activity was detected in pooled pregnancy serum.

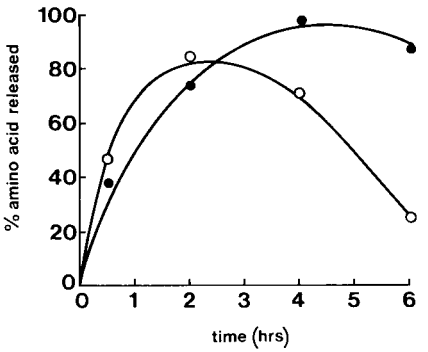


Figure 3: Relative rate of release of [^{3}H-tyrosine] (o——o) and ^{14}C-glycinamide (•——•) from radioactively labelled oxytocin by placental homogenates.

Table I

Effect of Storage (24 hr) and Inhibitors on Release of Glycinamide from C^{14}-oxytocin by Placental Extracts

Condition	*% Glycinamide released in 1 hr*
-20°	78
4°	64
20°	48
37°	13
EDTA ($10^{-4}M$)	11
Zn^{+}($10^{-4}M$)	1.6
NCDC	0
Pregnancy plasma	0

Table II

Chrymotryptic Activity of Various Tissue Samples

Sample [a] *(homogenate)*	*Activity (U/ml)*
Placenta	0.65[b] (0.03)[c]; 1.02 (0.01); 4.4 (1.4)[d];
Rat uterus	0.65 (0.03)
Rat kidney	0.74 (0.08)

Key to Table

(a) The tissues were extracted in ice-cold 0.9% (w/v) saline (1ml/100mg wet weight of tissue) and the supernatant, collected by centrifugation (105,000g for 1 hr) diluted with an equal volume of saline prior to enzyme assay

(b) Activity of homogenate

(c) Activity of homogenate plus NCDC

(d) Activity of homogenate plus EDTA ($10^{-4}M$)

Discussion

Oxytocinase, the cystine aminopeptidase associated with primate pregnancy has for almost 20 years been considered to be that placental constituent primarily responsible for the degradation of oxytocin. Recently, however, a specific glutathione-oxytocin-transhydrogenase enzyme system has been identified in human placental extracts.[6]

On incubation of placental extracts with [2-tyrosine-3-^{3}H]-oxytocin the rate of degradation of oxytocin was faster than the appearance of tritiated oxytocin. This is suggestive that more than one mechanism is involved in the inactivation of the peptide hormone. Detection of leucylglycinamide in the incubation mixture indicates the presence of an enzyme capable of cleaving the prolyl-leucine bond of oxytocin. The time-course profile of release of this dipeptide amide is similar to that observed when labelled oxytocin is inactivated by rat brain extracts.[9]

The appearance of glycinamide, on the other hand may result from the mechanisms:- (a) cleavage from the C-terminal of oxytocin by the action of endopeptidases and/or (b) hydrolysis of the leucylglycinamide fragment. Almost 95% of the radioactivity added to the incubation system appeared in the form of ^{14}C-glycinamide after 4 hr whereas only 5% of the radioactivity was accounted for in leucylglycinamide after 1 hr. This data clearly indicates the important role of endopeptidases in the release of glycinamide and the minimal contribution from the dipeptide amide. A similar pathway has been proposed for the degradation of the neurohypophysial hormones by extracts of kidneys, uteri and whole brains.[11-13]

That some of the placental enzymes responsible for the cleavage of glycinamide are chymotrypsin-like has been established in this work. Since chymotrypsin activity is also present in rat kidney and uterine extracts it is most probable that it is the presence of this enzyme in these extracts which aocounts for the cleavage of oxytocin at position 9.[17]

Partial purification of the placental enzyme liberating leucylglycinamide will be required in order to establish its identity with a similar enzyme system found in human uteri.[10] From Figure 3 it appears that, *in vitro*, at least, the role of placental endopeptidases and aminopeptidases contribute approximately equally in the degradation of oxytocin. However, whether this is also true *in situ* remains to be confirmed especially in the light of the work by Sjöholm[18] who concluded that oxytocin is taken up extensively by the liver and kidneys and probably completely inactivated at these sites.

Acknowledgment

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RELEASE OF MELANOCYTE-STIMULATING HORMONE BY NEUROHYPOPHYSEAL HORMONE FRAGMENTS

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Summary

Cys-Tyr-Ile(or Phe)-Gln-Asn,N-Tos-Cys(S-Bzl)-Tyr-Ile-Gln-Asn, Tyr-Ile-Gln-Asn and Ile-Gln-Asn at ng concentration decreased the pituitary MSH content of intact male rats to about 50% and increased serum MSH concentration indicating that MSH release from the pituitary had occurred. The MSH release induced by these peptides was not prevented in rats in which the pituitary gland had been disconnected from the central nervous system by lesioning the median eminence, thus revealing a direct action on the gland. No significant difference among the effect of the peptides on MSH release is apparent on a molar basis. The dimers (Cys-Tyr-Ile(or Phe)-Gln-Asn)$_2$ are ineffective in promoting MSH release, as are several other peptides.

Introduction

The release of melanocyte-stimulating hormone (MSH) is regulated by two factors[1-3] which are formed by hypothalamic enzymes using oxytocin as substrate.[4,5] One

of the factors, *i.e.*, Pro-Leu-Gly-NH_2 (MRIF) formed from oxytocin,[6-8] has the capacity to inhibit the release of pituitary MSH. In contrast, the N-terminal pentapeptide of oxytocin, Cys-Tyr-Ile-Gln-Asn, was found to be a potent stimulant for the release of MSH.[9]

In the current study we investigate whether other neurohypophyseal hormone fragments can stimulate MSH release *in vivo*. The effect of several peptides on pituitary and serum MSH levels in intact and median eminence (ME)-lesioned male rats was tested.

Materials and Methods

Male rats (180-200g) were used. The techniques used to study the decrease in pituitary MSH content[1] and MSH serum levels[9] were reported, as was the method for preparing rats with ME lesions.[10] Stock solutions were prepared by dissolving the peptides in 50% acetic acid, which had been carefully deaerated by prolonged flushing with nitrogen gas. Just prior to use aliquots of the appropriate stock solution were diluted with deaerated saline in order to obtain the desired peptide concentration in 0.20ml.

Results

The MSH releasing activity of Cys-Tyr-Ile-Gln-Asn was confirmed. In addition, the protected pentapeptide, N-Tos-Cys(S-Bzl)-Tyr-Ile-Gln-Asn, as well as Cys-Tyr-Phe-Gln-Asn, Tyr-Ile-Gln-Asn, and Ile-Gln-Asn decreased pituitary MSH content (Figure 1). The disulfide dimers of Cys-Tyr-Ile(or Phe)-Gln-Asn were inactive (Figure 1), as were Cys(S-Bzl)-Tyr-Ile-Gln-Asn-Cys(S-Bzl)-Pro-Leu-Gly-NH_2, Tyr-Ile-Gln-Asn-Cys(S-Bzl)-Pro-Leu-Gly-NH_2, Ile-Gln-Asn-Cys(S-Bzl)-Pro-Leu-Gly-NH_2, Cys(S-Me)-Tyr-Phe-NH_2·HCl, Met-Tyr-Phe-NH_2·HCl, and Gln-Asn.

The relative MSH releasing activities of Cys-Tyr-Ile-Gln-Asn, Cys-Tyr-Phe-Gln-Asn, Tyr-Ile-Gln-Asn and Ile-Gln-Asn have been compared *in vivo* by measuring the ability of the peptides to decrease pituitary MSH content and increase serum MSH levels in intact rats (Figure 2).

Next we studied the effect of increasing amounts of Cys-Tyr-Ile-Gln-Asn, Tyr-Ile-Gln-Asn or Ile-Gln-Asn on serum MSH levels of intact rats. As shown in Figure 3 a dose-dependent relationship is observed and all three peptides exhibit the same specific activity.

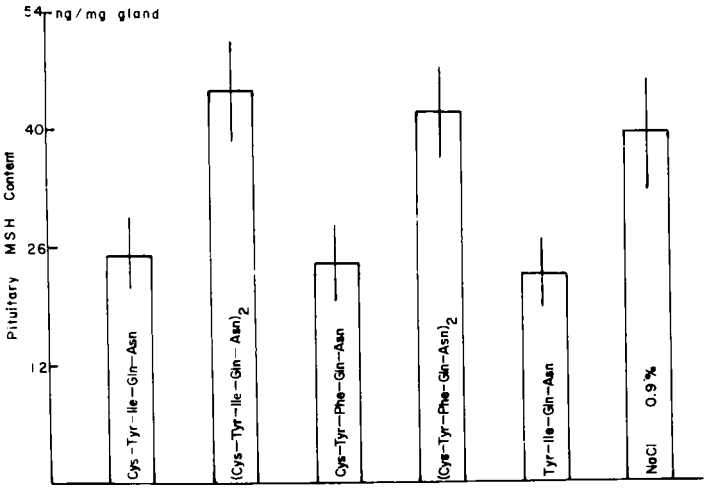

Figure 1: Effect of several neurohypophyseal hormone fragments on pituitary MSH content in intact rats. Peptides dissolved in ∿0.2 ml 0.9% NaCl were injected i.v. and the animals were sacrificed 20 min later. Animals injected with 0.2 ml of saline served as controls. A homogenate of two pituitaries was tested by an *in vitro* assay[1] for the MSH present. A synthetic α-MSH standard was used. The values represent the weighted mean of three experiments, except the control value is from five experiments. Mean and confidence limits were calculated according to the method of Bliss.[11]

Discussion

Some years ago we reported that the N-terminal pentapeptide of oxytocin, Cys-Tyr-Ile-Gln-Asn, possesses the activity profile expected for a MSH-releasing hormone.[9] The present work was performed in order to test whether this peptide is the only active fragment of oxytocin and thereby to evaluate the specificity of this compound. Prior to characterization of a compound as a new hormone, the principle would have to be first isolated from natural sources, its mechanism of formation would have to be understood and its mode of action be elucidated; however, the approach chosen by us can indicate the route for future work.

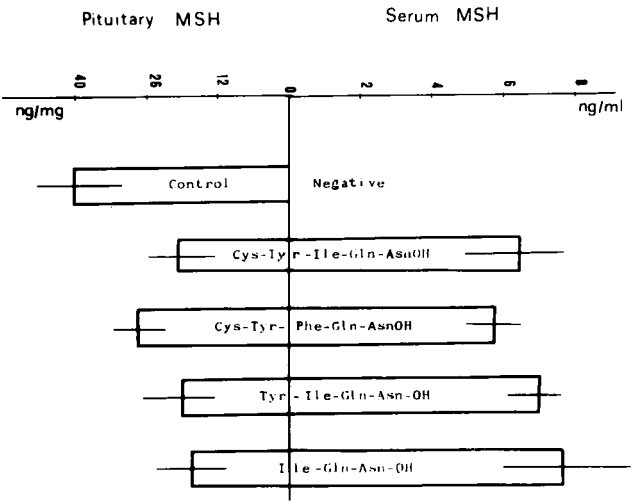

Figure 2: Effect of several hormone fragments on the pituitary and serum MSH levels. Experimental details are described in legend to Figure 1. For determination of serum MSH levels the animal was sacrificed 12 min after i.v. administration of peptide.

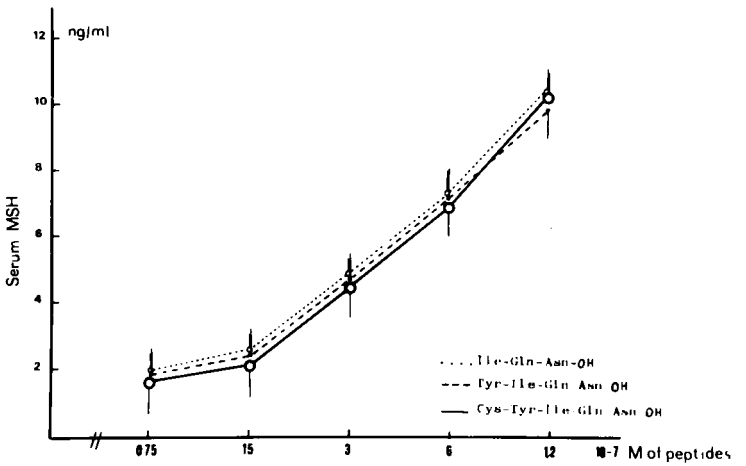

Figure 3: Dose-response relationship between amounts of oxytocin fragments injected into intact rats and MSH detected in serum. For details, see legends of Figures 1 and 2.

This study reveals that in addition to Cys-Tyr-Ile-Gln-Asn, its N-protected derivative as well as its close congener Cys-Tyr-Phe-Gln-Asn, are active in stimulating the release of MSH. More significantly, while the dimers of the N-terminal pentapeptides of oxytocin and vasopressin are inactive, analogs with fewer residues are active (Figures 1 and 2). In fact, all of the active peptides tested have the same dose-dependency (Figure 3). The tripeptide, Ile-Gln-Asn, seems to have both the active site and the binding capacity for maximal activity. The possibility cannot be excluded at this time that elongation of these active N-terminal hormone fragments in the C-terminal direction will yield active (or even more active) analogs. It will be recalled, however, that tocinoic acid, the cyclic N-terminal hexapeptide of oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys), did not deplete MSH from the pituitary *in vivo*[12] or *in vitro*[13]; the fact that several of the acyclic fragments of oxytocin tested in the present study are inactive lends further credence to the idea that elongation of the peptide chain at the carboxyl side of the Asn residue yields inactive analogs. In any event, information on this point should readily be forthcoming since the technical difficulties inherent in carrying out biological studies with a thiol such as the Cys-Tyr-Ile-Gln-Asn can now be circumvented by the use of the shorter peptides, the protected N-terminal pentapeptide or analogs of Ile-Gln-Asn (or possibly Phe-Gln-Asn).

Table I

Changes of Pituitary and Serum MSH Levels

Caused by Fragments of Oxytocin in Rats with ME Lesions*

	Pituitary MSH ng/mg	*Serum MSH ng/ml*
Cys-Tyr-Ile-Gln-Asn	23.5(19.6-27.3)	7.8(7.4-8.2)
Cys-Tyr-Phe-Gln-Asn	30.8(27.4-34.7)	6.5(6.0-7.0)
Tyr-Ile-Gln-Asn	25.7(20.6-31.8)	7.4(6.7-7.9)
Ile-Gln-Asn	15.0(12.0-19.3)	9.0(8.5-9.4)
Control	38.6(35.3-42.6)	negative

* For experimental detail see legends to Figures 1 and 2.

It is important to note that not only Cys-Tyr-Ile-Gln-Asn but also all of the other biologically active peptides studied stimulate the release of MSH by a direct action on the pituitary gland as shown in Table I above. Significantly higher levels of serum MSH as well as a decrease of pituitary MSH content were found as compared to controls in rats with ME lesions after injection with the peptides listed.

Acknowledgment

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EFFECT OF PRO-LEU-GLY-NH_2 AND TOCINOIC ACID ON THE SECRETION OF MELANOCYTE-STIMULATING HORMONE FROM RAT PITUITARIES INCUBATED *IN VITRO*

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IT HAS BEEN POSTULATED that the hypothalamus exerts an inhibitory control on secretion of melanocyte-stimulating hormone (MSH).[1] An hormonal factor, melanocyte-stimulating-hormone-release-inhibiting factor (MRIF) appears to be involved in such control.[2,3] There exists an enzymatic system in rat hypothalamic extracts which can form MRIF activity[4] on using oxytocin as a substrate.

Earlier it has been proven that Pro-Leu-Gly-NH_2, the C-terminal peptide released from oxytocin, at ng concentrations acts *in vivo* and *in vitro* as MRIF.[5,6] This tripeptide has subsequently been isolated from hypothalamic extracts.[7] Moreover, it has been shown that this tripeptide specifically inhibits the release of MSH and it has no effect on the release or inhibition of release of LH, LTH or FSH.[8,9]

On the other hand, Bower *et al.*[10] claim that tocinoic acid, the ring structure of oxytocin, has the capacity to inhibit MSH release *in vitro*. However, other authors do not find any activity with any of these peptides on the secretion of MSH in rats.[11]

These differences in results encouraged us to make a comparative study *in vitro* using a wide range of doses of Pro-Leu-Gly-NH_2 and tocinoic acid.

Materials and Methods

Male albino rats were decapitated and the pituitaries immediately removed and divided in half. Both halves were incubated at 37°C in a Dubnoff metabolic shaker, using the Krebs-Ringer-Bicarbonate buffer, pH 7.4. After 30 min of incubation the gland pieces were placed in 1 ml of fresh buffer.

Pro-Leu-Gly-NH_2, tocinoic acid or various hypothalamic extracts were added at the concentration mentioned in the text to one half of the gland; the other half was a control. The incubation was carried on for one hour more at 37°C. After this hour both halves of the gland were weighed and the amount of MSH released during incubation was determined by an *in vitro* method[12] through direct comparison with a standard of α-MSH. The design used was 2 x 2 (2 doses of reference preparation and 2 doses of the test preparation[13]) and content of MSH in the incubation medium was expressed in ng/mg/ml.

Extracts of median eminence (ME) were obtained from male albino rats and extracts of supraoptic nuclei (SON) from ovariectomized rats used three weeks after the operation. The animals were sacrificed, the brain was isolated and the hypothalamus was cut as shown earlier. The hypothalami were homogenized using 0.1 ml of 0.1 M phosphate buffer per hypothalamus. Pro-Leu-Gly-NH_2 and tocinoic acid solutions were prepared just prior to use.

Results

The mean value of MSH release from the pituitary halves incubated *in vitro* in buffer was found to be -4.6 with a range between -15 to 9.

Effect of Pro-Leu-Gly-NH_2 on the Secretion of MSH by Pituitaries Incubated *In Vitro*

Table I summarizes the effect of Pro-Leu-Gly-NH_2 on the release of MSH by pituitary halves incubated *in vitro*. Between 10 and 30 ng of tripeptide, the amount of MSH released by the gland decreased by about 60%. Doses between 10^3 to 10^4 ng induced release or inhibition of MSH release in individual preparations; however, the mean value from this group of experiments was no different from control. The dose of 10^5 ng induced release of large amounts of MSH.

One, two, or four SON extracts added to one of the half pituitaries induced a dose-dependent inhibition of MSH release (Figure 1).

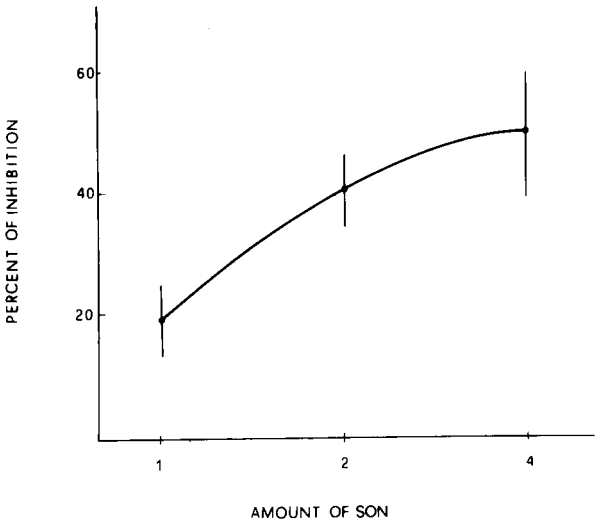

Figure 1: Effects of SON extracts of ovariectomized rats on the inhibition of MSH release by pituitaries *in vitro*.

To study whether high doses of Pro-Leu-Gly-NH_2 could reverse the inhibition of the release induced by SON extracts, 10^5 ng of the tripeptide was added to one of the halves that contained 4 SON extracts. The other half received only 4 SON extracts. The results in Table II show an increase in the MSH released into the medium in the pituitary half containing both the tripeptide and the SON extracts.

Fresh ME extracts of male rats induced pituitary MSH release *in vivo*.[12] Unpublished results have shown a similar behavior in pituitaries incubated *in vitro*. The effect of low doses of Pro-Leu-Gly-NH_2 on the release induced by the ME extracts was also studied by adding 40 ng of the tripeptide to one of two pituitary halves incubated in the presence of ME extract. The pituitary half with both ME and the tripeptide released less MSH into the medium.

Table I

Effect of Different Doses of Pro-Leu-Gly-NH_2 on the Release of MSH from Rat Pituitary Glands Incubated *In Vitro*

Tripeptide ng/ml	*Amount of MSH released ng/mg/ml*				*Percent of change*
	*Control**		*Experimental***		
10	42.7	(2.25)	16.2	(3.90)	-63
	39.1	(2.45)	9.5	(2.97)	-76.7
	44.0	(3.98)	29.1	(3.57)	-34
	41.9±1.46	2.59±0.24	18.2±5.75	3.48±0.26	-57.6±12.41
30	30.3	(5.27)	16.3	(3.96)	-47
	41.2	(5.88)	9.5	(5.68)	-77
	32.2	(5.27)	19.3	(4.22)	-41
	34.5±3.36	5.47±0.20	15.0±2.69	4.62±0.52	-55.0±11.13
1.10^2	32.6	(3.53)	25.8	(3.10)	-21
	29.8	(3.00)	29.9	(3.04)	0.33
	31.2±1.40	3.26±0.26	27.8±2.04	3.07±0.03	-10.3±10.66
1.10^3	33.7	(2.22)	31.2	(3.30)	- 8.5
	34.6	(3.18)	27.3	(2.81)	-21
	25.1	(2.22)	31.66	(2.21)	-25
	31.1±3.02	2.54±0.31	30.0±1.37	2.77±0.30	- 1.3±13.69

1.10^4	26.0	(2.70)	12.4	(2.77)	-53
	47.2	(2.87)	63.0	(3.08)	33
	25.1	(2.22)	31.0	(2.20)	23
	32.7±7.22	2.59±0.17	35.4±14.77	2.68±0.24	1.0±27.15
1.10^5	29.3	(2.45)	61.2	(2.59)	108
	48.8	(2.30)	62.5	(2.49)	28
	34.4	(2.77)	41.3	(3.00)	20
	27.2	(2.73)	29.3	(2.76)	7
	24.9	(3.10)	41.1	(2.84)	65
	32.9±4.26	2.67±0.10	47.0±6.41	2.73±0.09	45.6±18.33

* Control: pituitary half in buffer
** Experimental: pituitary half plus Pro-Leu-Gly-NH_2
In parentheses pituitary halves weight expressed in mg
⁘ MEAN ± S. E.

Table II

Effect of 100 µg of Pro-Leu-Gly-NH_2

on SON-Induced Inhibition of MSH Secretion

in Rat Pituitary Incubated *In Vitro*

No. of Experiment	*Amount of MSH released ng/mg/ml*				*Percent of Change*
	*Control**		*Experimental***		
3	28.6±0.7	(3.44)±0.4	46.5±1.2	(3.28)±0.5	62.0±0.1

* Control: pituitary half incubated with 4 SON

** Experimental: pituitary half incubated with 4 SON plus 100 µg Pro-Leu-Gly-NH_2

Effect of Tocinoic Acid on the MSH Release in Pituitary Halves Incubated *In Vitro*

Tocinoic acid in similar doses to that of Pro-Leu-Gly-NH_2 was used. No MRIF activity was found. The mean of 15 experiments was 5.7 ± 5.22% inhibition of the control.

Discussion

The inhibitory effect that Pro-Leu-Gly-NH_2 exerts at ng concentration on MSH release by pituitary gland incubated *in vitro* is lost when the amount of the tripeptide is elevated to µg concentrations. Doses from 1 to 10 µg can induce either MSH release or inhibition of MSH release, indicating that according to the peptide concentration the response can be reversed. The variability of the results found could be due to a different sensitivity of the various pituitaries to Pro-Leu-Gly-NH_2. A dose of 100 µg of tripeptide induced MSH release in all the experiments, although a wide range of values was observed. This may explain the finding of Grant *et al.*[11] who found MSH release in some samples using 10 to 50 µg of the tripeptide. Confirming Bower *et al.*[10] we found no MRIF activity with a dose of 100 ng of Pro-Leu-Gly-NH_2.

The results of the few additional experiments presented, although they represent no proof, are in line with the contention that Pro-Leu-Gly-NH_2 is the natural MRIF. First, the inhibition exerted by SON extracts on MSH release by pituitaries incubated *in vitro* is reversed in the presence of 100 µg of tripeptide, a concentration of Pro-Leu-Gly-NH_2 which can release MSH (Table II). Second, if low doses of Pro-Leu-Gly-NH_2 (40 ng) induced inhibition of MSH release in a system where the MSH is released, this could be taken as another evidence to support the possibility that both Pro-Leu-Gly-NH_2 and the natural MRIF are the same substance. The results (Table III) support the possibility that both substances could be the same.

Bower *et al.*[10] have previously reported that tocinoic acid, the ring structure of oxytocin could act as MRIF *in vitro*. In our hands, repetition and extension of their experiments using doses between 10 and 10^5 ng were unable to inhibit MSH release; although in one out of 15 experiments inhibition was observed, the mean value for all the experiments did not show any significant difference from the controls. Bower *et al.*[10] showed an inhibitory effect of hypothalamic extracts on MSH release by pituitary incubated *in vitro*, as well as an inhibitory effect of tocinoic acid. In comparing both results it is seen that the slope is very different for the two types of experiment. This finding suggests that the MRIF present in hypothalamic extracts and tocinoic acid may not be the same substance.

It will be recalled that the transition from inhibitory effect on release to releasing effect of Pro-Leu-Gly-NH_2 as a function of the concentration of tripeptide finds its analogy in studies with catecholamines.[14,15] The present results offer an explanation for the differences reported in the literature for the MRIF activity of Pro-Leu-Gly-NH_2 in rat.

In summary, a comparative study was made between the effect of Pro-Leu-Gly-NH_2 and tocinoic acid on MSH release by rat pituitaries incubated *in vitro*. The test agents were added to one half of a gland, and the other was used as control. MSH release into the medium was measured by a bioassay and the activity of the samples referred to a standard of synthetic α-MSH. Pro-Leu-Gly-NH_2 in doses of 10 to 30 ng inhibited MSH release by about 60%. Doses between 10^3 to 10^4 ng induced neither significant release nor inhibition of the release of MSH. A dose of 10^5 ng clearly induced release of MSH. Tocinoic acid used in the same doses as that of Pro-Leu-Gly-NH_2 did not produce any significant effect on the MSH release.

Table III

Effect of Pro-Leu-Gly-NH_2 on the MSH Release

Promoted by Median Eminence Extracts in Rat Pituitary Incubated *In Vitro*

No. of Experiment	*Amount of ME extract added*	*Amount of MSH released ng/ml*				*Percent of change*
		*Control**		*Experimental***		
2	1	56.2±11.3	(2.69)±0.13	25.5±2.7	(3.25)±0.18	-55.2±3.5

* Control: Median eminence extracts

** Experimental: Median eminence extracts plus 40 ng Pro-Leu-Gly-NH_2

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FREE SOLUTION CONFORMATION OF NEUROHYPOPHYSEAL HORMONES

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DETERMINATION OF THE FREE solution conformation of neurohypophyseal hormones in water and less polar solvents serves as a logical starting point for defining the conformation activity relationship of these molecules. Here we review studies of the free solution conformation in dimethyl sulfoxide (Me_2SO) and water of the neurohypophyseal hormones oxytocin, lysine vasopressin (LVP), and arginine vasopressin (AVP) by nuclear magnetic resonance (nmr) spectroscopy.

Limitations of Conformational Analysis in Solution

Molecular conformations in solution cannot be defined with nearly the precision and certainty attainable in the crystalline state by x-ray crystallography. Nmr spectroscopy is generally considered as the most comprehensive and promising method for studying conformations of biomolecules in solution. The chemical shifts of NH resonances usually permit identification of hydrogen bonded NH protons. However, consistently reliable criteria for distinguishing between peptide-solvent hydrogen bonds and intramolecular peptide hydrogen bonds have not yet been developed.[1-4] Four methods have generally been employed for this purpose: (1) rates of NH proton exchange with labile hydrogens of the solvent;[1,5] (2) temperature dependence of chemical shifts of NH resonances;[2,6] (3) dependence of NH chemical shifts on the composition of a suitable solvent mixture;[7] (4) degree of resonance broadening when a paramagnetic substance is added.[8] Each method has limitations; each can

yield useful information under favorable conditions. It is generally desirable to confirm internal hydrogen bonding by more than one method. Even when there is reasonable certainty that a specific NH hydrogen is internally bonded, identification of the specific acceptor cannot as yet be accomplished by reliable spectroscopic methods. Conformational averaging introduces ambiguities in the interpretations of coupling constants in terms of dihedral angles by means of the Karplus equation.[9] Because of the limitations imposed by existing spectroscopic techniques, the conclusions drawn in this review are tentative and subject to revision when more reliable methods for conformational analyses of peptides in solution become available.

Oxytocin in Dimethyl Sulfoxide

On the basis of spectra of oxytocin precursors and analogs and spin-decoupling experiments, Johnson *et al.*[10] assigned all the resonances in the 220 MHz ^{1}H nmr spectrum of oxytocin and deamino-oxytocin in Me_2SO to specific hydrogens of these hormones. Brewster *et al.*[11] confirmed most of the assignments[12] of oxytocin and deamino-oxytocin.

Urry and Walter[13] have proposed a model for the preferred solution conformation of oxytocin in Me_2SO. The essential features of their model are as follows: (1) The cyclic moiety of the hormone contains a 1→4 turn involving the sequence Tyr^2-Ile^3-Gln^4-Asn^5 with a hydrogen bond between the Tyr^2 CO and the Asn^5 peptide NH. An additional hydrogen bond between the CO of Asn^5 and the NH of Tyr^2 occurs in deamino-oxytocin. (2) A second β-turn was proposed for the Cys^6-Pro^7-Leu^8-Gly^9 (NH_2) sequence in the acyclic tail of the hormone. This structure is stabilized by hydrogen bonds between CO of Cys^6 and the peptide NH of Gly^9 and between the carboxamide CO of Asn^5 and the NH of Leu^8.

The model is consistent with the available ^{1}H nmr evidence. The low field positions of all the N$\underline{\text{H}}$ resonances of oxytocin indicate that in Me_2SO each of these hydrogens is hydrogen bonded. In the proposed model each NH is either internally bonded or is in an exposed orientation which permits hydrogen bonding with the solvent. Internal hydrogen bonding of the peptide NH hydrogens of Asn^5, Leu^8 and Gly^9 is suggested by the relatively slow rates of exchange of these hydrogens[14] and by the temperature independent chemical shift of the Asn^5 peptide NH.[15] Table I summarizes all the pertinent ^{1}H nmr data--chemical shifts (δ), temperature coefficients (dδ/dT) and peptide N$\underline{\text{H}}$-C$^{\alpha}\underline{\text{H}}$ coupling constants ($J_{N\alpha}$)--for oxytocin in Me_2SO. Peptide N$\underline{\text{H}}$ data

Table I

^{1}H Nmr Spectroscopic Parameters of
Oxytocin in Dimethyl Sulfoxide (at 22 ± 1°C)

Peptide NH	δ *ppm*	$10^3 x\ d\delta/dT$ *ppm/°C*	$J_{N\alpha}$ *Hz*
Tyr-2	8.35±.05*	-8.0±4.5,*-6**	7**
Ile-3	8.21*	-9.8,* -9.5**	4.4±.5,*5**
Gln-4	8.10*	-4.8,*-4**	6.6±.5,*6**
Asn-5	7.79*	.23,*0**	6.1±.2,*6**
Cys-6	8.74*	-11.1,*-11.5**	7.5±.1,*7.5**
Leu-8	8.06*	-6.8,*-7**	7.3±.2,*7.5**
Gly-9 (av.)	7.96*	-5.9,*-6.5**	5.6±.2,*5**
Primary Amide NH			
Asn, trans	7.40*	-4.5,*-2.5**	
Gln, trans	7.29*	-5.0,*-2.5**	
Gly, trans	7.12±.02*	-5.2±2.2,*-2.5**	
Gly, cis	7.09±.02*	-5.7±2.2,*-3**	
Asn, cis	6.88*	-5.7,*-3**	
Gln, cis	6.80*	-5.7,*-3**	

*Reference 29 (data at 20°C have been corrected to 22°C).
**Reference 11. Assignments of peptide NH resonances of Ile^3 and Gln^4 have been interchanged for reasons outlined in reference 12.

obtained in different laboratories agree within limits of experimental error. Urry and Walter[13,15] argued that the extreme high field position of the Asn^5 peptide NH peak relative to resonances of the other peptide hydrogens reflects the close proximity of the Asn^5 peptide hydrogen to the high field shifting region of the anisotropic field of the Ile^3-Gln^4 peptide bond--a constraint imposed by the geometry of the proposed 1→4 turn in the cyclic moiety of the hormone. Furthermore, these authors noted that all the observed values of $J_{N\alpha}$ were consistent with dihedral angles in their model. Its validity is further supported by subsequent x-ray data by Rudko *et al.*[16] on S-Benzyl-Cys-Pro-Gly(NH_2), which forms a β-turn with the same orientation as Urry and Walter proposed for the corresponding COOH-terminal moiety of oxytocin. A type I orientation[2] was proposed[13,15] for both β-turns in the oxytocin model, consistent with x-ray data showing that this orientation is

preferred unless a Gly or D-amino acid residue is at the i+2 position in the turn (the i+2 residues are Gln and Leu for the 1→4 turns in the cyclic moiety and acyclic tail, respectively, of the Urry and Walter model). However, the possibility remains that unique conformational constraints imposed by the cyclic moiety might stabilize a type II turn. That this may indeed be the case is suggested by the relatively small value of $J_{N\alpha}$ of the i+1 residue in the turn, Ile^3 (4-5 Hz). Nemethy and Printz[17] calculated a value of 4 Hz for this residue in a type II turn and about 8 Hz in a type I turn. Furthermore, the spectral parameters of oxytocin closely resemble those of two analogs--[D-Ala^4]oxytocin[18] and [^{3}Pro-^{4}Gly]-oxytocin[19]--in which a type II turn is expected. This tends to support a type II structure for the β-turn in the cyclic moiety, but a definitive proof would require conclusive evidence that the spectral characteristics of a type I structure would be measurably different from parameters of a type II structure. The side-chain orientations proposed by Urry and Walter are also tentative and require further refinement.

Concerning the chirality of the disulfide bond of oxytocin, Urry *et al.*[15] proposed a right-handed (P) helical screw sense from the circular dichroism (CD) spectrum of oxytocin in water.[20] However, a left-handed S-S bond could also be accommodated by the model. CD spectra of various cystine derivatives could be simulated assuming no contribution from a preferred screw sense of the S-S bond.[21a] The magnitude of the disulfide CD bands of oxytocin[20] argues against a significant preference for either screw sense, since rigid molecules exhibit much more intense S-S CD bands.[21] A dynamic equilibrium involving different orientations about the cystine S-S and α-β bonds of oxytocin appear likely.

Although the Urry and Walter model is consistent with available experimental data, it remains to be determined to what extent other structures may contribute to the time-average conformation of oxytocin in Me_2SO. Potential energy calculations[11,22,23] indicate that the model is a stable structure, but a number of other conformations with comparable stability are predicted. Brewster *et al.*[11] proposed that a rapid equilibrium exists between at least three conformations in Me_2SO which are all consistent with ^{1}H nmr data ($J_{N\alpha}$ and $d\delta/dT$) and potential energy calculations. In each of these structures (Figure 1), which differ with respect to the conformation of the cyclic moiety, the Asn^5 peptide NH is internally hydrogen bonded. Structure 1 is the Urry and Walter model, structure 2 contains a hydrogen bond between the Asn^5 peptide NH and the Gln^4 carboxamide CO, and

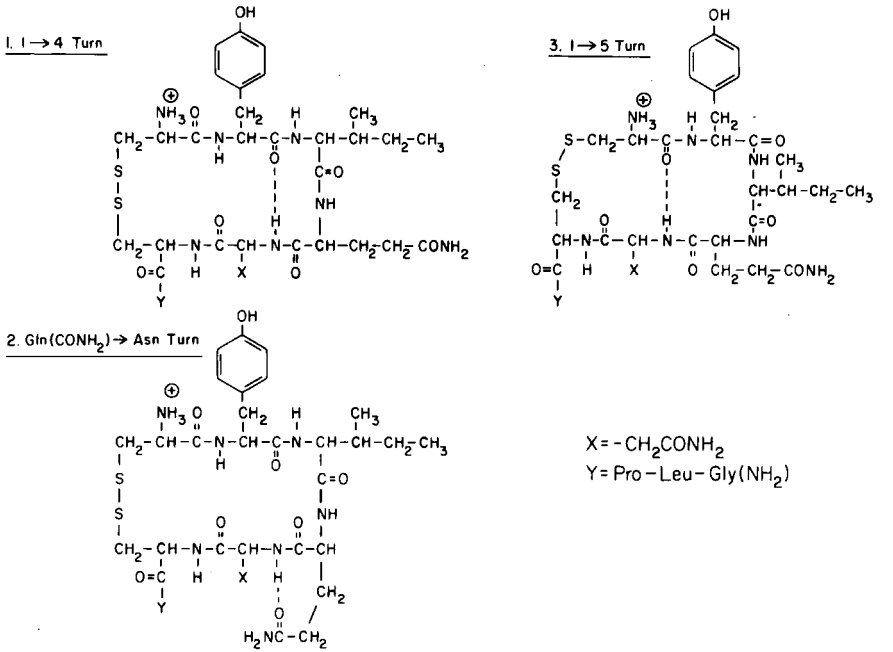

Figure 1: Schematic representation of proposed models for the conformation of the cyclic moiety of oxytocin: (1) the Urry and Walter model[13]; (2) model proposed by Brewster *et al.*[11] and by Honig *et al.*[23]; (3) another model proposed by Brewster *et al.*[11]

structure 3 has a 1→5 turn with a hydrogen bond between the CO of Cys^1 and the peptide NH of Asn^5.

Carbon-13 nmr spin lattice relaxation time (T_1) studies[24] based on ^{13}C resonance assignments from two laboratories[25,26] suggest that structure 2 is not a highly favored conformation in Me_2SO, but a small contribution from structure 2 to the conformational equilibrium cannot be excluded by this data.

There is also evidence against structure 3. Formation of the 1→5 transannular hydrogen bond requires that in structure 3 the Cys^1 carbonyl oxygen be directed into the ring and the Tyr^2 peptide NH point out of the ring (assuming a trans orientation for this peptide bond). This NH is therefore not available for formation of a second transannular hydrogen bond. Examination of space filling models also fails to indicate that any other internal hydrogen bond could readily form with this NH. Structure 1 (Urry and Walter model), however, places the Tyr^2 NH close to the Asn^5

CO so that a second transannular hydrogen bond involving these groups can form with minimal perturbation of the molecular architecture. Evidence for formation of an additional hydrogen bond involving the Tyr^2 peptide NH but not requiring extensive disruption of "oxytocin-like" structure has been obtained for deamino-oxytocin,[11,15] [Gly^4]oxytocin;[11] and [Pro^3,Gly^4]oxytocin.[19] The ^1H nmr spectral parameters of these derivatives closely resemble those of oxytocin except for a temperature-independent Tyr^2 peptide NH resonance. These data are consistent with structure 1, but not with structure 3.

There is at present no experimental method for determining how many different conformations are in rapid equilibrium in solution. Consequently, even though the Urry and Walter model is most consistent with experimental data, conformational averaging cannot be excluded and is even probable on the basis of potential energy calculations.[11,22,23]

Vasopressin in Dimethylsulfoxide

Consistent assignments of ^1H nmr resonances of LVP[27-29] and AVP[12] have been obtained by homonuclear spin decoupling in combination with comparative studies of LVP precursors and of oxytocin. Data from different laboratories agree within the limits of experimental error, and it is generally agreed that the 1→4 turn proposed for oxytocin in Me_2SO is maintained in LVP and AVP. This is supported by the temperature independence and high field position of the Asn^5 peptide NH resonance.

Conformationally most significant is the replacement of the hydrophobic Leu^8 residue in the tail of oxytocin by the polar Lys^8 or Arg^8 residues of the vasopressins. Integration of the Lys and Arg side chain resonances, which are observable in spectra of deamino-LVP[30] and AVP,[12] respectively, indicates that the ε-amino and guanido groups are charged in Me_2SO. The effect of the charged amino groups on the conformation of LVP in Me_2SO has been illustrated by comparative studies of LVP, deamino-LVP, and deamino-8-tosyl LVP.[30] Deamino-LVP lacks the NH_2-terminal amino group of LVP and deamino-8-tosyl-LVP also has its Lys ε-amino group tosylated. Deuterium exchange rates of LVP and deamino-LVP in 5% D_2O-Me_2SO are too rapid to follow by ^1H nmr suggesting a relatively exposed conformation. Deamino-8-tosyl-LVP yields measurable exchange kinetics; the slower exchange rates of the Asn^5 and Gly^9 peptide NH protons of this derivative are consistent with an "oxytocin-like" conformation since these protons are hydrogen bonded in the Urry and

Walter model. There is also a progressive increase in the similarity of ^1H nmr spectral parameters (δ, J_{N_α}, and $d\delta/dT$) of these LVP derivatives to those of oxytocin as one successively modifies the amino groups of LVP (*i.e.*, deamino-8-tosyl-LVP is most similar to oxytocin while LVP is most different). These data are consistent with the view that as a consequence of interactions involving its two amino groups, LVP assumes a more extended conformation than oxytocin in Me_2SO. Similar conclusions may also apply to AVP.[12]

The folding of the acyclic tail of the vasopressins has been subject to a minor controversy. A relatively low $d\delta/dT$ of the trans carboxamide NH of $Gly^9(NH_2)$ led Von Dreele *et al.*[28] to suggest formation of a 1→4 bend involving a hydrogen bond between this NH proton and the Pro^7 CO. Walter *et al.*[29] favor an "oxytocin-like" structure (*i.e.*, a 1→4 turn with a hydrogen bond between the Gly^9 peptide NH and the Cys^6 CO) consistent with slow deuterium exchange of the Gly^9 peptide $\underline{NH}$ of deamino-8-tosyl-LVP,[30] and with the similarity between primary amide $\underline{NH}$ resonances [particularly $Gly^9(NH_2)$] of LVP and oxytocin.

Oxytocin and LVP in Water

Proton nmr studies in water have been impeded by the difficulty of assigning the conformationally significant peptide NH resonances to their respective hydrogens. In aprotic solvents this is routinely accomplished by spin decoupling the corresponding $C^{\alpha}\underline{H}$ peaks, which can be assigned by standard techniques.[1-3] In water the $C^{\alpha}\underline{H}$ resonances are, however, buried beneath the intense H_2O peak. To overcome this difficulty, we attempted to assign the low field oxytocin resonances by starting with the assigned spectrum in Me_2SO and monitoring resonances as the solvent was progressively enriched with H_2O.[31] This solvent transition method proved successful for LVP[31,32] and various other molecules,[33] but in the case of oxytocin[31] led to a number of erroneous assignments as a result of extensive overlap of resonances. Brewster and Hruby[34] assigned the oxytocin peptide NH peaks by the "underwater decoupling" technique developed by Dadok *et al.*[32,35] This difficult procedure involves blind decoupling of $C^{\alpha}\underline{H}$ resonances underneath the water peak which have previously been identified in D_2O. The assignments of Tyr^2, Ile^3 and Leu^8 were confirmed by Bradbury *et al.*[36] on the basis of nmr spectral changes introduced by specific isotopic replacement ($^1H \rightarrow {}^2H$ and $^{14}N \rightarrow {}^{15}N$). We have recently repeated the underwater decoupling experiments of Brewster

and Hruby and confirmed all their assignments. Von Dreele *et al.*[32] also used this method in combination with the solvent transition technique to assign the LVP resonances in H_2O.* Agreement on the assignment of resonances in H_2O permits ^1H nmr analysis of the conformations of oxytocin and LVP in aqueous solution.

Figure 2 shows variations of chemical shifts of low field resonances of oxytocin[31] as the composition of the solvent is changed from Me_2SO to H_2O in small increments. The oxytocin assignments in H_2O are those of Brewster and Hruby.[34] Simultaneous perturbation of NH and CH resonances in the 70-90% H_2O solvent composition range reflects a change in oxytocin conformation. A similar conclusion is drawn

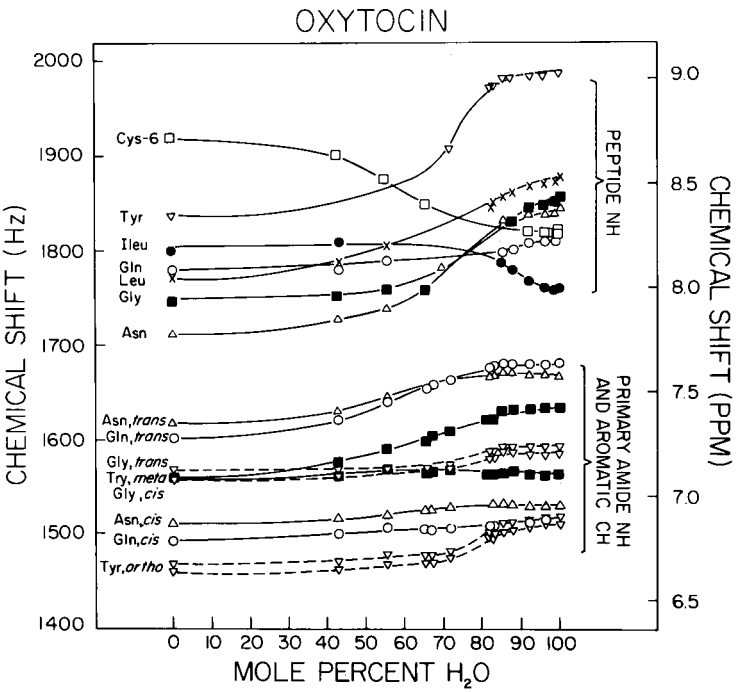

Figure 2: Chemical shifts of low field resonances of oxytocin in Me_2SO/H_2O mixtures.[31]

*Slight differences from assignments of Glickson *et al.*[31] (NH resonances of Phe^3 and Cys^6) have no significant bearing on this discussion.

from the solvent transition curve of LVP, which is qualitatively similar to that of oxytocin.[31] NH resonances might have been shifted by disruption of hydrogen bonds with the solvent, whereas this effect could not explain variation in chemical shifts of aromatic CH resonances. Displacement of H_2O and $(CH_3)_2SO$ resonances in the 70-90% H_2O region of solvent composition suggests that the change in hormonal conformation may be associated with a change in solvent structure.[31] Comparison of $J_{N\alpha}$ and $d\delta/dT$ values of oxytocin in Me_2SO (Table I) and H_2O (Table II) further indicates a conformational change in the course of the solvent transition. Similar observations have been made with LVP.[31,34,37] Particularly noteworthy is the change in $d\delta/dT$ of Asn^5 from a value close to zero in Me_2SO, consistent with internal hydrogen bonding, to a value of about -6×10^{-3} ppm/°C, which does not support the existence of an internal hydrogen bond. For oxytocin, a small value of $J_{N\alpha}$ (4-5 Hz) of Ile^3 is observed in Me_2SO but in H_2O only Gln^4 has a small $J_{N\alpha}$. This is consistent with the proposed β-turn in Me_2SO, but not with significant retention of this structure in H_2O.

There is considerable evidence for conformational averaging in oxytocin and LVP in aqueous solution. CD data suggests considerable internal rotation in the cystine residue (*vide supra*). Potential energy calculations indicate that a number of different orientations, many of which lack any internal hydrogen bonds, have comparable stabilities in water.[11,22,23] Values of $J_{N\alpha}$ close to 6 Hz, the value predicted for a random-coil,[38] are consistent with a flexible structure, as are the values of $d\delta/dT$, which show no evidence that any thermally stable intramolecular hydrogen bonds are formed.[36,37]

Additional insight into the dynamic state of oxytocin and LVP comes from measurements of the rotational correlation times (τ_c's) of these molecules. As noted above, ^{13}C NT_1 values suggest that in Me_2SO a single value of τ_c (8.8×10^{-10} sec) approximately describes the motion of the backbone carbons of the cyclic and acyclic portions of oxytocin.[24] In Me_2SO there appears to be little motion of the tail relative to the ring of oxytocin. In aqueous solution the protonated carbons of the ring moiety of oxytocin have NT_1 values of approximately 100 msec (τ_c about 5×10^{-10} sec),[39] whereas these values progressively increase as one moves along the backbone of Gly^9. This suggests that the tail of oxytocin is relatively more flexible than the ring in aqueous solution. This conclusion is also supported by ^2H nmr linewidth studies by Glasel *et al.*[40]

Table II

^{1}H Nmr Spectroscopic Parameters of Oxytocin in H_2O (at 22±1°C)

Peptide NH	δ *ppm*	$10^3 x\ d\delta/dT$ *ppm/°C*	J_{N_α} *Hz*
Tyr-2	8.99,*9.03,**9.04***	-5.5,*-6.5**	7.5,*6.5,**7.2±.1,***
Ile-3	7.94,*7.99,**8.01***	-7.6,*-7.0**	6.5,*6 ,**5.7±.3,***
Gln-4	8.20,*8.23,**8.26***	-4.3,*-5.5**	4.0,*4 ,**3.7±.5,***
Asn-5	8.32,*8.35,**8.38***	-7.3,*-5.0**	7.4,*8 ,**8.6±.3,***
Cys-6	8.19,*8.23,**8.24***	-5.0,*-5.5**	7.0,*7 ,**7.0±.2,***
Leu-8	8.47,*8.50,**8.52***	-10.8,*-9.50**	6.5,*6.5,**5.5±.1,***
Gly-9 (av.)	8.37,*8.39,**8.42***	-8.6,*-7.0**	5.4,*5.5,**6.1±.5,***
Primary Amide NH			
Asn, trans	7.64,**7.60***	6.5**	-
Gln, trans	7.59,**7.65***	6 **	-
Gly, trans	7.39,**7.44***	4.5**	-
Gly, cis	7.06,**7.11***	6 **	-
Asn, cis	6.90,**6.96***	6 **	-
Gln, cis	6.84,**6.91***	5 **	-

*Reference 38 employing spectral assignments of reference 34.
**Reference 34. Chemical shifts at 28°C corrected to 22°C.
***Reference 31. Chemical shifts at 24°C corrected to 22°C employing temperature coefficients of reference 34. Peptide NH assignments modified in accordance with reference 34.

Comparative ^{13}C T_1 measurements of oxytocin and LVP in water[39] indicate that the ring moieties of these molecules are about equally flexible, whereas the tail of LVP is significantly more mobile than that of oxytocin. Thin-film dialysis data[41] indicate that LVP has a slightly more extended structure than oxytocin in aqueous solution. Craig[42] estimated that the diameters of oxytocin and LVP are 14-15 Å and 16 Å, respectively. From ^{13}C T_1 measurements Deslauriers *et al.*[39] estimate a diameter of about 16 Å for both molecules. Craig *et al.*[41] also noted that deamino-LVP and oxytocin had essentially identical dialysis rates. This is consistent with the conclusion, also drawn from ^1H nmr data in Me_2SO (*vide supra*),[30] that the most significant difference between oxytocin and LVP may be a more extended conformation of the latter. As noted above, this may be a consequence of interactions between charged groups of LVP.

In order to delineate the extent of exposure to solvent of specific NH and CH hydrogens of oxytocin and LVP, we have recently been performing studies by the ^1H nmr solvent saturation method.[4,43] A typical experiment with oxytocin in H_2O is shown in Figure 3.

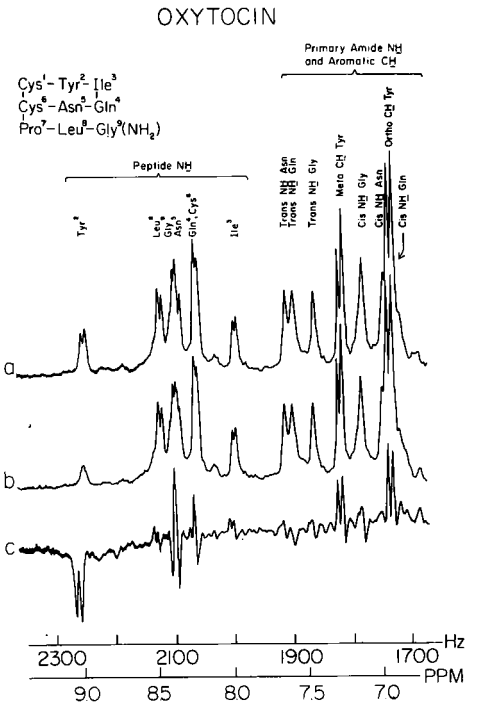

Figure 3: Solvent saturation study of 5% (w/v) oxytocin in H_2O at pH 3.8 and 30±1°C (accumulated in the correlation mode at 250 MHz).[43]

Spectrum 3a represents 80 scans accumulated by correlation ^{1}H nmr spectroscopy[44,45] at 250 MHz. Saturating rf power was continuously applied 4000 Hz off resonance so that only nonspecific effects of double irradiation are manifest in this spectrum. Spectrum 3b was accumulated under identical conditions except that saturating power was now applied at the resonance frequency of the solvent (1196 Hz from the reference methyl peak of 2,2-dimethylsilapentane-5-sulfonate). Saturation of the solvent resonance produces only a very small change in the population of the spin states of water and has a negligible effect on the molecular state of either the solvent or solute. It does, however, produce significant perturbations of resonances of a number of oxytocin hydrogens which are in intimate contact with water. This is most readily observed in the amplified difference spectrum shown in part c of Figure 3 (*i.e.*, 3c = [3b - 3a] x 2.68). Negative peaks correspond to a decreased intensity resulting from solvent saturation; positive peaks from an increased intensity. The complex pattern near 2100 Hz results from partial decoupling of $C^{\alpha}H$ protons whose resonances are close to the water peak.

Under these conditions only the Tyr^2 NH resonance exhibits decreased intensity (57±3%) as a result of transfer of saturation. This effect reflects rapid exchange with the solvent. Because of the inductive effect of the nearby NH_2-terminal amino group, the penultimate peptide NH hydrogen is expected to exchange more rapidly than other peptide hydrogens. By raising the pH, we have been able to monitor the exchange of the remaining NH hydrogens of oxytocin, and are now in the process of relating this to the extent of solvation of specific protons. To a first approximation, the data are consistent with a considerably solvated structure of oxytocin in water.

The Tyr^2 ortho-CH and meta-CH protons exhibit positive nuclear Overhauser effects (NOE's; increased resonance intensity) of 12±5% and 9±5%, respectively. It has been demonstrated that these NOE's result predominantly from direct dipole-dipole interaction between the respective aromatic CH protons and protons of the solvent.[43] Because of the inverse sixth power dependence of these interactions on internuclear distance, we may conclude that the aromatic hydrogens are in intimate contact with water protons.

Preliminary data suggests that the NH protons of LVP are somewhat more solvated than corresponding protons of oxytocin.

Summary

Current View of the Free Solution Conformations of Neurohypophyseal Hormones

1. The model of oxytocin proposed by Urry and Walter[13] probably represents at least one of the preferred conformations of oxytocin in dimethylsulfoxide solution. There is still a need to refine this model, especially with respect to the orientation of amino acid side chains and the type of β-turns in the structure.

2. In Me_2SO solution LVP and AVP appear to retain the general features of the oxytocin structure, except for a conformationally more mobile acyclic tail.

3. In aqueous solution there appears to be a rapid equilibrium between a number of different conformations of oxytocin and the vasopressins, whose detailed structures remain to be defined. Segmental motion, as revealed by ^{13}C T_1 of Deslauriers *et al.*[39] is more rapid and extensive in LVP than oxytocin, and may reflect a more extended conformation for vasopressin, as revealed by thin-film dialysis data of Craig *et al.*[41] in 1964.

It never ceases to amaze this reviewer that with the aid of a piece of sausage wrapping Lyman Craig was able to outline the general features of the conformations of oxytocin and vasopressin more than ten years ago. Today, numerous investigators are employing some of the most sophisticated spectroscopic techniques requiring some of the most expensive equipment to probe the molecular details of these hormones. To be sure, these methods define the structure and dynamics of these molecules with more precision and detail. Nevertheless, the overall conclusions remain consistent with those first reached by Lyman Craig. Truly, the durability of his work will remain a lasting tribute to this great scientist.

Acknowledgments

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CONFORMATIONAL STUDIES OF NEUROHYPOPHYSEAL HORMONES AND INHIBITORS USING HIGH RESOLUTION NMR SPECTROSCOPY

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A LARGE NUMBER OF CHEMICO-PHYSICAL STUDIES have been made in an effort to determine solution ([$^2H_6$]DMSO and aqueous) conformational properties of oxytocin and related peptides,[1,2] and based on a limited number of experimental parameters and/or conformational calculations, speculations regarding solution conformations have been made.[3-5]

The objective of the work reported here was to examine the experimentally determined NMR coupling constants

$$J_{\alpha CH-\beta CH}(J_{\alpha\beta}),\ J_{\beta CH-\beta CH'}(J_{\beta\beta'}),\ \text{and}\ J_{NH-\alpha CH'}$$

to test the assignments made and coupling constants determined by computer simulation, and then to utilize this more extensive information in conjunction with other published data to test for possible conformations. In the study discussed here a comparison of [1-L-penicillamine]oxytocin $S\text{-}C(CH_3)_2\text{-}CH(NH_2)\text{-}C(=O)\text{-}$Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 ([Pen^1]oxytocin), and oxytocin was made. [Pen^1]oxytocin was chosen for both its structural features and biological activities. On the biological side it is a known

antagonist[6] to oxytocin in several of the biological systems associated with the activity of oxytocin. On the structural side, the penicillamine residue was expected to have certain effects. Firstly, the replacement of two methylene hydrogens on one of the carbons of the 20-membered ring by two methyl groups would be expected to restrict the conformational possibilities and motions in that residue of the molecule and hence in the entire ring moiety. Secondly, since the two methyl groups are α to the disulfide bridge, the allowed dihedral angle of the disulfide is more restricted than in oxytocin, and from X-ray studies[7] and UV spectral data,[8] is expected to be greater than 90° (∿100° is estimated). In this derivative then, CD should provide rather specific information on disulfide helicity.

In Figure 1 we show the 300 MHz NMR spectrum of oxytoxin (A) and [Pen^1]oxytocin (B) in D_2O(0 to 6 ppm) and H_2O(6-10 ppm), of [Pen^1]oxytocin in [$^2H_6$]DMSO (C), and the 220 MHz NMR spectrum of oxytocin in [$^2H_6$]DMSO (D). The spectrum of [Pen^1]oxytocin in aqueous solution is particularly noteworthy since essentially every αNH, αCH and βCH or βCH_2 proton has a distinct and separate chemical shift making it an ideal polypeptide for determining coupling constants and chemical shifts and carefully checking them by computer simulation. The situation for oxytocin in aqueous solution is nearly as good and use of the spectra of specifically deuterated derivatives[9] further aided these studies. After a brief consideration of the results in [$^2H_6$]DMSO, we will return to a more complete discussion of the results in aqueous solution.

Several aspects of the NMR spectrum of oxytocin in [$^2H_6$]DMSO are puzzling. In the first place at comparable temperatures and solute concentrations, the proton and to some extent carbon-13 NMR spectra in DMSO solution are broader and less defined than the aqueous solution spectra.[4,9-13] Secondly, carbon-13 T_1 values[13,14] are about 1.2- to 3.5-fold larger in D_2O than in [$^2H_6$]DMSO. Several explanations might be used to account for these observations, but we have experimental evidence from various FT NMR spectroscopy, tlc, etc. that oxytocin and [Pen^1]oxytocin associate in DMSO solutions at concentrations generally used in previous studies. In Figure 1 note the severely broadened and poorly defined spectrum of [Pen^1]oxytocin in [$^2H_6$]DMSO (C) compared to that in H_2O (B). The only significant experimental differences between the two spectra is that different

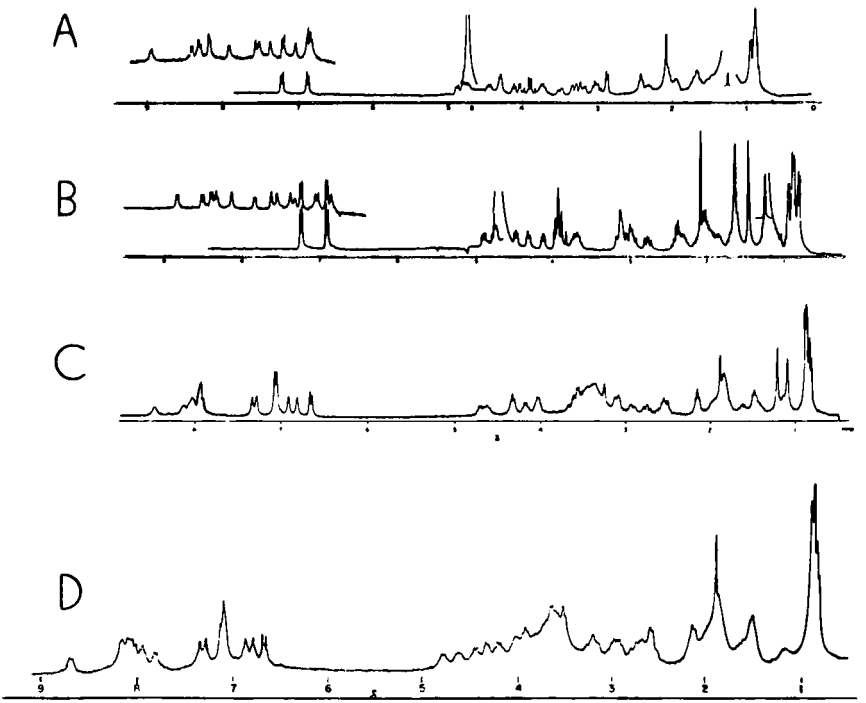

Figure 1: NMR spectra of (*A*) oxytocin in D_2O (0 to 6 ppm) and H_2O (6-10 ppm) at 300 MHz, 28°, pH = 3.8; (*B*) [Pen^1]-oxytocin in D_2O (0-6 ppm) and H_2O (6-10 ppm) at 300 MHz, 30°, pH = 3.2; (*C*) [Pen^1]oxytocin in [$^2H_6$]DMSO, at 300 MHz, 28°; (*D*) oxytocin in [$^2H_6$]DMSO at 220 MHz, 28°.

solvents are used. The [Pen^1]oxytocin spectrum in [$^2H_6$]DMSO reminds one of a protein in some respects. However when the temperature is raised to 62° a comparatively well-defined spectrum is obtained (Figure 2). In view of the need for further study of this association phenomena and its possible effects on conformational parameters nothing further will be said here regarding the situation in DMSO. We return instead to the studies in aqueous solution.

The 300 MHz NMR spectra of [1-penicillamine]oxytocin (A) and oxytocin (C) in D_2O (0-6 ppm) and H_2O (6-10 ppm) together with their respective partial (2.5-10 ppm) computer simulated spectra (B) and (D) are shown in Figure 3. In the spectral region of 2.5 to 6 ppm we did not consider the

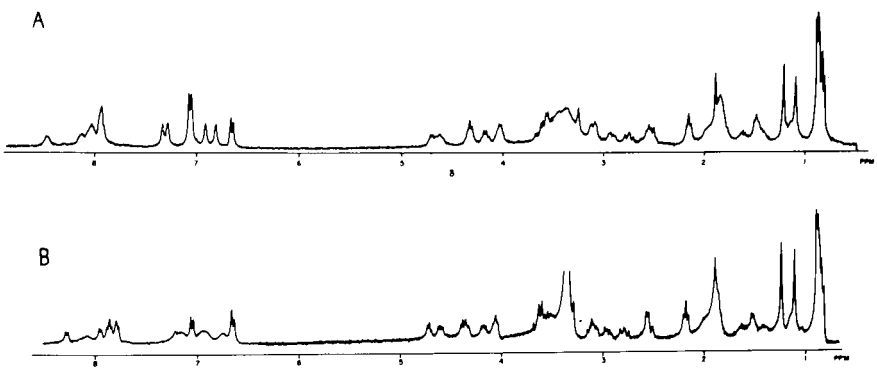

Figure 2: The 300 MHz NMR spectra of [Pen^1]oxytocin in [$^2H_6$]DMSO at 28° (*A*) and 60° (*B*).

δ-methylene protons of proline-7, or the aromatic and carboxamide protons in the simulation. The assignments of the resonances of [Pen^1]oxytocin were obtained in the same manner as previously done for oxytocin.[9]

Table I summarizes the spectral parameters used in the simulations (B) and (D) of Figure 3. As can be seen from Figure 3 the simulated and experimental parameters are in excellent agreement. In Table II the temperature dependence of the peptide NH proton chemical shifts of [Pen^1]oxytocin are presented and compared with those previously obtained for oxytocin.[9]

The CD spectra of [Pen^1]oxytocin and oxytocin were found to be qualitatively the same. The positive CD band at about 250 nm in oxytocin and oxytocin analogs was previously assigned[15,16] to the disulfide chromophore, and we assign the positive CD band at 245 nm in [Pen^1]oxytocin to this chromophore. On the basis of the quadrant rule[17] the disulfide chirality in [Pen^1]oxytocin is assigned as left-handed helical, and we used this chirality in constructing a conformation for [Pen^1]oxytocin, though essentially the same conformation but with the opposite disulfide chirality can be readily constructed.

From previous studies[9,18-20] of oxytocin in aqueous solution, it was known that oxytocin had a flexible structure in this solvent with no apparent intramolecular hydrogen bonding, and the further data reported here confirms these findings. The spectral features for [Pen^1]oxytocin summarized in Tables I and II suggest that the cyclic

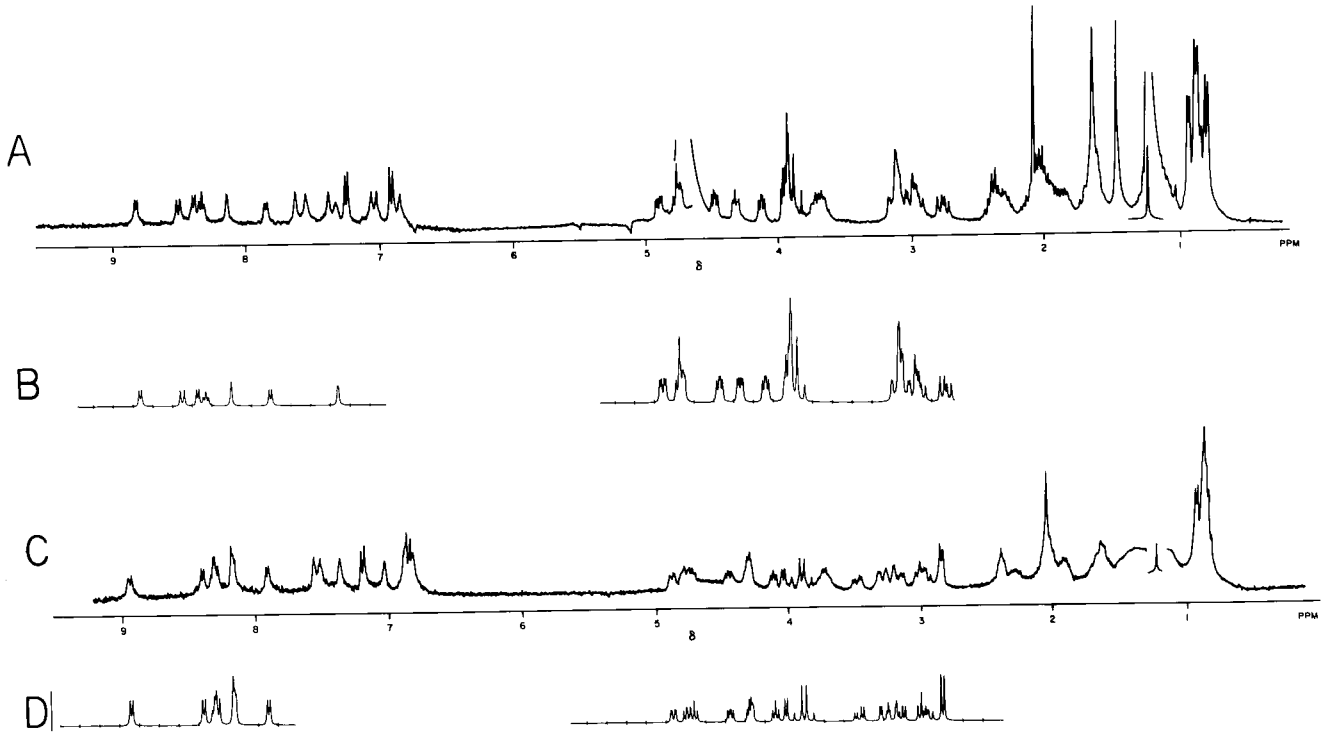

Figure 3: The 300 MHz NMR spectrum of [Pen^1]oxytocin in D_2O (0 to 6 ppm) and H_2O (6-10 ppm) (*A*), and partial (2.5-10 ppm) computer simulated spectrum (*B*), and the 300 MHz NMR spectrum of oxytocin in D_2O (0 to 6 ppm) and H_2O (6-10 ppm) (*C*) and partial (2.5-10 ppm) computer simulated spectrum (*D*).

Table I

Chemical Shifts (ppm) and Coupling Constants (Hz)

Used in Simulations (B) and (D) of Figure 3, pH = 3.8, T = 28°C

Residue Number	*[1-penicillamine oxytocin]*					*oxytocin*				
	δ (ppm)	$J_{NH-\alpha CH}$	$J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$	*δ (ppm)*	$J_{NH-\alpha CH}$	$J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$
Half Cys-1 or Half Pen-1	δ_{NH}=—— δ_α =3.92 δ_β =—— $\delta_{\beta'}$=——					δ_{NH}=—— δ_α =4.30 δ_β =3.31 $\delta_{\beta'}$=3.49	——	3.8	6.0	15.
Tyr-2	δ_{NH}=8.82 δ_α =4.74 δ_β =2.95 $\delta_{\beta'}$=3.13	6.2	10.0	3.1	14.7	δ_{NH}=8.99 δ_α =4.79 δ_β =3.02 $\delta_{\beta'}$=3.17	6.4	8.1	6.2	14.
Ile-3[a]	δ_{NH}=7.33 δ_α =3.94	3.0	5.8			δ_{NH}=7.96 δ_α =4.04	6.	6.		
Gln-4[a]	δ_{NH}=8.14 δ_α =4.11	2.2	8.4	5.2		δ_{NH}=8.22 δ_α =4.12	4.	6.6	6.8	
Asn-5	δ_{NH}=7.84 δ_α =4.76 δ_β =3.08 $\delta_{\beta'}$=3.11	8.6	7.5	7.3	14.5	δ_{NH}=8.34 δ_α =4.74 δ_β =2.86 $\delta_{\beta'}$=2.86	8.	7.5	7.5	——

Half Cys-6	δ_{NH}=8.51 δ_{α} =4.88 δ_{β} =2.75 $\delta_{\beta'}$=3.0	8.6	10.0	4.0	15.8	δ_{NH}=8.21 δ_{α} =4.89 δ_{β} =2.98	$\delta_{\beta'}$=3.24	6.5	9.6	3.0	15.9
Pro-7[a]	δ_{α} = 4.46		8.4	5.2		δ_{α} =4.6			6.5	5.0	
Leu-8[a]	δ_{NH}=8.39 δ_{α} =4.3	5.8	9.6	3.6		δ_{NH}=8.45 δ_{α} =4.32		6.	8.6	4.4	
Gly-9	δ_{NH}=8.33 δ_{α} =3.85 $\delta_{\alpha'}$=3.93	6.6 5.2	$J_{\alpha\alpha'}$=16.8			δ_{NH}=8.36 δ_{α} =3.87	$\delta_{\alpha'}$=3.94	6.0 4.5	$J_{\alpha\alpha'}$=17.0		

[a] The simulation was made without knowing the exact $\delta\beta$ and the exact $J_{\beta\beta'}$. This does not affect the parameters determined.

Table II

Temperature Dependence of the Chemical Shifts δ of the Peptide NH Protons of [1-Penicillamine]Oxytocin and Oxytocin

Residue Number	$-d\delta/dT \times 10^3$	
	oxytocin	*[Pen^1]oxytocin*
Half Cys-1	--	--
Tyr-2	6.5	6.5
Ile-3	7.	0.5
Gln-4	5.5	9.
Asn-5	5.	0.5
Half Cys-6	5.5	4.5
Pro-7	--	--
Leu-8	9.5	6.5
Gly-9	7.	8.

moiety of this compound possesses a fairly rigid conformation in aqueous media. Thus as shown in Table I, values of the $J_{NH-\alpha CH}$ coupling constants for residues in the ring moiety of [Pen^1]ocytocin are going to more extreme values (<4Hz, >8Hz) while in oxytocin they are all in the intermediate range (4-8 Hz). In Table II we note that both the Ile-3 and Asn-5 NH protons have a near zero temperature dependence of their chemical shifts ($-d\delta/dT$) in [Pen^1]oxytocin. These results show that these two amide protons are not exposed to the solvent, and we interpret these results to indicate that [Pen^1]oxytocin has a conformation stabilized by two intramolecular H-bonds. A search for the fractional populations of rotational isomers around the C_α-C_β bonds using the description of Pachler[21] and the data in Table I, reveals that the Tyr-2, Gln-4, and Leu-8 side chains have more hindered rotations in the case of [Pen^1]-oxytocin than in oxytocin indicating less conformational flexibility in the former compound. This latter data can be used also in restricting the conformational possibilities available to the ring moiety of [Pen^1]oxytocin.

Table III summarizes the torsional angles for a proposed conformation for the backbone of the cyclic moiety of [Pen^1]-oxytocin in aqueous solution at pH 3.8. The ϕ angles were

Table III

Torsional Angles of a Proposed Conformation for the Backbone of the Cyclic Moiety of [1-Penicillamine]oxytocin in Aqueous Solution, pH 3.8

Residue Number	$\phi^{a,b}$	ψ^{c}	χ^{b}
Half-Pen-1	--	-30	-150
Tyr-2	93	-60	
Ile-3	180	150	
Gln-4	-50	70	
Asn-5	-165	60	
Half Cys-6	-75	--	90

Disulfide bridge: ∿100°; chirality (M).
H-bonds: 3-1 and 5-3.

[a] The various angles follow the IUPAC-IUB nomenclature (24).

[b] The calculated angles are understood to have a range of 30° due to the physical origin of the coupling constants.

[c] Angles read on the model.

deduced from the relationship of the vicinal coupling constant $J_{NH-\alpha CH}$ and the dihedral angle αCH-NH as suggested by Bystrov *et al.*[22], as was the χ angle of the side chain of half Cys-6 from a similar relationship.[23] As indicated in Table III the conformation contains two seven-membered rings (within the 20-membered ring of the cyclic moiety), a conformational arrangement which previously was found to be not only theoretically energetically favorable[25] but also experimentally observable.[26] An attempt to accomodate the two H-bonds into a γ-turn like conformation[27] was not successful due to a lack of correlation between the side chain experimental parameters of Table I and the conformational requirements for this conformation. As for the behavior of the side chain moiety of oxytocin and [Pen^1]-oxytocin, the data suggests that they may be similar, but further studies will be necessary to establish this more fully.

Conclusions

Careful examination of high resolution ^1H NMR backbone and side chain coupling constants and chemical shift data in $H_2O(D_2O)$ solution, reveals that [Pen^1]oxytocin has a greatly reduced degree of freedom in the ring moiety compared to the ring in oxytocin. It, therefore, appears that the mobility of the ring moiety of oxytocin is enhanced by a lack of restriction to rotation about the C_α-C_β bond of the half cystine-1 residue. The relative lack of conformational mobility for [Pen^1]oxytocin perhaps may be used to explain its antagonist biological activity to oxytocin in that the conformation allows it to interact (bind) with the same receptors as oxytocin, but once bound it apparently cannot assume the conformation necessary to initiate an agonist response. Oxytocin, on the other hand, has a more flexible structure and, once bound to the receptor, readily assumes the conformation necessary to initiate an agonist response.

Acknowledgments

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THE β-TURN IN THE CYCLIC MOIETY OF OXYTOCIN: PMR STUDIES OF [PRO^3, GLY^4]OXYTOCIN IN DIMETHYLSULFOXIDE

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ON THE BASIS OF PMR STUDIES, Urry *et al.*[1] proposed in 1970 that oxytocin in DMSO-d_6 contains a β-turn[2-5] involving residues 2-5 in the tocin-ring moiety and that this β-turn is stabilized by an intramolecular hydrogen bond between the backbone NH of Asn^5 and the C=O of Tyr^2. Several years later, on the basis of PMR studies combined with conformational energy calculations, Brewster *et al.*[6] proposed that oxytocin in DMSO-d_6 consists of an equilibrium mixture of three different conformers of comparable energy. One of these three conformers corresponds to the structure proposed by Urry *et al.*, but the other two proposed

conformers manifest a different pattern of intramolecular hydrogen bonding and do not contain a β-turn. We also believe that conformational averaging is more prevalent in oxytocin than originally thought, but believe that a β-turn involving residues 2-5 is a major conformational component.

β-Turns in which both corner residues have an L configuration may, in theory, be of either type I or type II,[3,5] and indeed, examples of both have been found in proteins.[5,7] The values of the coupling constants between vicinal amide and C^{α} protons for the corner residues (Ile^3 and Gln^4) of the proposed β-turn in oxytocin are compatible with both type I and type II β-turns,[5,8,9] and thus it is necessary to use other spectroscopic parameters as criteria for determining which particular type of β-turn is manifest in the ring moiety of this peptide.

A priori it should be possible to synthesize oxytocin analogs with such residues in the corner positions to create steric restrictions that assure that a particular type of β-turn is energetically favored. For example, in theory, a type II β-turn is favored when a residue with a D configuration is placed in the second corner position (residue 4 in oxytocin), while a type II' β-turn is favored when such a residue is placed in the first corner position (residue 3).[3,5]

From the study of a series of oxytocin analogs possessing various types of β-turn in the ring moiety, it is hoped that certain NMR parameters for some of the nuclei in oxytocin will be quite similar to those for the corresponding nuclei in analogs possessing the same type of β-turn as oxytocin, but quite different from those in analogs possessing different types of β-turn. In the first study in this series, Walter and Glickson[8] found that certain PMR parameters for oxytocin more closely resembled those for [D-Ala^4]oxytocin -- which, in theory, should possess a type II' β-turn--than those for [D-Ala^3]oxytocin--which, in theory, should possess a type II' β-turn. Thus, one reasonable but unproven working hypothesis is that oxytocin possesses a type II'β-turn in the ring moiety.

In a continuation of this work, we have synthesized [Pro^3, Gly^4]oxytocin (PGO) and studied some of its conformational properties by PMR. The relatively fixed conformation between the backbone N and C^{α} atoms in prolyl residues is compatible with the conformation required for the first corner position of both type I and type II β-turns.[3,5] Furthermore, all of the type I and type II conformations accessible to residues with both L and D configurations in the second corner position should, at

least in theory, also be accessible to a glycyl residue in this position.[3,5] Indeed, Pro-Gly sequences as consecutive corner residues for both type I and type II β-turns have been found in proteins.[5,7]

In this paper, we utilize the PMR parameters of PGO to determine the type of β-turn manifest at corner positions 3 and 4. In a subsequent paper,[10] we shall compare the PMR parameters of PGO and oxytocin and conclude that the data do nothing to suggest that these peptides have grossly different backbone conformations in DMSO-d_6.

Biological Studies

PGO showed extremely weak agonistic rat pressor activity (0.004 ± 0.001 U/mg)[11] and had no rat antidiuretic activity when a dose of 0.06 mg was tested.[12] A single dose of 0.72 mg demonstrated no rat uterotonic activity *in vitro*,[13,14] as was the case with 2.41 mg in the avian vasodepressor assay.[15,16] Furthermore, PGO did not inhibit the action of oxytocin in these assays, suggesting that the various receptors have a low affinity for binding PGO.

Spectral Assignments

The assigned 220-MHz PMR spectrum of PGO in DMSO-d_6 at 41° is shown in Figure 1. The details of the assignment process will be described in a subsequent paper.[10] Only the assignments relevant to this paper -- *viz.*, those of the proton resonances of the two glycyl residues and the single asparaginyl residue -- will be discussed below.

Two multiplets of glycyl NH resonances were detected -- one centered at 8.00 ppm and the other, at 8.76 ppm at 41°. The latter multiplet is a quartet rather than a triplet, which is characteristic of the NH of Gly^9 in oxytocin. On the basis of this difference, the glycyl NH resonances centered at 8.76 ppm at 41° are assigned to Gly^4. Furthermore, the remaining glycyl NH resonances centered at 8.00 ppm at 41° occur only 0.15 ppm from the NH resonances of Gly^9 in oxytocin at the same temperature (centered at 7.85 ppm), and these glycyl multiplets in both peptides are triplets (the data from oxytocin was calculated from Table I of Walter and Glickson[8]). Thus, it is reasonable to assign the glycyl NH resonances centered at 8.00 ppm at 41° to Gly^9.

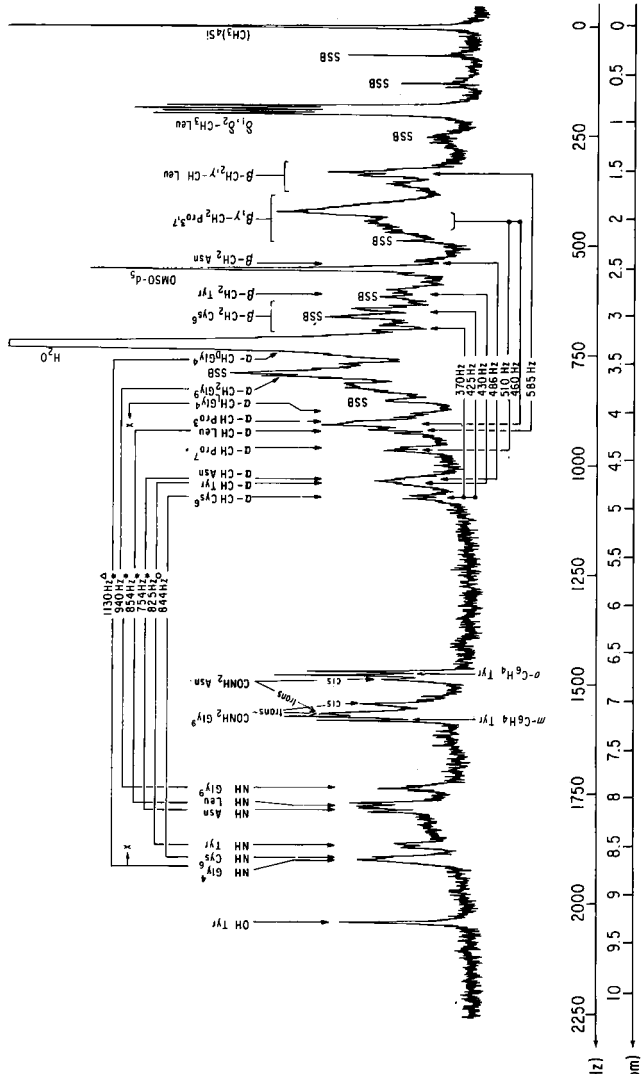

Figure 1: The assigned 220-MHz PMR spectrum of [Pro^3, Gly^4]oxytocin in DMSO-d$_6$. SSB denotes spinning sideband. Couplings detected by double-resonance experiments performed at 28° (O), 41° (*), and 58° (Δ) are indicated by bridges, and separations of coupled resonances are given in Hz. The coupling between the amide and C$^\alpha$ protons of Gly^4 indicated by x was detected on a 270-MHz spectrometer.

The identification of the NH resonances of Asn^5 was unequivocal (see Figure 1). The $C^{\beta}H_2$ resonances were assigned on the basis of expected chemical shift, and this assignment was confirmed by comparison with the corresponding resonances in oxytocin in DMSO-d_6.[17] In the classical manner,[18] the $C^{\alpha}H$ resonances were assigned by decoupling from the $C^{\beta}H_2$ resonances, and the NH resonances were then assigned by decoupling from the $C^{\alpha}H$ resonances.

The Type of β-Turn

As mentioned above, it is known that a Pro-Gly sequence can fill the role of either a type I or type II β-turn. The observed sum of coupling constants ($\Sigma\ ^3J_{NH-\alpha CH}$) for Gly^4 is 11.2 ± 0.4 Hz, a value that is compatible with either type of β-turn. For example, for a type I β-turn, this value could correspond to a value of ϕ of -130.5° ± 12.5°, and for a type II β-turn, to one of either +58° ± 6° or +130.5° ± 12.5°.[5,9]

The observed quartet resonance pattern for the NH of Gly^4 indicates that the chemical shifts of the two C^{α} protons of Gly^4 are different and that these two protons have different couplings to the vicinal NH. Classical continuous-wave INDOR spectroscopy has been previously used to reveal hidden transitions in PMR spectra of amino acids and peptides.[19,20] A 270-MHz INDOR study of PGO in DMSO-d_6 revealed hidden proton transitions of the C^{α} protons of Gly^4 in the neighborhood of 3.5 ppm. The amide (X) and two C^{α} (AB) protons of a glycyl residue form an isolated ABX spin system, and an analysis of the INDOR spectra for such a system can be performed as described previously.[19] Such a spectral analysis revealed that one value of $^3J_{NH-\alpha CH}$ is 8.0 ± 0.4 Hz, but the other value is too small to be revealed by the technique used. Inasmuch as the sum of couplings is known to be 11.2 ± 0.4 Hz, however, the other value of $^3J_{NH-\alpha CH}$ must be 3.2 ± 0.6 Hz. The pair of values (3.2 Hz, 8.0 Hz) is compatible with fixed ϕ values of either +60° ± 4.5° or -60° ± 4.5°.[9] In the second corner position of a β-turn, ϕ values of +60° and -60° are compatible only with type II and II' β-turns, respectively.[5] If it is assumed that Gly^4 in PGO is indeed in the second corner position of a β-turn, then we conclude that the β-turn is of type II inasmuch as a type II' β-turn is not compatible with an L-Pro→Gly sequence.[5]

It should be noted that our conclusion that the consecutive prolyl and glycyl residues in PGO play the roles of corner residues in a type II β-turn is in agreement with the conclusions of others that these consecutive residues play similar roles in the model cyclic hexapeptides *cyclo*(Ser-*Pro*-*Gly*)$_2$ and *cyclo*(Gly-*Pro*-*Gly*)$_2$.[21,22]

Stabilization of the β-Turn

If the Pro^3 and Gly^4 residues do indeed occupy the consecutive corner positions of a β-turn and if this β-turn is a true reverse turn rather than a near reverse or open turn,[7] then the backbone NH of Asn^5 is intramolecularly hydrogen bonded to the C=O of Tyr^2. The temperature dependence of the chemical shift for the NH of Asn^5 is only -1.6 ppb/°C. The magnitude of this dependence ($|\Delta\delta_{NH}/\Delta T|$) is much smaller than the magnitudes of the dependences for the other backbone amide protons in the molecule. The small value of $|\Delta\delta_{NH}/\Delta T|$ for Asn^5 indicates that the NH of Asn^5 is relatively shielded from solvent, possibly because it is involved, at least part of the time, in the formation of an intramolecular hydrogen bond.[4,23,24] If the backbone NH of Asn^5 is indeed hydrogen bonded to the C=O of Tyr^2, then this bond would help to stabilize the proposed β-turn in the ring moiety of PGO in DMSO-d_6.

Conclusions

We believe that Pro^3 and Gly^4 are consecutive corner positions in a type II β-turn when PGO is in DMSO-d_6.

We also believe that the isolated comparison of various PMR parameters for oxytocin with those for PGO is only of marginal value in itself and that it is merely a part of the larger problem of obtaining information on the proposed β-turn in the ring moiety of oxytocin. We believe that the real value of the study of oxytocin analogs with modifications at positions 3 and 4 will become evident only when a whole series of analogs representing all four types -- *viz.*, I, II, I', and II' -- of β-turn in the ring moiety have been prepared, studied, and compared with oxytocin.

Acknowledgments

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CARBON-13 NUCLEAR MAGNETIC RESONANCE STUDIES OF THE BINDING OF SELECTIVELY ^{13}C-ENRICHED OXYTOCIN TO ITS NEUROHYPOPHYSEAL CARRIER PROTEIN, BOVINE NEUROPHYSIN II

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^{13}C-NMR SPECTROSCOPY HAS ONLY RECENTLY BEEN APPLIED to the study of peptides and peptide hormones.[1,2] A major obstacle to such studies has been the very low sensitivity of ^{13}C-NMR. Fourier transform NMR techniques have extended the useful sensitivity of the method to study solutions containing peptide concentrations of 0.05*M* to 0.5*M*. Since the natural abundance of ^{13}C is only 1%, isotopic enrichment is capable of further enhancing the sensitivity of ^{13}C NMR spectroscopy. Selective enrichment of peptide hormones offers the additional advantage of giving the absolute assignment of the observed chemical shift values to particular carbon atoms in the peptide as recently shown in studies on the ^{13}C resonances of gramicidin-S[3] and oxytocin[4]. Such definite assignments can

then be used in the interpretation of ^{13}C experiments which utilize the ^{13}C natural abundance spectra. Moreover, ^{13}C enriched hormone molecules offer a new approach for characterizing conformational and microdynamical properties of neurohypophyseal peptides bound to their physiological carrier proteins, the neurophysins.[5] The synthesis and the ^{13}C NMR spectra of (85% ^{13}C enriched Gly^9) oxytocin in DMSO have been described[4] and the results demonstrated the usefulness of selective ^{13}C enrichment of oxytocin for extending the working sensitivity of ^{13}C NMR spectroscopy to the millimolar concentration range and for assigning unambiguously the ^{13}C resonances which can be observed in the natural abundance spectrum. We present herein several observations from studies of the binding of ^{13}C-enriched oxytocin molecules to neurophysin which will be published elsewhere.[6]

The ^{13}C NMR spectrum of (85% ^{13}C enriched Gly^9) oxytocin at 5 m*M* in D_2O is seen in Figure 1. This spectrum shows how ^{13}C-enrichment extends the working sensitivity of ^{13}C NMR by at least an order of magnitude above natural abundance studies.[2] Both the carbonyl and alpha carbon regions near 174 ppm and 42 ppm, respectively, exhibit three resonance peaks as seen for 85% ^{13}C-enriched free glycine.[7] The center peak in each case gives the true chemical shift values and it arises from glycine molecules which contain one ^{13}C and one ^{12}C atom. The two side peaks represent the doublet arising from ^{13}C-^{13}C coupling of the carbonyl and α-carbons. In Figure 1, the observed value of $^1J_{13Co-C\alpha}$ is 50.5 Hz.

The effects of adding highly purified[8] bovine neurophysin II to a solution of (^{13}C-Gly^9) oxytocin on the ^{13}C-NMR parameters of the enriched hormone are shown in Table I. Oxytocin has been shown to exchange rapidly between bound and free states[9] and, thus, for a titration of hormone with protein, the observed NMR parameters represent the weighted averages of the parameters for the bound and free states. The data in Table I show that addition of up to 0.24 equivalents of neurophysin II does not change the observed chemical shift or line width values of the Gly in position 9 of the hormone. The relaxation time, T_1, of the alpha carbon of the ^{13}C-enriched glycine[9] of oxytocin was 0.23 ± 0.02 sec in the absence of any protein and 0.23 ± 0.03 sec in the presence of 0.25 equivalents neurophysin II. The relaxation time, T_1, of an individual carbon atom reflects the relative mobility of that particular atom and, thereby, of a specific region of a molecule.[10] Control

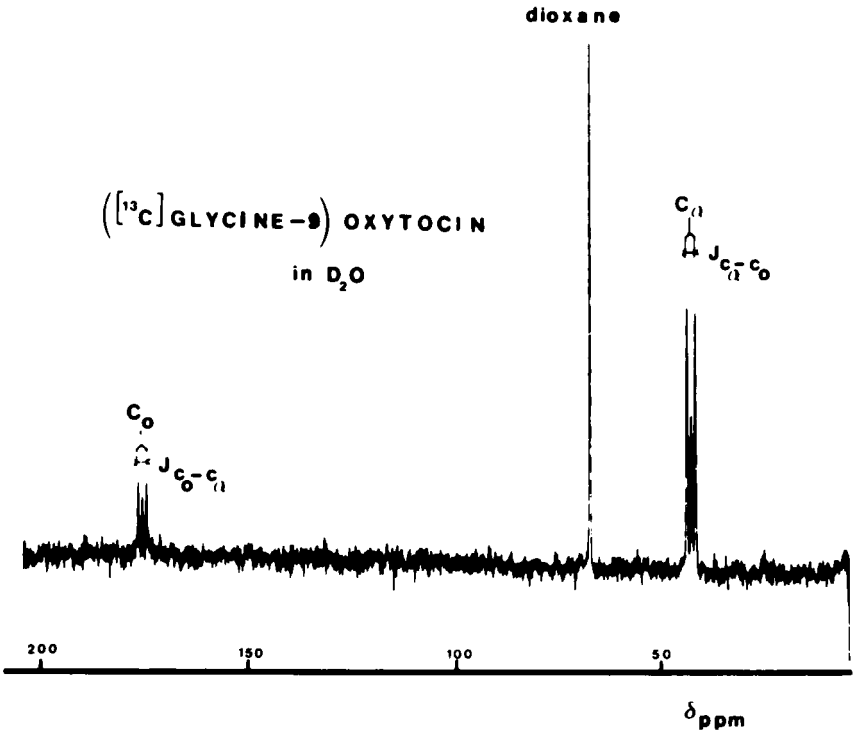

Figure 1: ^{13}C NMR spectrum of (85% enriched ^{13}C-Gly^9) oxytocin at 5 m*M* in D_2O, 0.1 *M* NaCl, pH 6.90, 30°. Obtained on a Varian XL-100 12 WG spectrometer at 25.5 MHz with proton noise decoupling, 40,000 transients, acquisition time 0.8 sec., 5000 Hz spectral width, and D_2O deuterium lock signal. Internal dioxane provided an internal reference of 67.4 ppm downfield from external TMS.

experiments based on the tyrosyl UV absorbance differences caused by hormone binding[11] showed that the (^{13}C-Gly^9)oxytocin was bound to neurophysin II under the conditions of the ^{13}C NMR experiments. Consequently, the binding of oxytocin to neurophysin II does not perturb the ^{13}C chemical shift, T_1, or T_2 values of the hormone's glycine^9 residue. This

Table I

^{13}C-NMR of (^{13}C-Gly^{9}) Oxytocin in Absence and Presence of Bovine Neurophysin II

	pH	C_o		C_α	
		δ *ppm*	$\Delta_{v1/2}$ *ppm*	δ *ppm*	$\Delta_{v1/2}$ *ppm*
(^{13}C-Gly^{9}) Oxytocin	6.90	174.95	0.25	42.94	0.28
+0.05 equiv. Neurophysin II	6.96	174.90	0.25	42.94	0.33
+0.10 Neurophysin II	7.05	174.94	0.23	42.99	0.29
+0.16 " "	7.05	174.90	0.20	42.99	0.34
+0.24 " "	6.98	174.92	0.27	42.94	0.30

suggests that neither the microenvironment nor the local mobility of glycine^{9} is altered upon hormone binding and, therefore, that the glycine^{9} residue of oxytocin is not directly involved in the binding reaction.

Oxytocin molecules containing ^{13}C-enriched isoleucine or leucine at residue 3 were synthesized[6] in order to study the involvement of that residue in the hormone binding reaction. ^{13}C NMR studies of the binding of (^{13}C-Leu^{3}) oxytocin and (^{13}C-Ile^{3}, Gly^{9}) oxytocin to bovine neurophysin II provided clear evidence that the ^{13}C NMR parameters of residue 3 are perturbed upon hormone binding.[6] For example, the chemical shift of the C_β of Ile^{3} of oxytocin is shifted 1.2 ppm downfield, *i.e.* from 36.9 ppm to 38.1 ppm, for a 1:1 complex of oxytocin:neurophysin II.[6] This result shows that the microenvironment of residue 3 is changed upon hormone binding. Additionally, using the analog (^{13}C-Leu^{3}) oxytocin, we found that the T_1 value of the C_δ of Leu^{3} was changed from 0.58 sec. in the absence of neurophysin to 0.19 sec. in the presence of 0.35 equivalents of neurophysin. Assuming rapid exchange obtains[9], this implies that the extrapolated value of T_1 for the bound hormone in a 1:1 complex is 80 msec. This sevenfold reduction in T_1 suggests that the local mobility of the side chain of residue 3 is greatly restricted in the hormone:neurophysin complex, presumably through direct interactions with neurophysin II.[6]

A simple schematic representation of a hormone binding site on neurophysin is seen in Figure 2. Previous evidence supporting this model proposed by us[11] has been discussed

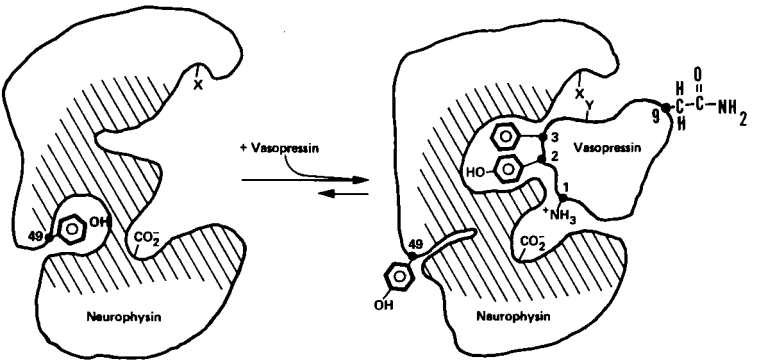

Figure 2: Schematic representation of the binding of Lys^8-vasopressin to neurophysin. The parallel diagonal lines indicate hydrophobic regions in the protein where residues 1-3 are bound. The hormone's glycine^9 residue remains freely exposed to solvent in the complex.

in detail elsewhere.[12,13] The particular contributions of the enriched ^{13}C NMR studies have been to show that leucine^3 or isoleucine^3 but not glycine^9 is involved in the hormone-protein interaction.

This approach of using specific ^{13}C enrichment of particular amino acids in peptide hormones is of general applicability for sensitivity enhancement, specific assignments, and ligand binding studies, and it merits further exploration.

Acknowledgments

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KINETICS OF OXYTOCIN BINDING TO NEUROPHYSIN MEASURED BY TEMPERATURE-JUMP RELAXATION

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A LARGE BODY OF THERMODYNAMIC INFORMATION is available describing the interaction between neurophysin and oxytocin.[1] The binding of oxytocin and vasopressin to bovine neurophysins I and II has been investigated utilizing techniques such as circular dichroism,[2-4] pH titration,[2] equilibrium dialysis,[5,6] and nuclear magnetic resonance.[7-9]

The ease of preparation of neurophysin[2] and the availability of the hormones make this an ideal system in which relaxation kinetic techniques[10] can be utilized to determine the dynamic nature of the binding process. Accordingly, we have begun a systematic study of the kinetics of neurophysin binding to hormones utilizing relaxation techniques. The binding of neurophysin and oxytocin is the first of these systems we have examined. We present a preliminary report of this work here.

Materials and Methods

Bovine neurophysin was prepared from a posterior pituitary acetone powder (Pel-Freez) according to the procedure of Breslow, *et al.*[2] Bovine neurophysin II (NP) was further purified using a shallow pH gradient (6.08 → 5.90) on Sephadex DEAE-A50 with pyridine-acetate buffer as the developing solvent. Traces of pyridine were removed by successive lyophilization.

The purity of the neurophysin was tested using disc gel electrophoresis at pH 9.5 (Figure 1) according to Breslow, *et al.*[2] The gels were stained with Coomassie Blue and scanned at 560 nm with a Gilford Spectrophotometer.

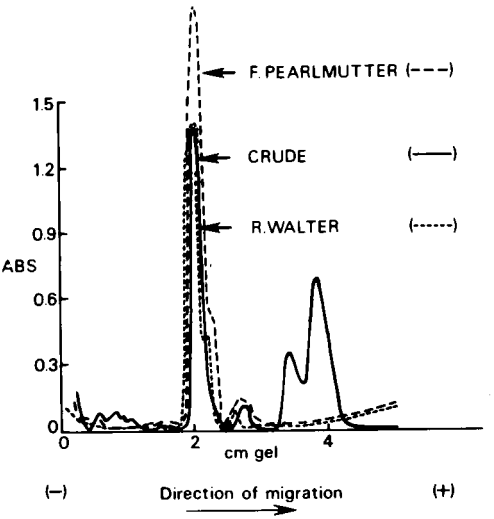

Figure 1: Spectral scan of analytical polyacrylamide gels of crude and purified bovine neurophysin II. Fifty micrograms of material was applied per gel. The Electrophoresis was performed with a 7.5% gel and a running pH of 9.5.[14] The gels were stained with Coomassie brilliant blue and scanned at 560 nm.

The purified material showed a single protein band which coincided with that of a sample of bovine neurophysin II, generously donated by Dr. Roderich Walter, which had been purified[11] using preparative disc gel electrophoresis (Figure 1). The pattern of the crude neurophysin is shown for comparison. A sample of porcine neurophysin I was donated by Drs. Ting-chi Wuu and D. H. Coy.[12] Oxytocin (OXY), donated by Dr. Guttman, Sandoz, Switzerland, was purified on Sephadex G-15 using ^{3}H-oxytocin (Schwarz-Mann) of high biological activity as a tracer, according to the procedure of Manning *et al.*[13] Pilot studies were made possible by a gift of oxytocin from Dr. M. Manning. All materials were stored as solids in a vacuum dessicator at 4°.

Kinetic measurements for porcine neurophysin and oxytocin were made on a temperature-jump in the laboratory of Dr. John Stuehr. All other measurements were made on a Gibson-Durrum Stopped-Flow Temperature Jump. For both

instruments, initial temperatures of the solutions were adjusted so that the final temperature was 25°.

Known amounts of oxytocin and neurophysin were dissolved in degassed 0.1 *M* KNO_3 containing 1 x 10^{-5} *M* phenol red. The pH was adjusted using measured amounts of KOH and HNO_3. All relaxation spectra were monitered at 558 nm, corresponding to the absorbtion maximum of phenol red.

In the presence of indicator alone, oxytocin and indicator, and neurophysin and indicator, only the relaxation effect due to proton transfer of the indicator itself was seen (∿100 μsec). Difference spectra of phenol red with either oxytocin or neurophysin showed no appreciable interaction with the indicator.

Results and Discussion

When a mixture of neurophysin and oxytocin containing 1 x 10^{-5} *M* phenol red was tested in the temperature-jump, a relaxation time in the msec time region was observed for each system (Tables I and II). Each relaxation time was

Table I

Relaxation Times for Porcine Neurophysin I and Oxytocin in .1 *M* KNO_3 Containing 1 x 10^{-5} *M* Phenol Red

NP° x $10^4 M$	OXY° x $10^4 M$	*pH*	τ *(msec)*
6.0	6.0	7.00	19
5.0	5.0	7.68	16
2.5	2.5	7.68	28
2.0	2.0	7.65	37
1.2	1.2	7.68	38
1.0	1.0	7.63	34
0.5	0.9	7.82	36

evaluated from at least two oscilloscope tracings by enlarging the photograph and plotting the log amplitude versus time to determine half times. An example of one such effect is shown in Figure 2. Half times were converted to relaxation times, τ, using the relationship

Table II

Relaxation Times for Bovine Neurophysin II and Oxytocin in .1 *M* KNO_3 Containing 1 x 10^{-5} *M* Phenol Red

NP^o x 10^4M	OXY^o x 10^4M	pH	τ(msec)
4.05	5.25	7.61	12
4.84	3.49	7.67	10
2.88	2.88	7.45	17
2.02	2.62	7.51	14
2.23	1.61	7.67	23
.96	1.23	7.49	19,22
.95	.95	7.52	28
.53	.80	7.43	30
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.33	.42	7.60	28
.31	.40	7.41	37
.44	.32	7.45	38

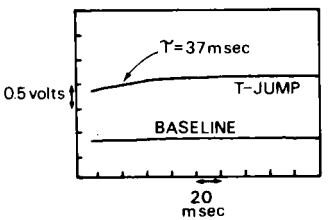

Figure 2: Temperature-jump reaction trace for a mixture of bovine neurophysin II (3.1 x 10^{-5} *M*) and oxytocin (4.0 x 10^{-5} *M*) in .1 *M* KNO_3 at pH 7.41 containing 1 x 10^{-5} *M* phenol red. Time scale is 20 msec per horizontal division (time constant, 100 μsec) and vertical scale is 0.5 volts (5% transmittance) per division. The temperature-jump was Δ15C (5kv), from 10 to 25°. The relaxation time was determined to be 37 msec.

$\tau = t^{1/2} / \ln 2$ (ref. 10).

The mechanism which fit the data shown in Tables I and II was

$$NP + OXY \underset{k_r}{\overset{k_f}{\rightleftharpoons}} NP - OXY \qquad [1]$$

Fast pre-equilibrium steps corresponding to protonation of the neurophysin, oxytocin, and indicator are coupled to the binding reaction and were included in the mathematical analysis. Only those proton transfer steps known to influence neurophysin-oxytocin binding were considered.

The complete expression for the relaxation time including correction factors for the pre-equilibrium proton transfers is

$$\frac{1}{\tau} = k_f \left(\frac{NP}{1+\gamma} + \frac{OXY}{1+\beta} \right) + k_r \qquad [2]$$

where

$$\gamma = \frac{H}{K_{HNP} + NP/\alpha}$$

$$\beta = \frac{H}{K_{HOXY} + OXY/\alpha}$$

$$\alpha = \frac{IN}{K_{IN} + H}$$

The correction factors γ and β were less than 0.04 in all cases and could be neglected.

Figures 3 and 4 show a plot of $1/\tau$ versus the sum of the equilibrium concentrations of unbound neurophysin and oxytocin. In both cases, straight lines are obtained whose slopes correspond to $k_f = 2.2 \times 10^5\ M^{-1}\ sec^{-1}$ and $1.3 \times 10^5\ M^{-1}\ sec^{-1}$ and whose intercepts correspond to $k_r = 18\ sec^{-1}$ and $12\ sec^{-1}$ for bovine and porcine neurophysin, respectively. In both cases, the binding constant used to determine equilibrium concentrations was that of Breslow and Walter ($1.21 \times 10^4\ M^{-1}$) determined at pH 7.4.[6] The ratio of k_f to k_r gives back the binding constant used and is a measure of the internal consistency of the interpretation of the data.

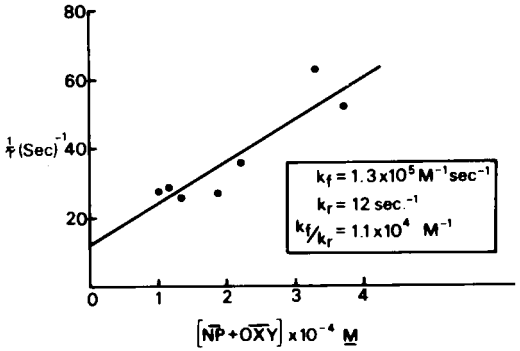

Figure 3: Plot of $1/\tau$ versus the sum of the equilibrium concentrations of porcine neurophysin I and oxytocin. The forward and reverse rate constants determined from this plot are shown in the figure.

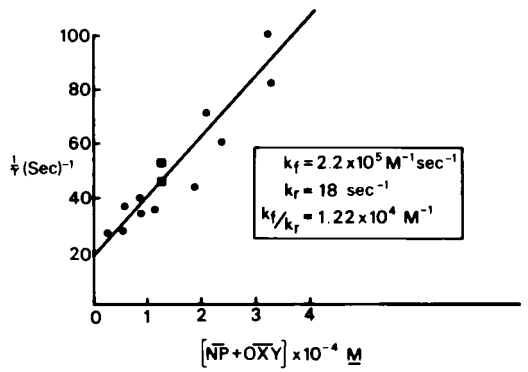

Figure 4: Plot of $1/\tau$ versus the sum of the equilibrium concentrations of bovine neurophysin II and oxytocin. The points shown as squares (■) were determined using bovine neurophysin II donated by Dr. R. Walter.

The existence of a slow dimerization of neurophysin would not affect the kinetic analysis described above, since any kinetic step slower than that under analysis (the binding interaction) will not affect the observed relaxation time.

The addition of a proton to the NP-Oxy complex has been considered. If this step is slow compared with the formation of NP-Oxy, then, as for the dimerization, it need not be included in the kinetic analysis. If, on the other hand, this step it occurs at a time comparable to or faster than the interaction of neurophysin and non-protonated oxytocin, it must be considered.

When the formation of NP-Oxy is considered as a fast pre-equilibrium, then

$$\frac{1}{\tau} = k_f\left(\frac{NP}{1+x} + OXY\right) + k_r\left(\frac{1}{1+y}\right) \qquad [3]$$

where x and y are complex functions of the pre-equilibria of the system.[10] A computation of x and y shows that $x < .045$ and $y < .008$. Therefore protonation of the complex, if faster than complex formation itself, would not affect the kinetic analysis shown above.

We can also rule out direct interaction between protonated oxytocin (α-NH_3^+) and neurophysin at this pH. When the data are analyzed assuming this mechanism, regardless of the magnitude of the binding constant utilized, the ratio of k_f to k_r is an order of magnitude larger than the binding constant used to determine equilibrium concentrations.

Our results may be compared with the exchange rates of neurophysin-hormone complexes determined by ^{1}HNMR. Alazard *et al.*[9], assuming fast exchange limit for $1/T_2$, calculate that the exchange rate of the complex for either oxytocin or vasopressin at pH 6.75 in .1 *M* NaCl is >60 sec^{-1}; Balaram *et al.*[8], assuming a slow exchange limit for $1/T_2$, determined that the exchange rate of the neurophysin-vasopressin complex at pH 6.8 in .16 *M* KCL is <125 sec^{-1}. Although our results, 18 sec^{-1} for the reverse rate constant, appear to be more consistent with an exchange rate of <125 sec^{-1}, the difference in pH, 6.8 to 7.4, makes it unlikely that a direct numerical equivalence between the relaxation and NMR data would occur. Furthermore, the predominant species in solution may change at higher pH values.

Our results strongly indicate that the major species present in a one to one mixture of oxytocin and neurophysin is a complex in which one hormone molecule is bound to one neurophysin molecule, and that complex formation at pH 7.4 occurs via an initial interaction between neurophysin and oxytocin in which the α-amino group is unprotonated.

Acknowledgments

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SECTION VII

HYPOTHALAMIC PEPTIDES

HYPOTHALAMIC HORMONES: A FLUID STORY

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Summary--The present state of knowledge will be contrasted to that in 1969. Special topics of discussion will be: the versatility of the thyrotropin releasing hormone (TRH); the dual nature of the gonadotropin releasing hormone (GnRH); the synthesis and biological properties of analogs of TRH and of GnRH; the activities of TRH, MIH and GnRH in the central nervous system; the isolation of the growth hormone release inhibiting hormone (SRIH or somatostatin); the medical uses of the four known hormones; and the status of the other hypothetical hormones. Finally, principal topics of present interest in the field and of the opportunities for future work will be discussed with special regard for the clinical utilization of these substances.

"FLUID" IS AN APPROPRIATE ADJECTIVE to use in describing the course of research in the hypothalamic releasing hormones over the past few years. As could be expected, greater consensus has been achieved regarding the two older hormones, thyrotropin releasing hormone (TRH) and gonadotropin releasing hormone (GnRH). However, for all of these substances there are still many points of controversy which will only be cleared up after a great deal of additional experimentation has been done.

This review will not touch upon the historical background for the concept of hypothalamic control of the adenohypophysis. That was admirably done by Guillemin[1] at the 3rd American Peptide Symposium in 1972 and more recently by Sawyer[2] at the 1974 Milan Conference on Hypothalamic Hormones. At this time an effort will be made only to

review the major accomplishments in the field since the Tucson Conference on "Hypophysiotropic Hormones of the Hypothalamus" which was held in January of 1969. Since there are literally hundreds of publications on the subject, this review will be selective rather than comprehensive and will center upon research which reflects the attitudes and interests of our group at Abbott Laboratories and our colleagues at Takeda Chemical Industries in Japan.

As the year 1969 opened no structures of hypothalamic factors were known although considerable work had already been done in attempts to separate and purify them. Table I shows a list given by McCann and Porter[3] in 1969 of substances for which physiological evidence had been presented and which were presumed to be present in hypothalamic extracts.

Table I

Hypothalamic - Hypophyseal
Stimulating and Inhibiting Hormones:

Corticotropin-releasing factor
Thyrotropin-releasing factor
Luteinizing hormone-releasing factor
Follicle stimulating hormone-releasing factor
Prolactin-inhibiting factor
Growth hormone-releasing factor
Growth hormone-inhibiting factor
MSH-releasing and inhibiting factors

In the same review, McCann and Porter adduced three properties which qualify a compound for classification as true neurohumoral regulators of the adenohypophysics. In an abbreviated form, these properties are listed in Table II.

To date four peptides are known which appear to satisfy in varying degrees the criteria of McCann and Porter. Since one of these peptides, referred to as the gonadotropin releasing hormone, controls the release of both LH and FSH, more than half of those listed in Table I have been accounted for. In this review the four known compounds will be discussed in the order of their discovery, emphasing the properties which are in accord with early physiological work but point out the unexpected properties.

Table II

Proof of Neurohumoral Regulation
of the Adenohypophysis Requires that the Substance:

1. Is elaborated in secretory elements of the brain within diffusion distance of the hypophyseal portal vessels.
2. Can be identified and characterized in portal vessel blood.
3. Can be shown to affect anterior pituitary cells in such a way as to cause them to alter the release of at least one trophic hormone.

Thyrotropin Releasing Hormone (TRH)

This hormone, which was characterized as the tripeptide <Glu-His-Pro-NH_2 in 1969[4,5], was originally thought to be specific for the release of thyrotropin[6]. Thus, the prolactin releasing activity of TRH, noted in cultures of GH_3 cells by Tashjian *et al.*[7] and in man[8,9] cows[10] and rats[11] was unexpected in view of the widely held theory that the hypothalamic factors would each be responsible for the control of a single pituitary hormone. Hinkle *et al.*[12] have recently shown that the release of prolactin and TSH are closely related in GH_3 cells and have postulated that the same receptors are involved. This conclusion, however, is difficult to accept in view of the dissociability of the two effects in man, as shown by Gautvick *et al.*[13]. These investigators report that nursing induces a marked increase in serum prolactin but has no measurable effect on TSH. Perhaps these apparent anomalies will be resolved by the discovery in the pituitary of local regulatory systems which control the availability of TRH to two populations of identical binding sites.

Although more research continues to be done in TRH, this peptide has generally fulfilled the criteria of McCann and Porter for a neurohumoral regulator. Table III shows some of the more pertinent evidence for this conclusion.

TRH has been widely studied clinically as a diagnostic tool. In a recent review of this work Besser[14] states that TRH provides a safe, reliable and simple test for primary thyroid disease but that in pituitary of hypothalamic disease less valuable data are obtained since the results are variable. The commercial introduction of TRH as a diagnostic agent has been slow in coming, partly because of the restricted availability of the TSH radioimmunoassay.

Table III

Authentication of TRH as Hypothalamic Hormone

1.	Elaborated in hypothalamus: In rat: (Reichlin and Mitnich, 1973); In guinea pig: (Grimm-Jorgensen and McKelvey, 1974)
2.	Presence in portal vessel blood: (Wilber and Porter, 1970)
3.	Proof of action in pituitary:
	a) Binding to receptor sites: (Wilber and Seibel, 1973)
	b) Release from pituitary cells: (Vale *et al.*, 1972)

Gonadotropin Releasing Hormone (GnRH)
<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

By the time of the Tucson Conference it had become clear to this investigator that the postulated LH and FSH releasing hormones were remarkably difficult to separate. Subsequently, after a series of specially designed experiments failed to show any physical or chemical separation of the two activities, the hypothesis was advanced that the two activities were due to a single chemical substance[15]. After the hormone had been isolated from porcine hypothalamic extracts and characterized by the New Orleans group[16], the dual nature of the decapeptide was confirmed[17]. Therefore, it has seemed appropriate to designate this hormone "gonadotropin releasing hormone" and to use the abbreviation "GnRH". The use of this nomenclature in the new edition of "Textbook of Endocrinology" edited by R. H. Williams suggests that it is gaining wide approval and recognition.

The decapeptide format of GnRH has offered synthetic chemists a much greater latitude in designing analogs than did TRH. At Abbott we have worked in collaboration with a group of chemists in Japan under the leadership of Dr. Masahiko Fujino, who have achieved outstanding success in this field. A preliminary probe of the GnRH molecule with single substitutions of amino acids did not show any unexpected results[18]. Position 3 was not investigated. However, substitution at all other positions by related amino acids retained some biological activity, but no analogs showed potency greater than the natural hormone. In a later study it was found that deletion of the glycine residue in position 10 presented an opportunity to design a variety of substituted amides on the proline residue in position 9[19,20] which,

in the ethyl amide, showed an activity of approximately five times that of GnRH[21]. Subsequently the strategic nature of position 6 in the molecule was exploited to give doubly modified analogs with ovulation inducing activities up to 80 times that of the natural hormone[22]. The surprisingly intense biological activity of the des-Gly^{10}-[D-Leu^{6}]-GnRH ethylamide analog was reported independently by Vilchez-Martinez *et al.*[23]. The reason why these highly active ovulation-inducing analogs show much less activity *in vitro* is not fully understood. However, in confirmation of the high ovulatory activity, we have recently shown[24] that the des-Gly^{10}-[D-Leu^{6}]-GnRH ethylamide analog is at least 50 times more active than GnRH in releasing LH in both the mid luteal and the anestrus ewe.

Among the almost 300 analogs of GnRH tested in our laboratory none has shown significant dissociation of the LH- and FSH-releasing activities. These data greatly diminish the possibility of the existence of a hypothetical FSH-releasing factor with close structural similarity to GnRH. However, Johansson *et al.*[25] have reported the existence of a specific FSH-releasing factor without as yet giving any details as to the chemical structure. Table IV summarizes the evidence for GnRH as a true neurohumoral regulator of the adenohypophysis. The evidence for its

Table IV

Authentication of GnRH as Hypothalamic Hormone

1.	Elaboration in hypothalamus:
	a) Formed from amino acids: (Reichlin and Mitnich, 1973; Johansson *et al.*, 1973)
	b) Presence shown by immunochemical means: (Zimmerman *et al.*, 1974; Kordon *et al.*, 1974, Setalo *et al.*, 1975)
2.	Presence in portal vessel blood: (Eskay *et al.*, 1974)
3.	Proof of action in pituitary:
	a) Binding to receptor sites: (Spona, 1973)
	b) Release from pituitary cells (Vale *et al.*, 1972)

direct elaboration from amino acids by hypothalamic tissue is not quite as convincing as that for TRH. However this deficiency is balanced by detailed immunochemical documentation of the presence of GnRH in what appear to be neurosecretory channels which contact the portal blood

vessels. Thus GnRH also qualifies under the McCann and Porter definition.

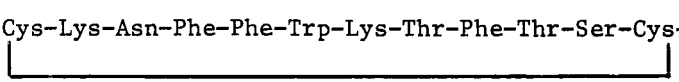
Like TRH, GnRH has shown clinical usefulness as a diagnostic agent, although the results are considerably more difficult to interpret. In the course of this work, the possible application of GnRH as a therapeutic agent is becoming evident. Besser[14] reports that of 150 patients with apparent gonadotropin deficiency, both LH and FSH were released following the initial dose in 128 cases and that most of the others responded after extended dosing. Besser also describes detailed cases of improvement in both male and female infertility after extended treatment with GnRH. Other clinicians presented similar results at the Milan Conference on Hypothalamic Hormones in October of 1974.

MSH Controlling Factors

The controversy surrounding the identity of the two factors which were postulated as controllers of MSH release, alluded to in the review of Guillemin[1], has continued. Recently, however, Walter *et al.*[26] have confirmed chemically the earlier biological data for the formation of the Pro-Leu-Gly-NH_2 (PLG) from oxytocin by enzyme systems in hypothalamic particulates. The yield of the tripeptide from oxytocin is not impressive, although it is probably greater than the overall yield shown for TRH in biosynthetic experiments using hypothalamus tissue. Thus research on the possible genesis of PLG from oxytocin might eventually serve as a model for the other hypothalamic hormones even though there have as yet been no claims for the existence of prohormones for TRH or GnRH.

Growth Hormone Release Inhibiting Hormone (Somatostatin)

In 1973 Brazeau *et al.*[27] announced the isolation from ovine hypothalamic extracts of the cyclic tetradecapeptide:



for which they proposed the name "somatostatin". Isolation of the peptide was monitored on the basis of its ability to inhibit secretion of growth hormone from rat pituitary cells in culture. Elucidation of the structure was quickly followed by synthesis[28] and testing in animals and in man. Surprisingly, in man somatostatin not only inhibits growth hormone release, but it appears to show a number of extrapituitary effects since it suppresses insulin and glucagon

secretion by direct action on the pancreas and perhaps even suppresses gastric secretion[14]. These diverse and complicated effects of somatostatin will require a great deal of study before the compound can be used therapeutically. However, the possibilities for human use now appear to extend beyond the most obvious theoretical application to the treatment of acromegaly.

Factors Controlling Prolactin Release (PRF, PIF)

It has already been mentioned that TRH releases prolactin in several species, including humans. However, since TSH is not released under natural conditions leading to prolactin release, it is presumed that there is still another factor awaiting isolation. There has also been little information in the literature regarding the purification and properties of the postulated prolactin release-inhibiting factor.

Other Postulated Releasing Factors

Up to this point allusion has been made to seven of the nine factors listed by McCann and Porter in 1969. Both of the remaining two, the corticotropin releasing factor (CRF) and the growth hormone releasing factor (GRF) were the objects of intensive search in the past. However, little has been published in either subject recently.

In the case of growth hormone release, we started a collaborative study with Drs. J. F. Wilber and T. Nagel of Northwestern University in 1971. Initial tests of Sephadex G-25 fractions from crude porcine extracts revealed the presence of a fraction which caused the release of radioimmunoassayable growth hormone from rat hemipituitaries *in vitro*[29]. The active fraction emerged near the included volume, widely separated from the hexapeptide described earlier by Schally *et al.*[30]. A systematic search for the active substance was made in our porcine fractions. As purification proceeded it was obvious that the growth hormone releasing activity was not being separated from TRH. The most active fraction, releasing growth hormone at a level below one nanogram, contained about 30% TRH by direct radioimmunoassay. Further efforts to separate the active fraction from TRH were unavailing and comparative tests revealed that TRH did release growth hormone in the same dose as the porcine fraction. These results were confirmed by Carlson and Mariz at Washington University in St. Louis[31]. These results, together with reports that TRH

causes release of growth hormone in several species *in vivo* suggest that TRH may be involved in the physiological mechanism regulating growth hormone.

Presence of Releasing Factors in the CNS

In another paper in this meeting, Plotnikoff and Kastin[32] will document some central nervous system activities of the releasing hormones. Such functions suggest the presence of the releasing substances in brain tissues outside the hypothalamic area and evidence is accumulating that such is indeed the case. Several investigators[33,34] have measured the amounts of TRH in the various brain segments and have discovered to their surprise that there is more TRH outside the hypothalamus than within it, although the hypothalamus possesses the highest concentration. We and our associates at Northwestern University, have recently shown[35] that both TRH and GnRH are present in the pineal gland in concentrations comparable to those in the hypothalamus. This was perhaps not surprising in view of the reported sequestration of systemically administered TRH[36] and GnRH[37] by the pineal. These results encouraged us to check for the presence of GnRH in the brain segments. Using the hot 2 *N* acetic acid extration method described some years ago by Schally *et al.*[38] and measuring GnRH concentrations by specific radioimmunoassay[39], we detected substantial amounts of GnRH in all of the conventional brain segments. Table V shows the values for both GnRH and TRH in the tissues from male and female rat litter mates. It is clear that the total amount of GnRH is higher than that of TRH.

Table V.

Intact Litter Mates

	(GnRH (ng/gland)		*TRH (ng/gland)*	
Tissue	*Male*	*Female*	*Male*	*Female*
Pinela	.2	.32	1.1	.14
Hypothalamus	8.8	4.8	<.2	.27
Pituitary	1.5	1.6	0.14	.18
Brain Stem	1.7	3.6	0.45	.77
Cerebellum	9.5	2.7	1.4	.26
Cerebral Cortex	6.2	2.6	2.8	1.2
Midbrain	32	4.2	3.6	1.6
Total Per Brain	60	20	9.7	4.5

The question of the origin of these substantial quantities of extrahypothalamic releasing hormones next occupied our attention. The concentrations of GnRH and TRH in the brain segments from chronically pinealectomized rats were measured by the same methods. From Table VI it is apparent

Table VI

Pinealectomized Females; 29 Days Post-Surgery

	GnRH (ng/gland)		*TRH (ng/gland)*	
Tissue	*Operates*	*Sham*	*Operates*	*Sham*
Pineal		19.0		0.85
Hypothalamus	4.4	5.9	.53	0.58
Pituitary	3.0	4.8	.16	0.23
Brain Stem	25	3.6	1.4	2.3
Cerebellum	29	16	0.4	1.0
Cerebral Cortex	4.0	18	2.4	4.0
Midbrain	3.7	16	1.6	2.3
Total Per Brain	69	83	6.5	11.3

that there are still substantial amounts of both hormones in the brain segments. Thus the pineal gland does not appear to be vital to the maintenance of brain levels of GnRH and TRH.

This subject is presently in a very fluid condition. We have not discovered the role of the pineal gland nor have we shown that this gland is a source of the releasing hormones in the CNS. However, there now appears to be abundant evidence that peptides, traditionally considered "hypothalamic-releasing hormones" may subserve CNS functions unrelated to pituitary hormone secretion and biosynthesis.

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POSSIBILITIES FOR THE DUAL CONTROL ROLE OF THE HYPOTHALAMIC PEPTIDES

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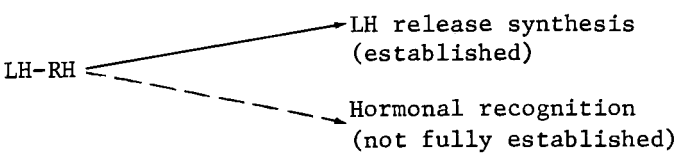
RECENTLY AN ENORMOUS INCREASE of interest has occurred in the hypothalamic regulatory peptides (Walter *et al.*,[1]; Schally *et al.*[2]; Kastin *et al.*[3]). It has been established that the hypothalamic peptides have extraendocrine effects on the CNS (Kastin *et al.*[4]).

The main point of our present study is an attempt to answer the questions: Is the release and the possible synthesis of protein hormones of central origin (Schally *et al.*[5]) the only main function of the hypothalamic peptides? What is the possible role of these peptides in hormonal information transfer and recognition at the target level? In other words, can the central regulatory peptides produce the hormonal signal and at the same time mediate hormonal recognition and hormonal information transfer? The results we obtained (Vassileva-Popova *et al.*[6]) on the phase shift between the maxima of LH concentration and LH-LHTR complex formation suggest that for hormone-receptor binding initiation the elevation of the hormone concentration is not absolutely obligatory. Possibly another kind of signal should be expected.



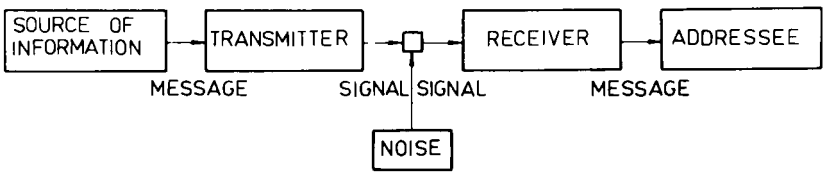


Figure 1: Basic scheme of the communication system.[7]

The scheme of Shannon[7] (Figure 1) may be applied for classical description of hormonal regulation (Figure 2). This allows the logic of information transfer to be used to define luteinizing hormone (LH) and follicle stimulating hormone (FSH) regulation.

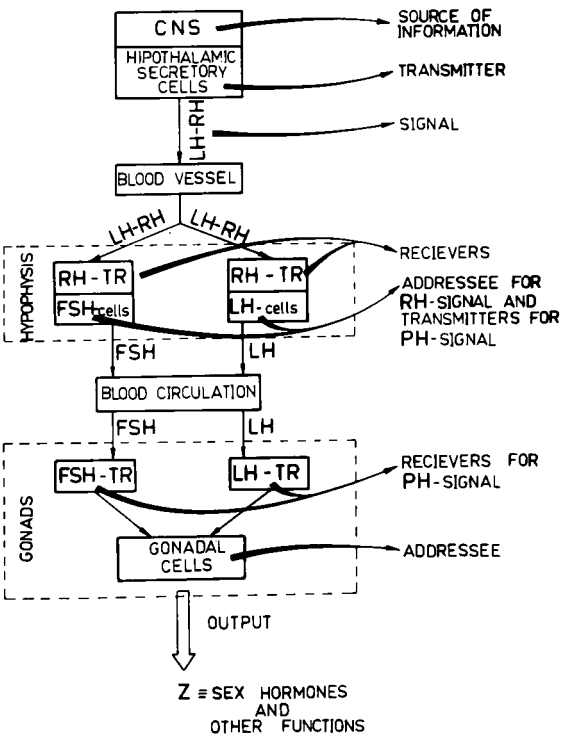


Figure 2: Scheme of hormonal information transfer.

In the classical idea of releasing hormones, the pituitary receives the LH-RH information and possibly LH-RH action is then mediated by cyclic 3',5'-adenosine monophosphate (cAMP), which leads to LH and/or FSH release:

LH-RH ⟶ ↑cAMP ⟶

$\begin{cases} \text{LH} \\ \text{FSH} \end{cases}$ release (Schally *et al.*[5]; Borgeat *et al.*[8])

In the classical case of hormonal regulation for the polypeptide hormones (PH) (Figure 3), we have:

Z (final action) = f(PH) and, in the concept suggested here on the basis of preliminary results described below, Z = f(RH,PH).

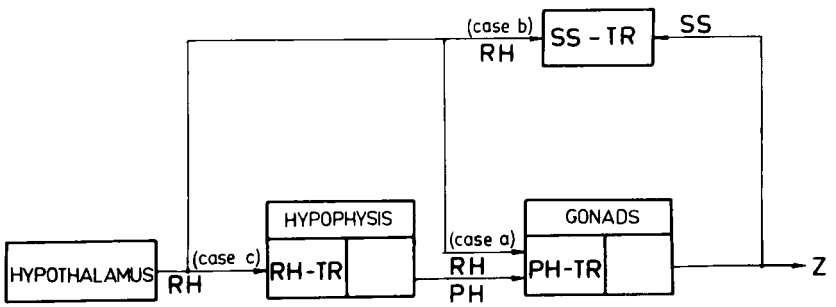


Figure 3:

The possible formal combinations of the mediation of the hormonal information transfer will be:

The Classical Case

RH ⟶ release PH (+)

RH ⟶ inhibit PH (−) for prolactin

The Concept Suggested

Case a-RH $\underset{(\text{control}\pm)}{\text{mediate}}$ PH-TR (gonadal or brain originated)

Case b-RH $\frac{\text{mediate}}{(\text{control} \pm)}$ SS-TR (gonadal or brain originated)

Case c-RH $\frac{\text{mediate}}{(\text{control} \pm)}$ RH-TR (brain originated)

Which are the negative and positive sides of these assumptions? In cases a and b the possibility of the releasing hormones (RH) mediating or controlling (stimulating or inhibiting) the interaction of protein hormones (PH) or sex steroids (SS) with their receptors on gonadal level is unlikely because of the low concentration of the releasing hormones (RH) in the circulation, although there is evidence that RH reach the targets[9-11]. It may be that for this kind of mediation only trace quantities of RH are necessary to amplify the hormonal signal.

Our preliminary results obtained with the application of the radio receptor assay (Vassileva-Popova)[12] indicate an increase in the binding of LH to testicular plasma membrane in the presence of traces of LH-RH, but there is a possibility of a nonspecific stimulation of the PH-TR binding. The function of RH in controlling hormone-receptor interaction would be an additional complication of this already complex regulation network.

It is more likely that the RH are involved in hormone-receptor interactions at the central level where the RH concentration is higher than in the peripheral circulation. The amount of RH in the hypothalamic area was estimated a decade ago[13,14] and more recently it was confirmed by Arimura and coworkers.[11]

Recent results indicate that the SS modulate the gonadotropin releasing action of LH-RH,[15] as is illustrated by case c in our scheme, where it is postulated that RH can be the mediator or controller (stimulate or inhibit) of its interaction with its own target receptors at the hypothalamic and pituitary level. The concept suggested for an additional action of RH seems to be a complication of the hormonal regulation, but will probably be a synchronizing mechanism for regulation. There are other cases where hormonal control is also duplicated or triplicated via the stimulation of release (RF), stimulation of inhibition (IF), and hormonal self inhibition. This or a similar kind of complex control is observed in prolactin regulation.[16,17,18]

Our previous results indicating the phase-shift between the maxima of the hormonal concentration and the binding receptor affinity[12] and the corresponding rhythm between the hypothalamic peptides and the pituitary hormones[6] lead

to the concept of the dual control role of the hypothalamic peptides: to produce the polypeptide hormonal signal and as a second possible action to be involved in the hormonal recognition.

Abbreviations for Figure 2

RH	Releasing hormone
PH	Polypeptide hormone
SS	Sex steroids
TR	Target receptor
RH-TR	Releasing hormone target receptors
PH-TR	Polypeptide hormone target receptors
SS-TR	Sex hormone target receptors
RF	Releasing factor
IF	Inhibiting factor
LH	Luteinizing hormone
LH-RH	Luteinizing hormone - releasing hormone
LH-TR	Luteinizing hormone - releasing hormone
FSH	Follicle-stimulating hormone
FSH-RH	Follicle-stimulating hormone - releasing hormone
CNS	Central Nervous System

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SYNTHESIS OF SOMATOSTATIN

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THE NEW PERSPECTIVES for clinical endocrinology deriving from the use of hypothalamic hypophysiotropic hormones led us to set up a research program involving a synthesis of somatostatin[1] (GIF) besides earlier syntheses of GHRH and analogs[2].

The structure of the growth hormone release-inhibiting factor (GIF)

H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH
1 2 3 4 5 6 7 8 9 10 11 12 13 14

appeared of particular synthetic interest for evaluation of the practical applicability of pyrazoline active esters [3,4,5,6] (HOMMP and HOPMP esters)* in peptide synthesis. The molecule was assembled, *via* the azide procedure, from two fragments of equal size both synthesized by the classical solution method (*Figure 1*). Fragment 1-7 was obtained from tripeptide 5-7 and tetrapeptide 1-4. The tripeptide was prepared by stepwise elongation from phenylalanine methyl ester using pyrazoline esters for activation of the N-protected amino acids to be incorporated. The tetrapeptide 1-4 intermediate was prepared starting from N^{ε}-Z-Lys-OMe which was coupled with Boc-Cys(MeOBzl)-OH activated as OMMP ester. The resulting dipeptide, after the deblocking step, was reacted with OMMP ester of Z-Ala-Gly-OH which, in its turn, was obtained by coupling

*HOMMP = 1,3-dimethyl-4-nitroso-5-aminopyrazole
HOPMP = 1-phenyl-3-methyl-4-nitroso-5-aminopyrazole

Z-Ala-OMMP with glycine. Hydrazinolysis of the ensuing tetrapeptide followed by azide-condensation with the deblocked tripeptide 5-7, afforded fragment 1-7, which was subsequently used as hydrazide.

The hexapeptide 9-14 intermediate was obtained by coupling two fragments, corresponding to the sequences 9-11 and 12-14, *via* the azide procedure. Tripeptide 9-11 was built up by stepwise elongation from the C-terminal H-Phe-OMe, threonine being introduced *via* the azide method and lysine as OMMP ester. Tripeptide 12-14 was synthetized by the azide procedure from cysteine and Z-Thr-Ser-$NHNH_2$, obtained through hydrazinolysis of the corresponding methyl ester which was in turn prepared according to the HOBt/DCC method. The OMMP activation of the hydroxylated amino acids was unsuitable due to the high reactivity of the hydroxyl function towards pyrazoline active esters during the activation process. The introduction of tryptophan-8 as OMMP ester completed the 8-14 fragment.

Recoveries were satisfactory in all steps (ca. 80%). All couplings were carried out under conditions known to avoid racemisation and were carefully chosen to minimize side chain protection which was limited to the sulphydryl group of cysteine (as MeOBzl) and to the ε-amino group of lysine (as benzyloxycarbonyl derivative). During the synthesis, the α-amino groups of amino acids were masked as benzyloxycarbonyl or *t*-butyloxycarbonyl derivatives, while the C-terminal amino acids were used as methyl ester except for cysteine-14 which was unprotected at its carboxyl function.

Protecting group removal was performed with anhydrous hydrogen fluoride and cyclisation was obtained by air oxidation. Ion-exchange and partition chromatography yielded a highly purified product possessing full biological activity.

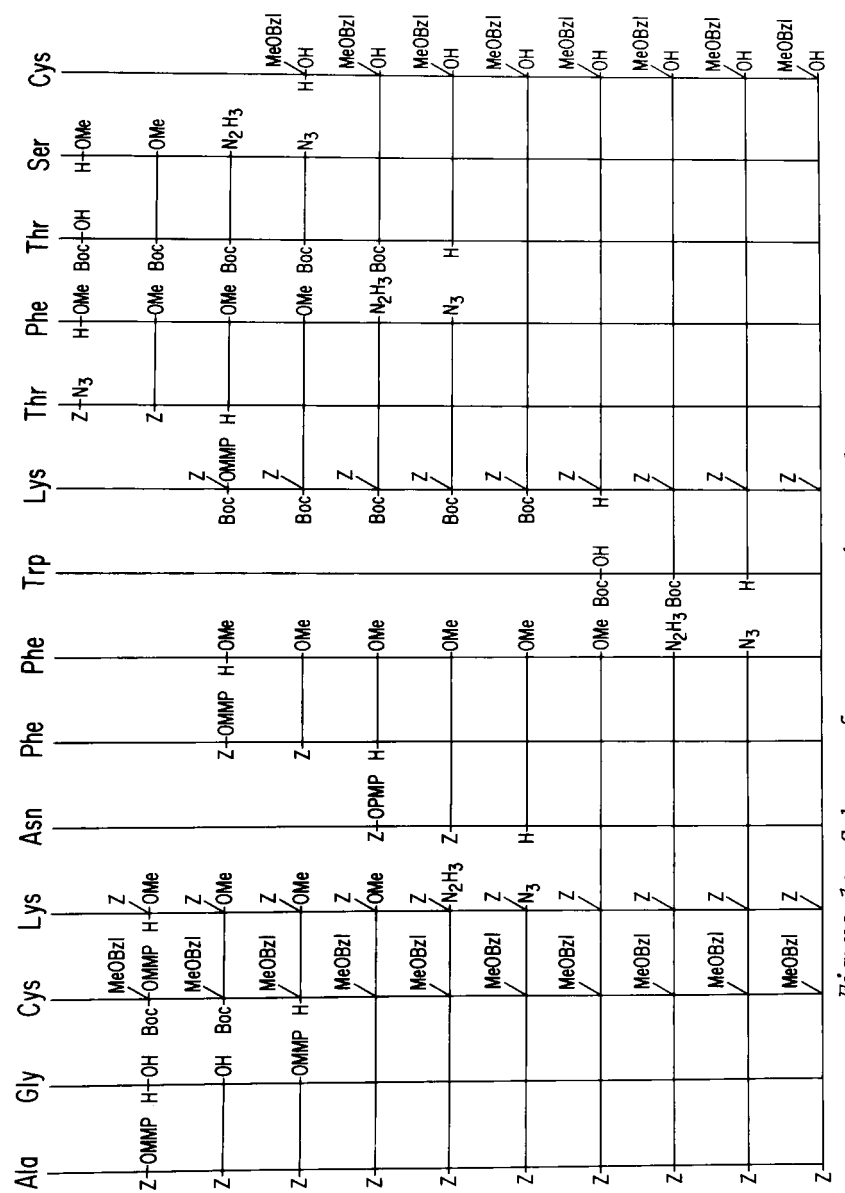

Figure 1: Scheme of somatostatin synthesis.

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LUTEINIZING HORMONE RELEASING FACTOR AND SOMATOSTATIN ANALOGS

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SYSTEMATIC STUDIES INVOLVING the substitution of the His^2, Gly^6 and Gly^{10}-NH_2 residues in luteinizing hormone releasing factor (LRF: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) by L-, D- and unnatural amino acids led to the synthesis of highly potent LRF agonists and antagonists.

Systematic studies involving the substitution of each amino acid in somatostatin (H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) by L-Ala led to analogs of higher potency than somatostatin in the case of Ala^2- and Ala^5-somatostatin (120% and 190% respectively) while Ala^8-somatostatin was less than .01% when tested *in vitro* for inhibition of growth hormone and less than 10% when tested *in vivo* on inhibition of arginine stimulated release of glucagon and insulin in the rat.

Luteinizing Hormone Releasing Factor (LRF) and Analogs

Since our first report on the importance of the histidine 2 residue in LRF (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) for full intrinsic activity and the evidence that its replacement by glycine or better, its deletion, yielded compounds showing competitive antagonism[1,2], emphasis has been put by us (Table I) and others[3] on analogs with modifications at that position. Soon after this basic contribution, we found[4] that substitution of the glycine residue at the 6 position by D amino acids yielded compounds with greater potencies (Table II). Concomitantly, Fujino *et al.*[5] reported that the des-Gly^{10}-Pro^9-NEt modification also yielded a compound with increased potency (3-fold).

Table I

In Vitro Activities* of LRF Analogs with Substitutions at the 2 Position

Compound	*Intrinsic Activity*	*Agonist Potency*	*Antagonist Potency*
LRF	100	100	-
des-His^2-D-Ala^6-LRF	<5%	-	100
des-His^2-LRF	<5%	-	10-25
Gly^2-LRF	20-50	-	-
Ala^2-LRF	20-50	-	-
D-Ala^2-LRF	<5%		9
β-Ala^2-LRF	20-50	-	-
D-Leu^2-LRF	<5	-	15
Pro^2-LRF	<5	-	12
D-Pro^2-LRF	<5	-	13
Phe^2-LRF	100	40	
D-Phe^2-LRF	<5	-	22-50
Tyr^2-LRF	100	8	
D-Tyr^2-LRF	<5	-	6
Trp^2-LRF	100%	38	-
D-Trp^2-LRF	<5%	-	120
D-Glu^2-LRF	20-50	<0.1	<5
Lys^2-LRF	<5	<0.1	<5
D-Lys^2-LRF	<5	<0.1	<5
Met^2-LRF	75	0.1	
D-Met^2-LRF	<5	-	17

* The *In Vitro* antagonist potencies determined relative to des-His^2-D-Ala^6-LRF = 100 according to Vale and Grant[19]

Table II

In Vitro Activities* of LRF Analogs with Substitutions at the 6 position

	In Vitro Potencies		*In Vitro Potencies*
LRF	100		
β-Ala^6-LRF	60	D-Lys^6-LRF	383
γ-ABu^6-LRF	3	D-Arg^6-LRF	387
Cyclo-Leu^6-LRF	11	D-Glu^6-LRF	92
D-Leu^6-LRF	300	δ-Ac-D-Orn^6-LRF	101
D-Phe^6-LRF	750	δ-Bzl-D-Orn^6-LRF	<30
D-Tyr^6-LRF	1350	δ-lauryl-D-Orn^6-LRF	290
D-Met^6-LRF	300	ε-Ac-D-Lys^6-LRF	275
D-Orn^6-LRF	182	ε-lauryl-D-Lys^6-LRF	164
Lys^6-LRF	1.8	ε-DiMe-D-Lys^6-LRF	802

*The *in vitro* potencies of LRF analogs were determined according to Vale and Grant[19].

Obviously, all kinds of combinations taking place at those three positions were synthesized and tested[6,7] (Table III). We rapidly found that the increased potency obtained by combining Fujino's modification to that of a D amino acid at the 6 position were cumulative[7,8] but that only the contribution of the D amino acids at the 6 position would hold when the des-His^2-modification was introduced to make a competitive antagonist[6,8]. More recently, while systematically investigating the influence of substitution of the histidine^2 and/or glycine^6 residues by L-, D- and

Table III

In Vitro Activities* of LRF Analogs
with Substitutions at the 2,6 and 10 positions

Agonists:	
des-Gly^{10}-D-Ala^{6}-Pro-NEt	1350
des-Gly^{10}-D-Lys^{6}-Pro-NEt	1726
des-Gly^{10}-D-Arg^{6}-Pro-NEt	1668
des-Gly^{10}-D-Orn^{6}-Pro-NEt	682
des-Gly^{10}-D-Leu^{6}-Pro-NEt	1510
des-Gly^{10}-ε-lauryl-D-Lys^{6}-Pro-NEt	1038
des-Gly^{10}-δ-lauryl-D-Orn^{6}-Pro-NEt	490
Antagonists:	
des-His^{2}-D-Ala^{6}	100
des-His^{2}-D-Lys^{6}	123
des-His^{2}-D-Orn^{6}	136
des-His^{2}-D-Arg^{6}	113
des-His^{2}-D-Leu^{6}	110
des-His^{2}-ε-lauryl-D-Lys^{6}	366
des-His^{2}-D-Ala -N$^{\alpha}$Me-Leu^{7}	246
des-His^{2}-Gly^{10}-Pro-NEt	28
des-His^{2}-Gly^{10}-D-Ala^{6}-Pro-NEt	100
des-His^{2}-Gly^{10}-D-Lys^{6}-Pro-NEt	105
des-His^{2}-Gly^{10}-D-Arg^{6}-Pro-NEt	75
des-His^{2}-Gly^{10}-D-Leu^{6}-Pro-NEt	130
des-His^{2}-Gly^{10}-ε-lauryl-D-Lys^{6}-Pro-NEt	266
D-Phe^{2}-D-Ala^{6}	220
D-Phe^{2}-D-Lys^{6}	225
D-Phe^{2}-D-Ala^{6}-N$^{\alpha}$Me-Leu^{7}	879
des-Gly^{10}-D-Phe^{2}-D-Lys^{6}-Pro-NEt	185

*The *in vitro* potencies of LRF agonists (LRF = 100%) and LRF antagonists (des-His^{2}-D-Ala^{6}-LRF = 100%) were determined according to Vale and Grant[19].

unnatural amino acids having hydrophobic, aromatic or charged side chains with combinations thereof, Rees *et al.*[9] presented biological evaluation of LRF analogs with substitutions at the 2 position. Our earlier observation that D-Ala^2-LRF[10] was an antagonist was acknowledged and D-Phe^2-LRF was reported to be 5 times a better antagonist than des-His^2-LRF while D-Trp^2 was as active an antagonist as des-His^2-LRF. The physical constants and biological potency of D-Trp^2-LRF reported by Rees *et al.*[9] are, however, quite different from ours; $^{litt}[\alpha]_D^{25}=-43.4^0$ our value $[\alpha]_D^{25}=-62.7^0$. Furthermore, D-Phe^2-LRF was found to be only twice as good an antagonist as des-His^2-LRF (see Tables I and II). Coy *et al.*[11] confirmed the inhibiting properties of D-Phe^2-LRF analogs in compounds being further substituted at the 6 position. Our more recent work (Table III) on substitutions at the 6 position has shown that large aliphatic side chains introduced at the 6 position as alkylating agents of ε-D-Lys or δ-D-Orn still retain high levels of biological activity. Even more striking is the very high potency of LRF analogs with aromatic amino acids at the 6 position D-Trp^6-LRF being the most active (not reported). Another interesting observation is that methylation of the Leu^7 α amino group in [D-Ala^6]LRF yields a molecule slightly more potent *in vitro* than [D-Ala^6]LRF, and a very potent antagonist in D-Phe^2-D-Ala^6-N$^{\alpha}$CH$_3$-Leu^7-LRF (Table III).

Somatostatin and Analogs

We recently reported the synthesis of a series of short chain analogs of somatostatin[13,14] which consisted essentially of the ring structure of somatostatin and acylation thereof. The high potency of these analogs points out that the 38 membered ring contains all the information necessary for recognition by the pituitary receptor. In our search for an even smaller active fragment, we synthesized des-Ala^1-des-Gly^2-desamino-somatostatin which is also highly active (Table IV). L-Ala^2 and D-Ala^2-somatostatin were synthesized to find out whether stabilization of the backbone would increase or decrease the affinity of the analog for its receptor. Both compounds are statistically equipotent *in vivo* and about twice as active as somatostatin. Very soon after we had realized that the Ala^1-Gly^2-side chain could be replaced by almost any acylating agent and that high levels of biological activity were still observed, we replaced the N-terminal alanine with tyrosine which can be iodinated[15]; subsequently others[16] reported the use of this analog in a somatostatin radioimmunoassay. Tyr^{11}-somatostatin

Table IV

Potencies of Somatostatin Analogs*

	In Vitro	*In Vivo*
Somatostatin	100	100
des-Ala-Gly-somatostatin	65	-
Ac-des-Ala-Gly-somatostatin	39	-
des-Ala-Gly-desamino-som.	60	-
Tyr^1-somatostatin	37	-
Ala^2-somatostatin	190	135
D-Ala^2-somatostatin	55	227
Ala^5-somatostatin	130	112
Ala^6-somatostatin	1	<10
Ala^7-somatostatin	3	<10
Ala^8-somatostatin	<0.5	<10
Ala^{10}-somatostatin	25	<10
Ala^{11}-somatostatin	2	29
Tyr^{11}-somatostatin	50-100	-
Ala^{12}-somatostatin	4	22
Ala^{13}-somatostatin	6	<10

*The *in vitro* potencies relative to somatostatin (100%) are on inhibition of spontaneous release of growth hormone (GH) by monolayered pituitary cells in culture[19]. The *in vivo* potencies are on inhibition of insulin release induced by arginine[20].

was also synthesized to obtain an iodinatable molecule. It is notable that even with the added functional phenol group, this analog is very active. The remaining alanine substituted analogs (Table IV) allow an appreciation of the role of side chains of the various amino acids in determining the potency of somatostatin while the backbone is left intact. In most cases, the potency is decreased as a result of the loss of groups which are important either in maintaining the conformation of the molecule or in the binding and

activation of the receptor[15,17-19]. Thus, as is the case for other peptide hormones, there appears to be strict structural requirements within the 38 membered ring for high potency.

Acknowledgements

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STRUCTURE - ACTIVITY RELATIONSHIPS IN LUTEINIZING HORMONE - RELEASING HORMONE

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SINCE THE ISOLATION and structural elucidation of porcine[1] and ovine[2] hypothalamic luteinizing hormone releasing hormone (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2: LH-RH) many analogs of LH-RH have been synthesized and tested for their agonist and antagonistic activities. Most of the analogs have one or more amino acid residues substituted by other protein amino acids. We wish to report the synthesis and biological assay of three LH-RH analogs having non-protein amino acids in positions 1, 3 and 8: pyro-L-α-aminoadipic acid (<Aad) in place of <Glu providing [<Aad^1]-LH-RH [I]; 3-(2-naphthyl)-L-alanine in place of Trp giving [3-(2-naphthyl)-L-ala^3]-LH-RH [II], and δ-N-isopropylornithine in place of arginine leading to [δ-N-isopropyl-Orn^8]-LH-RH [III].

Synthesis

<Aad was prepared by the procedure of Greenstein *et al.*[3]. 3-(2-naphthyl)-DL-Ala was prepared[4] and resolved, both as the N-acetyl derivative, using hog kidney acylase, and as the N-acetyl methyl ester, using α-chymotrypsin[5]. It was used as Boc- 3-(2-naphthyl)-L-Ala in peptide

synthesis. δ-N-Isopropyl-α-N-Boc-L-Orn was obtained in quantitative yield by hydrogenation of δ-N-Z-α-N-Boc-L-Orn with Pd/C in aqueous acetone at pH 10.5. Reaction of this with tosyl chloride gave the δ-N-Tos-δ-N-isopropyl-α-N-Boc-L-Orn used in the synthesis of analog III.

The peptides were synthesized by the solid phase method[6] on 1% cross-linked polystyrene containing 0.37 mmole Gly per gram. The protected peptides were removed from the resin by ammonolysis and deprotected either by Na/NH_3 reduction[7] or by HF[8]. The LH-RH analogs were purified by desalting over Sephadex G-15 followed by gel filtration and/or partition chromatography over Sephadex G-25. Experimental details will be published elsewhere.

Biological Results and Discussion

The LH-RH activities of the peptides I, II and III were determined as described previously[9] and are shown in Table I. The LH/FSH-RH activities of [3-(2-naphthyl)-ala^3]-LH-RH were also assayed against synthetic LH-RH by subcutaneous injection in immature male rats[10] and are shown in Figure 1. The integrated amounts of LH released after 100ng of II were approximately 1.2 times greater than those after 100 ng of LH-RH. The integrated amounts of FSH released after 100 ng of II were approximately 0.8 times as great as those after 100 ng of LH-RH.

Variations in position 1 of LH-RH[11-17] have resulted in either complete loss of activity or in retention of only low activity. Des-<Glu^1-LH-RH has low activity[9] and [Pro^1]-LH-RH,[11] which differs from the natural hormone only in lacking a carbonyl group has negligible activity. Okada *et al.*[15] suggested that the minimum structural requirement, for biological activity of LH-RH, in the N-terminal residue is -CO-NH-CH-CO-. From our results, the low activity of [<Aad^1]-LH-RH (3.6%) indicates that the size of the lactam ring is also important for the retention of full biological activity.

Deletion of Trp or replacement of Trp in LH-RH by aliphatic amino acids,[14-18] by D-Trp[19] or by other aromatic amino acids[13,14,20,21] results in low activity except for [pentamethyl phenylalanine^3]-LH-RH[21] which exhibits FSH and LH releasing potencies of 34 - 70% of natural LH-RH. The high activity was attributed to the ability of the pentamethyl phenylalanine residue to donate electrons in a charge-transfer complex with an election acceptor in the receptor site. However, the high activity of analog II indicates that position 3 does not require a good

Table I

LH-RH Activity of the Analogs Compared with that of Natural LH-RH in Ovariectomized, Estrogen-Progesterone Treated Rats

Sample	*Dose (ng/rat)*	*Mean Serum LH (ng/ml±SE)*	*P vs Control*	*Potencies vs Pure natural LH-RH*
Saline		5.2 ± 0.9		
Natural LH-RH	1	15.5 ± 1.5	0.01	
	5	67.7 ± 8.6	0.01	
[<Aad^{1}]-LH-RG [I]	1000	116.5 ± 0.5	0.01	3.6 %
	5000	143.0 ± 14.7	0.01	(1.1 % - 55.8 %)
[δ-N-Isopropyl-Orn^{8}]-	25	37.1 ± 8.6	0.05	3.7 %
LH-RH [III]	125	43.6 ± 2.9	0.01	(1.4 % - 9.1 %)
Saline		6.8 ± 0.7		
Natural LH-RH	1	39.6 ± 0.5	0.01	
(AVS 77-33 215-269)	5	83.2 ± 2.6	0.01	
[3-(2-Naphthyl)-ala^{3}]-	2	37.1 ± 8.1	0.05	51.8 %
LH-RH [II]	10	87.8 ± 3.3	0.01	(33.8 % - 79.8 %)

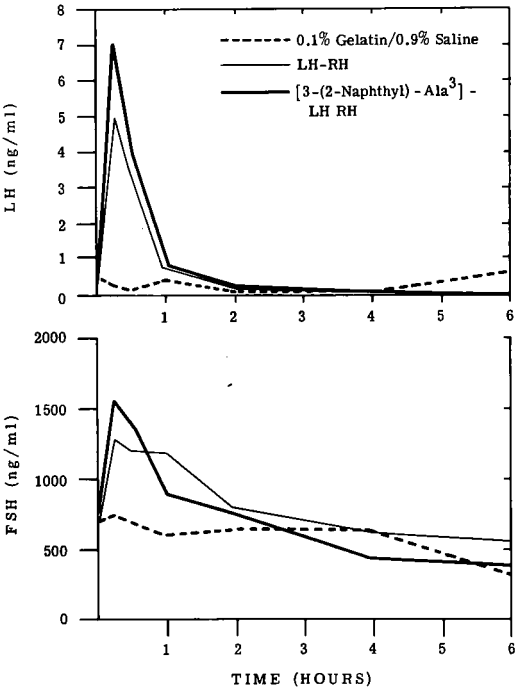

Figure 1: Mean serum LH and FSH levels at various time intervals after sc injection of LH-RH and [3-(2-Naphthyl)-ala^3]-LH-RH in 25 day old male rats. LH expressed as NIH-LH-S17. FSH expressed as NIAMD-Rat-FSH-RP-1.

charge-transfer donor. The association constants of pentamethyl benzene, indole and naphthalene with tetracyanoethylene in CH_2Cl_2 are 7.88, 2.8 and 0.75 M^{-1}, respectively.[22]

The δ-N-isopropylornithine residue has almost the same steric and electrostatic properties as an arginine residue but lacks the potential to form four hydrogen bonds. This is apparently not essential for activity since [δ-N-Isopropyl-Orn^8]-LH-RH has 3.7 % of the activity of natural LH-RH. A number of other substitutions[14,23,24-29] have been made in position 8; in general analogs with a protonated residue in position 8 have considerable activity, but even the uncharged [Gln^8]-LH-RH[26] and [Cit^8]-LH-RH[28] have high activities. It is surprising to note that acidic [Glu^8]-LH-RH[28] retained about 1 % of the activity of natural LH-RH.

Acknowledgments

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STRUCTURE-ACTIVITY STUDIES ON SOMATOSTATIN

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SOMATOSTATIN, A TETRADECAPEPTIDE, was isolated from the hypothalamus of ovine origin by Guillemin *et al.*[1] who determined its sequence as:

H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH

The linear disulfhydryl form was synthesized as soon as the structure was known,[1] and shortly afterwards the oxidized cyclic form was prepared by a solid phase[2] and a conventional method.[3]

Initially, somatostatin was identified by its ability to suppress growth hormone release from the pituitary gland. However, it soon became evident that the compound had several other activities; it inhibited glucose-induced insulin secretion,[4] glucagon secretion,[5] TRH-induced TSH secretion,[6] and gastrin secretion,[7] and it lowered plasma glucose levels in hyperglycemia.[5a]

In order to understand the structural requirements of somatostatin for the expression of its activity against release of growth hormone, insulin and glucagon, we have synthesized the analogs shown in Table I.

The synthetic procedures for analogs (I), (II) and (III) are represented on the following pages immediately after Table I.

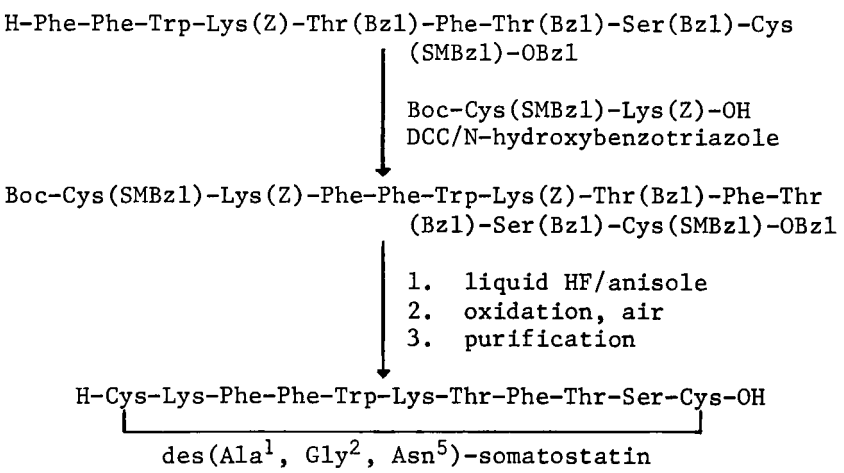
The analogs (IV), (V) and (VI) were prepared by solid phase methodology. Esterification of the chloromethylated resin with Boc-Cys(SMBzl)-OH was carried out by Gisin's cesium salt method.[8] Addition of the amino acid residues was carried out with diisopropylcarbodiimide (DIC) in the

Table 1

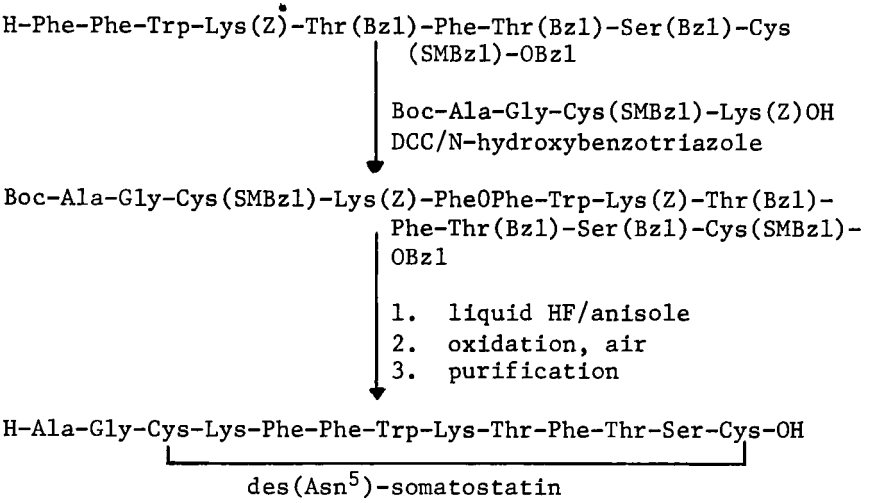
Suppression of Growth Hormone Release
by Somatostatin Analogs in Rats

Compound	*Relative Potency 15 Minutes after S.C. Injection*
Somatostatin	100%
des-Ala^1, Gly^2, Asn^5-somatostatin (I)	15%
des-Asn^5-somatostatin (II)	20%
des-Ala^1, Gly^2, Lys^4-somatostatin (III)	12%
des-Ser^{13}-somatostatin (IV)	55%
Nle^9-somatostatin (V)	0.5%
D-Lys^9-somatostatin (VI)	0.5%

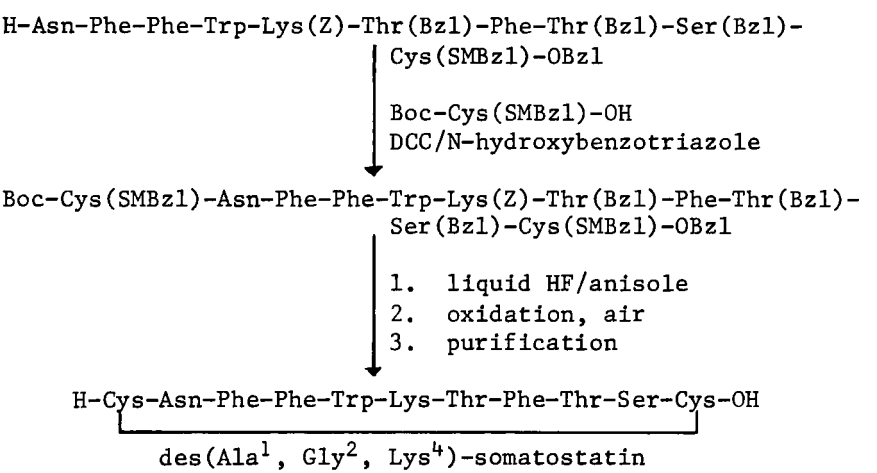
Analog (I)



Analog (II)



Analog (III)



presence of N-hydroxybenzotriazole. The by-product N, N'-diisopropylurea is more soluble in organic solvents then N,N'-dicyclohexylurea. Consequently it is easier to remove during the washing steps of the synthesis and precipitation of insoluble urea byproduct on the resin bead is avoided, thus facilitating the diffusion of the reactants.

Deprotection was carried out with 30% TFA in dichloromethane for 30 minutes in the presence of dithioerythrytol (0.5%).

Removal from the resin and deprotection was accomplished with liquid HF in the presence of anisole. Oxidative disulfide bond formation was carried out by air at pH 7.4. Purification included gel filtration and partition chromatography through Sephadex G-25.

The significant activities of analogs (I), (II), (III), and (IV) which possess a 35 membered ring instead of the 38 membered ring of somatostatin, suggest that the size of the ring is not crucial for biological activity at least for growth hormone. In the same light it is apparent that the serine at position 13, the asparagine at position 5 and lysine at position 4 are not involved in the catalytic core of somatostatin. The "tail" Ala-Gly- of somatostatin can also be deleted with a minor loss of potency and this is in agreement with Rivier's results.[9]

Lysine at position 9 exhibits a striking sensitivity to changes. Substitution of the ε-amino group with hydrogen providing L-Nle^{9}-somatostatin and inversion of the α-L-carbon of the lysine-9 giving D-Lys^{9}-somatostatin result in practically inactive molecules, suggesting a significant role for this lysine residue. This is in contrast to the indifference for activity when eliminating lysine-4.

Further work is in progress for the delineation of the structure-activity relations of somatostatin.

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STRUCTURE-FUNCTION RELATIONSHIPS OF LH-RH/FSH-RH

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DURING OUR INVESTIGATIONS on structure-function relationships of LH-RH/FSH-RH we discovered that by introduction of another positive charge in position 7 the intrinsic activity was diminished to a great extent. [Lys^7]-LH-RH and [Arg^7]-LH-RH have about 1% of the biological activity of LH-RH. However an intermediate product, [$Lys(Boc)^7$]-LH-RH still showed a considerable amount of activity (32% of LH-RH). In the meantime other laboratories also modified Leu^7 in LH-RH.[1-4] The activity of the recently published LH-RH analogs decreases in the following order: LH-RH (100%), [Ile^7]-LH-RH (45%), [Nle^7]-LH-RH (30%), [Val^7]-LH-RH (16%), [Ser^7]-LH-RH (10%), [Ala^7]-LH-RH (5-6%), [Gly^7]-LH-RH (3 bzw. 0.2%), [D-Leu^7]-LH-RH (1%). These results show that in position 7 an L-amino acid with a branched alkyl side chain is required. But the protons of α-CH and β-CH_2 should be sustained.

A representative bulky alkyl side chain is the *tert*-butyl group, which may be well introduced as ethers in cysteine, serine, and threonine, as esters in glutamic acid and aspartic acid and as Boc-groups in lysine and ornithine. We synthesized a number of LH-RH-(1-9)-nonapeptide ethylamides, in which position 7 was substituted by such *tert*-butyl containing moieties.

Table I shows that leucine can be substituted without substantial loss in activity by $Glu(OBu^t)$ and $Ser(Bu^t)$. A very strong increase in activity could be observed when Leu^7 was exchanged by $Cys(Bu^t)$. The 100-fold weaker activity of the [$Thr(Bu^t)^7$] analog is interesting because one of the obviously essential β-protones is substituted by a methyl radical.

Table I

Substitution of Position 7
in Des(10-glycinamide)-LH-RH-nonapeptide ethylamide

Compound	$[\alpha]_D^{20}$ *(c=1, in H_2O)*	*Ovulation (LH-RH=1)*
Des-Gly^{10}-LH-RH-nonapeptide ethylamide	-63.5°	6.7[6]
[$Lys(Boc)^7$]	-48.9°	2.0
[$Orn(Boc)^7$]	-55°	2.0
[$Glu(OBu^t)^7$]	-50.1°	6.2
[$Asp(OBu^t)^7$]	-59°	2.2
[$Cys(SBu^t)^7$]	-57.8°	0.5
[$Cys(Bu^t)^7$]	-62°	40
[$Ser(Bu^t)^7$]	-68.6°	4.1
[$Thr(Bu^t)^7$]	-62.3°	0.06

In the meantime it was known from literature, that D-amino acids in position 6 increase the intrinsic activity of LH-RH[5-9]. Above all [D-amino $acid^6$]-LH-RH-(1-9)-nonapeptide ethylamides exhibit high biological activity[6-10]. [D-Leu^6]-LH-RH-(1-9)-nonapeptide ethylamide was the most potent compound[6,8]. We adopted the principle found with *tert*-butyl containing amino acids in position 7 also for position 6. With [D-$Ser(Bu^t)^6$]-LH-RH-(1-9)-nonapeptide ethylamide we found an analog which surpassed even the D-Leu^6 compound (see Table II). As may be seen from the less active D-$Thr(Bu^t)^6$, D-*tert*-Leu^6 and D-$penicillamine^6$ analogs the β-CH_2-group is essential for optimal intrinsic activity, as observed above with 7-position analogs.

Combination of *tert*-butyl containing amino acids in position 7 with D-amino acids in position 6 did not result in the expected or desired potentiation of activity. In most cases the biological activity decreased (see Table III).

These compounds were synthesized by fragment condensation (see reaction scheme I and II). Side chain unprotected arginine was used because of the instability of *tert*-butyl groups in acidic medium. Another possibility for the cleavage of tosyl or benzyl protecting groups

Table II

Substitution of Position 6
in Des(10-glycinamide)-LH-RH-nonapeptide ethylamide

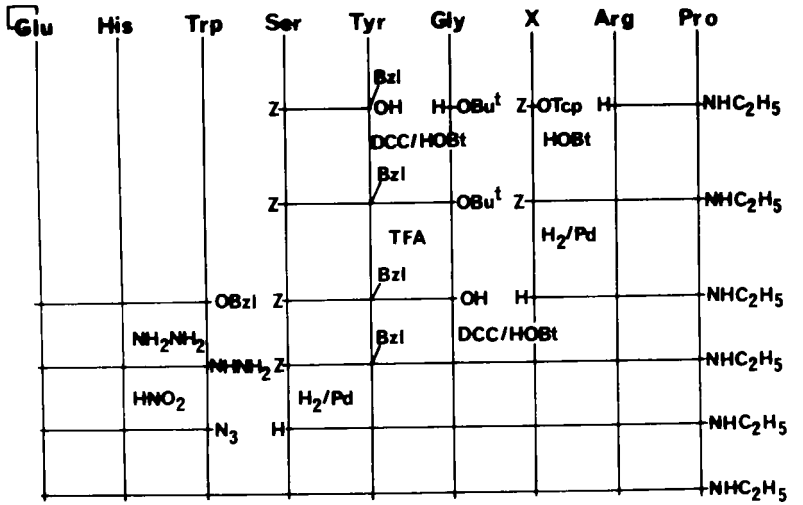
Compound	$[\alpha]_D^{20}$ *(c=1, in H_2O)*	*Ovulation (LH-RH=1)*
Des-Gly^{10}-[D-Leu^6]LH-RH-nonapeptide-ethylamide		82 (^{6})
[D-Lys(Boc)^6]	-50°	45
[D-Glu(OBu^t)^6]	-38.8°+)	40
[D-Glu^6]	-56.2°	12.5
[D-Asp(OBu^t)^6]	-64.2°	47
[D-Cys(Bu^t)^6]	-60.2°	43
[D-Ser(Bu^t)^6]	-53.2°+)	140
[D-Ser^6]	-56.4°	20
[D-Thr(Bu^t)^6]	-49.7°	13
[D-*tert.*-Leu^6]	-28.6°	1.6
[D-Penicillamin (Bu^t)^6]	-49.0°	1.7
[D-Penicillamin(Me)^6]	-73.7°++)	2.2

+) in dimethylacetamide, ++) in methanol

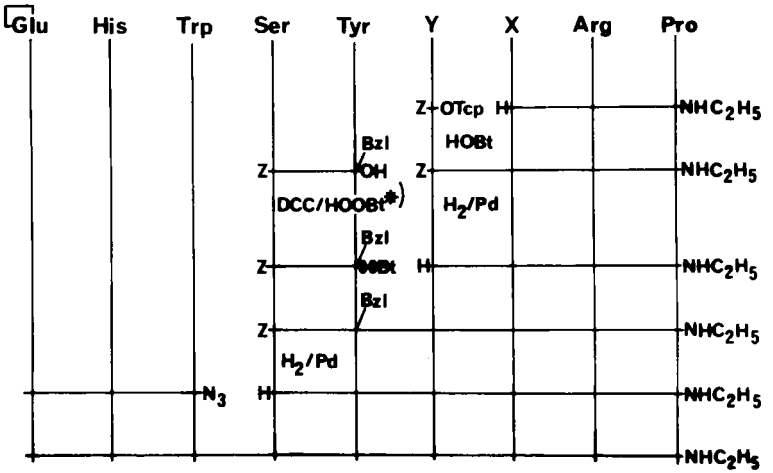
Table III

Substitution of Position 6 and 7
in Des(10-glycinamide)-LH-RH-nonapeptide ethylamide

Compound	$[\alpha]_D^{20}$ *c=1, in H_2O*	*Ovulation (LH-RH=1)*
[D-Leu^6,Ser(Bu^t)^7]	-42.9°	48-58
[D-Leu^6,Cys(Bu^t)^7]	-34.8°	40
[D-Ala^6,Cys(Bu^t)^7]	-48.3°	40
[D-Ser(Bu^t)^6,Cys(Bu^t)^7]	-39.8°	20
[D-Ser(Bu^t)^6,Ser(Bu^t)^7]	-35.6°	42
[D-Cys(Bu^t)^6, Ala^7]	-51°	0.3



Reaction Scheme I: Synthesis of (1-9)-LH-RH-nonapeptide-ethylamide, Substituted in Position 7



Reaction Scheme II: Synthesis of (1-9)-LH-RH-nonapeptide-ethylamides, Substituted in Position 6 and 7

*) HOOBt = 3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine

without affecting the *tert*-butyl groups is the use of sodium in liquid ammonia. This possibility was excluded because preliminary experiments with intact LH-RH showed that under these conditions LH-RH was cleaved between arginine and proline. Manning *et al.*[12] however reported with this deprotection method a 60% yield of LH-RH from a benzyl and tosyl protected LH-RH-decapeptide amide. The unprotected intermediate LH-RH-(4-9)-hexapeptide ethylamides were purified by partition chromatography on Sephadex LH 20 (acetic acid/*n*-butanol/water). The endproducts were finally chromatographed as acetates on carboxymethyl cellulose and/or on Sephadex LH 20 (acidic acid/*n*-butanol/water).

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SECTION VIII

ANTIBIOTICS, ENZYME INHIBITORS, AND TOXINS

STRUCTURAL AND MECHANISTIC STUDIES OF ANTIBIOTICS

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THE WIDE RANGE OF molecular structures of antibiotics - aminoglycosides, macrolides, tetracyclines, polyenes, β-lactams, peptides and others - is most reasonably interpreted as a reflection of the versatility of various species of micro-organisms in evolving means of inhibiting the growth of other competing species in their environment. As we learn more of the mechanism of action of antibiotics, it becomes increasingly worthwhile to make this the basis of classification, rather than molecular structure; different classes of organic compounds may exert their activity by similar mechanisms. The emphasis on mechanism has another significance. If the antibiotic is a weapon evolved by one species of micro-organism to deal with another, it cannot be expected to have built-in characteristics which optimise it for human therapy. The antibiotic will only be effective in man if it survives metabolism in human tissue and reaches its target in effective concentration. It follows that if an important mechanism of antibiotic action becomes understood and structure-activity relationships of antibiotics with this mechanism are defined, it may then become possible to tailor molecules better suited to human therapy than the natural products themselves.

Excellent earlier reviews[1] of peptide antibiotics have been based largely on a classification of molecular structures. They have distinguished a wide range of types of linear and cyclic peptides, many incorporating unusual amino acids. This survey cannot hope to do justice to all of the almost 300 peptides and peptide congener families of antibiotics which are known - in spite of the impressive recent advances in knowledge of mechanism of action.[2] The

following major families of peptide antibiotics may be distinguished: (i)bacterial cell-wall biosynthesis inhibitors, (ii)inhibitors of nucleic acid synthesis and function, and (iii)membrane active compounds.

Bacterial Cell Wall Biosynthesis Inhibitors

Antibiotics with this mode of action include (peptides being underlined): natural and semisynthetic penicillins and cephalosporins, D-cycloserine, phosphonomycin, bacitracin, vancomycin diumycin, janiemycin, and closely related congeners. The outer covering of *Eubacteriales* consists of peptidoglycans[3] comprising alternate residues of 2-N-acetylamino-2-deoxy-D-glucose(N-acetylglucosamine, GlcNAc) and 2-N-acetylamino-3-O-(D-1-carboxyethyl)-2-deoxy-D-glucose(N-acetylmuramic acid, MurNAc) crosslinked by peptide chains of three main types involving both D and L-aminoacids (Figure 1). The peptide chains attached to each

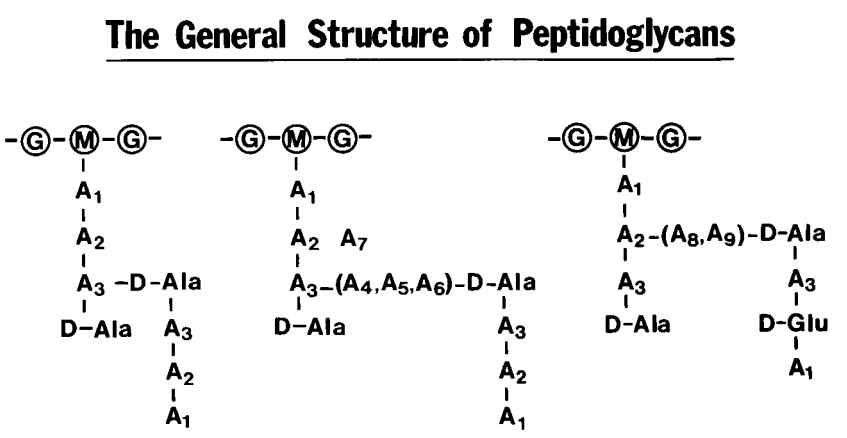


Figure 1:

carboxyl of the muramic acid residue (except *Micrococcus lysodeikticus*) vary, but only in a limited way for a given species. Other polymers are attached covalently to part of the peptidoglycan and may be a lipopolypeptide (*E. coli*), teichoic acid (many Gram +ve species) or protein (*e.g.* protein A of *S. aureus*).

The general outline of the biosynthesis is shown in Figure 2, which also indicates the points of inhibition of various antibiotics. The investigations of Strominger and others[4] have established that penicillins inhibit the

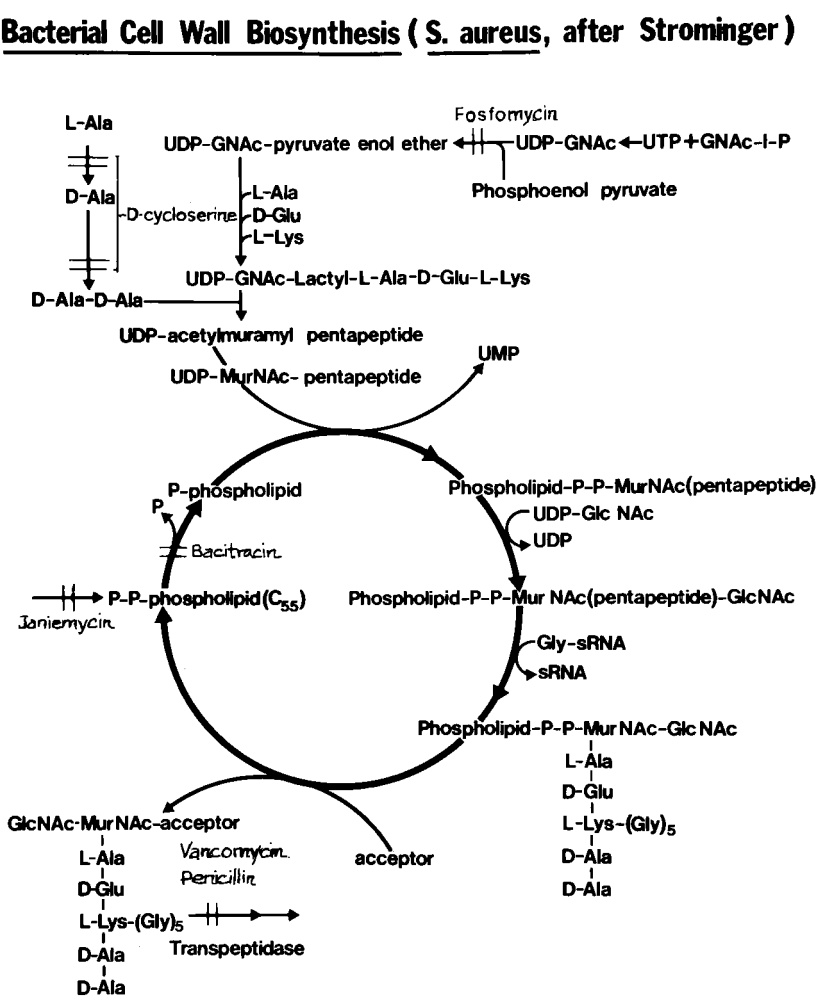
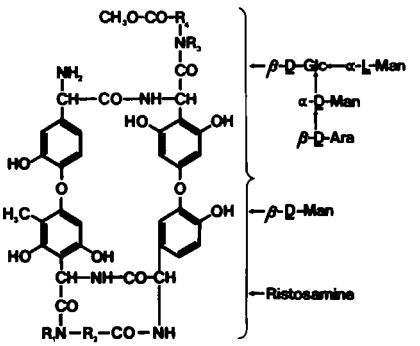


Figure 2:

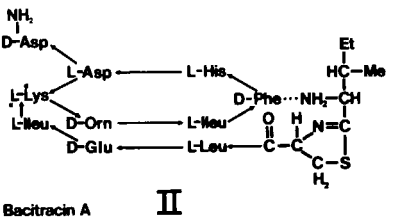
important transpeptidation step involving -D-Ala-D-Ala-OH residues and probably, less importantly, the D-Ala-carboxypeptidase. It is proposed that the action arises from the envisaged structural similarity of penicillins to -D-Ala-D-Ala-OH.

Although, for human therapy, the penicillins and cephalosporins dominate this group because of their favourable characteristics and long established position, there are others with this mode of action that have found practical use; they include the peptides bacitracin (for topical use and as a veterinary growth promoter) and vancomycin (human therapy, Gram +ve organisms). The recently discovered polypeptide janiemycin has an antibacterial spectrum very similar to the phosphoglycolipid group of animal growth promotants; these antibiotics, which include diumycin, moenomycin, macarbomycin, prasinomycin and others, have a remarkably prolonged duration of activity. They appear to act by inhibition of a step in cell wall biosynthesis involving linkage of the lipid in the peptidoglycan biosynthesis sequence.[5]

Ristomycin A (a member of the vancomycin group which also include ristocetin A,B; actinoidin and others) has the partial structure (I)[6] but the biological activity is with the aglycone. Members of the group have a remarkable ability[7] to bind specifically to peptides with C-terminal-D-Ala-D-Ala-OH (but not L-Ala-L-Ala). The precise mechanism of inhibition of cell wall biosynthesis by these compounds is still unknown.



Ristomycin A (Lomakina et al., 1970) I



Bacitracin A II

Structures I and II:

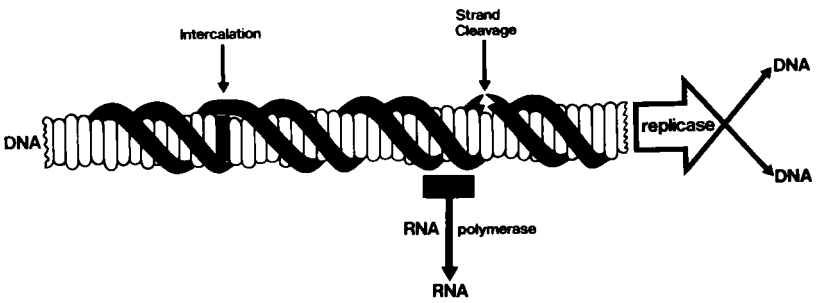
More is known of the mechanism of action of the cyclic peptide bacitracin (II). It appears to interfere with cell wall synthesis largely by complexing with bacterial C_{55}-isoprenylpyrophosphate[8] and, through this, inhibiting recycling of the lipid carrier. The following specific reaction is inhibited:

$$C_{55}\text{-isoprenyl-PP} \rightleftharpoons C_{55}\text{-isoprenyl-P+P}$$

Structural features for optimum binding are relatively specific. An intact thiazoline ring and histidine residue are essential.[9]

Antibiotics that Inhibit Nucleic Acid Synthesis and Function

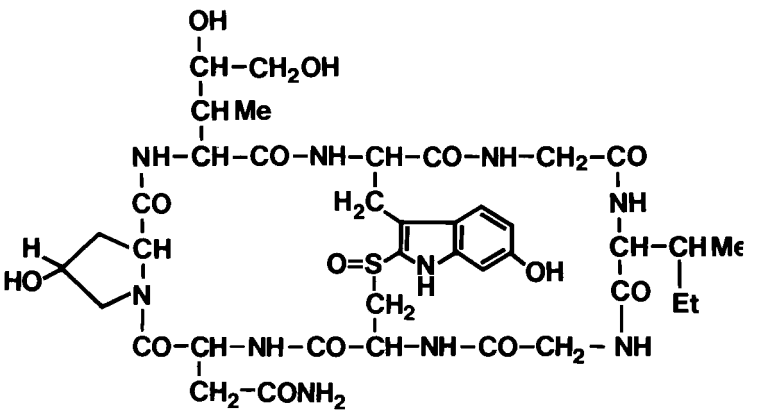
These may be further divided into: (i) inhibitors of synthesis and metabolism of nucleotides: (ii) agents that interfere with enzymes for nucleic acid synthesis; (iii) agents that interfere with DNA template function by intercalation (echinomycin, actinomycins) or strand breaking; and, (iv) antibiotics that inhibit protein synthesis by ribosomes, a large group containing macrolides, aminoglycosides, tetracyclines, chloramphenicol, lincomycins, and the peptides streptogramins, edeines, gougerotin, viomycin, thiostrepton. The mechanism of action of these antibiotics is illustrated in Figure 3.



Representation of interference with nucleic acid function

Figure 3:

Only one peptide, α-amanitin, is included in categories (i) and (ii) if gliotoxin and related epidithio-2, 5-dioxopiperazines (*e.g. Penicillium turbatium* metabolites)[10], with antiviral activity, are excluded. Moreover, α-amanitin (III), typical of the toxic principles from *Amanita phalloides*, can hardly be described as an antibiotic; it is of interest, however, as it specifically inhibits DNA-dependant RNA polymerase in animal cells but not in bacteria.[11] Such evidence of differences in RNA polymerases encourages efforts to seek species-specific inhibitors.

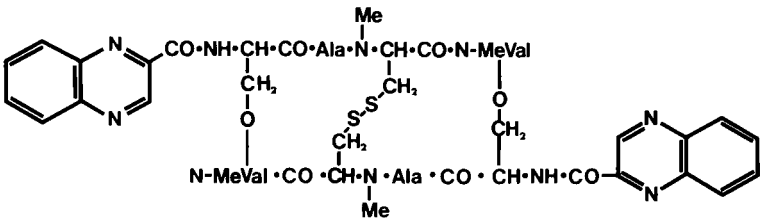


α-Amanitin III

Structure III:

The Group (iii) includes the quinoxalines and actinomycines which act against a wide range of organisms and cells, but they have a relatively low degree of specificity. The quinoxalines[12], derived from various *Streptomyces* species, comprise triostins and quinomycins (IV) involving 4 and 6 congeners respectively. They have interesting antitumour, antiviral and antibacterial activities but relatively high mammalian toxicity. The structure assigned to quinomycin A was recently revised.[13]

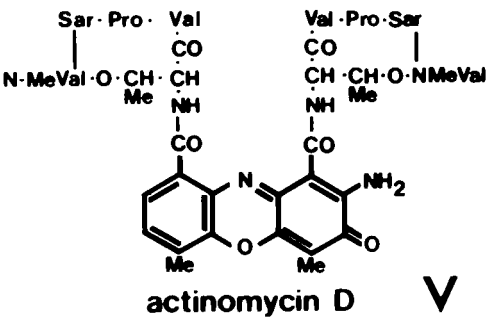
The use of actinomycin (V), particularly in the treatment of Wilm's tumour in man, has encouraged extensive studies[14,15] towards improvement of the therapeutic index. Unfortunately, toxicity continues to limit more general application in tumour therapy. More than 30 natural actinomycins have been identified and numerous synthetic



Triostin A (Otsuka and Shoji, 1967) IV

Structure IV:

and semisynthetic analogues and derivatives have been prepared. The structure-activity relationships resulting from these studies have been reviewed.[16] X-Ray analysis of the crystalline complex between actinomycin D and deoxyguanosine led Sobell and Jain to propose a model[17] for the intercalation of actinomycin into DNA which was supported by nmr data.[18]

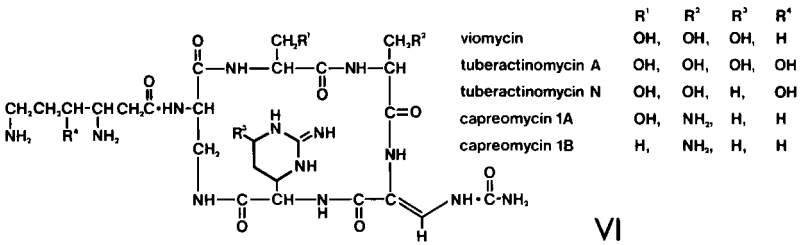


actinomycin D V

Structure V:

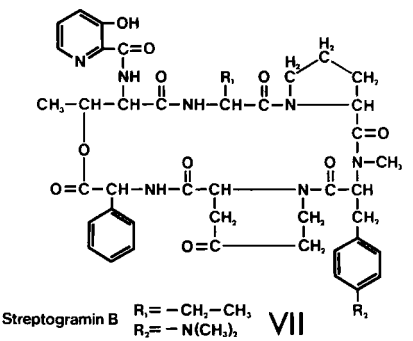
The antibiotics that interfere with protein synthesis by inhibiting ribosome function may do so by acting either on larger (*e.g.* streptogramin-B) or smaller ribosomal subunits (*e.g.* edeine-A). They are being used increasingly as biochemical tools to elucidate protein biosynthesis processes. Recent knowledge of this field has been reviewed.[19] The viomycin, tuberactinomycin, capreomycin

group with general structure (VI)[20] have interesting activity against the tubercle bacillus. Two compounds, pristinamycin-I and staphylomycin-S, of the streptogramin group (VII)[21] have found limited use for human therapy in Europe, and the related peptide mikamycin[22] is used as a veterinary growth promotant in Japan. The cyclic peptides of the streptogramin group are synergised by a second group of cyclic lactones which are not peptides (*e.g.* streptogramin A). The complex cyclopeptide, thiostrepton (VIII),[23] and related compounds,[24] have not found a place in clinical medicine but thiostrepton itself has been used for the treatment of bovine mastitis. Edeines[25] and gougerotin[26] are relatively toxic, broad spectrum peptide antibiotics. Little is known as yet about the effect of changes in molecular structure on biological activity in this group.



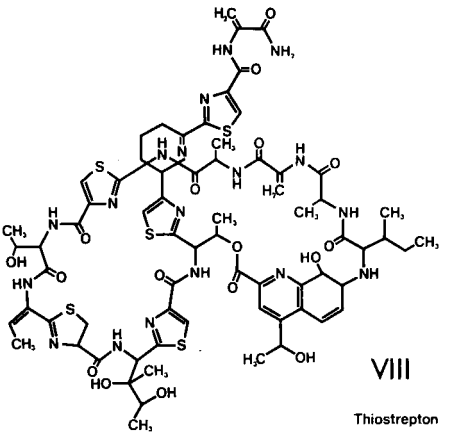
VI

Structure VI:



VII

Structure VII:



VIII

Thiostrepton

Structure VIII:

Membrane-Active Peptide Antibiotics

In spite of the very active interest in the molecular basis of the behaviour of bacterial and synthetic membranes and, consequently, the remarkable increase in knowledge of the nature of membranes during the last ten years,[27] there is still insufficient understanding to enable much to be said about the detailed mechanism of action of membrane-active antibacterial compounds. In fact, there is no generally agreed model for the structure of membranes although there is a considerable measure of agreement that the membrane is a thin structure, no more than a few molecules thick, held together by hydrophobic and polar interactions. They are probably minimum energy, self assembling systems. The antibiotics that owe their effect to membrane-activity may be divided into at least two distinct categories: (i) compounds that modify the permeability of cytoplasmic membrane to cations, as gramicidins A,B,C and D, valinomycin, enniatin and alamethicin; and (ii) compounds that cause damage to the membrane, as gramicidin S, tyrocidins, polymyxins, circulins, colistins and monamycins.

(i) Of the peptides in the first class, only the gramicidins A-D (IX) have found some use in human therapy and this is limited to topical application against Gram +ve organisms; however, all have served as interesting tools for the investigation of transport across natural and artificial

lipid bilayer membranes. The linear gramicidin A, which has cation-binding properties, facilitates transport of alkali metal cations and hydrogen ions across membranes.[28] Some structure-activity relationships have been elucidated by synthesising variants[29] but this has not, as yet, been rationalised in terms of molecular interactions.

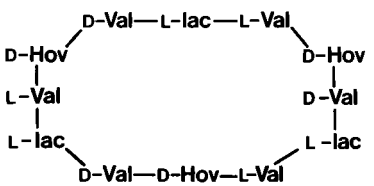
Gramicidins A–C **IX**

Val-gramicidin A	HCO-Val Gly Ala Leu Ala Val Val Val Trp Leu Trp Leu Trp Leu Trp NH·CH_2CH_2OH
Val-gramicidin B	HCO-Val Gly Ala Leu Ala Val Val Val Trp Leu Phe Leu Trp Leu Trp NH·CH_2CH_2OH
Val-gramicidin C	HCO-Val Gly Ala Leu Ala Val Val Val Trp Leu Tyr Leu Trp Leu Trp NH·CH_2CH_2OH

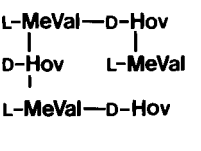
[Sarges and Witkop, 1965]

Structure IX:

Valinomycin, cyclododecadepsipeptide (X), and the enniatins, cyclohexadepsipeptides (XI), have been the subject of extensive chemical studies and a large variety of synthetic analogues of both families have been synthesised and investigated for complexation with cations and for antibacterial activity. Ovchinnikov, Ivanov and co-workers have synthesised some 59 and 85 analogues of enniatin and valinomycin, respectively. Convincing evidence of the conformations of free and cation-complexed compounds has been obtained by nmr and X-ray crystallographic studies.[28] It is clear, too, that the antibacterial properties of these cyclodepsipeptides as well as of serratamolide[30] is intimately related to cation complexing. Analogues unable to complex had no antibacterial properties.



Valinomycin X



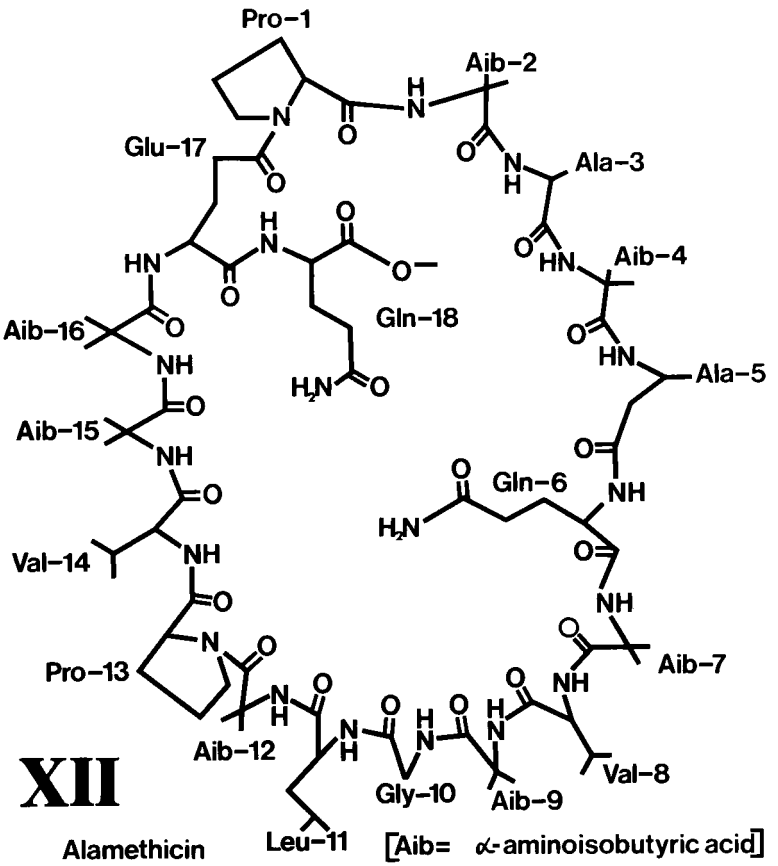
Enniatin-B XI

(lac = lactic; MeVal = *N*-methylvaline; Hov = α-hydroxyisovaleric acid)

Structures X and XI:

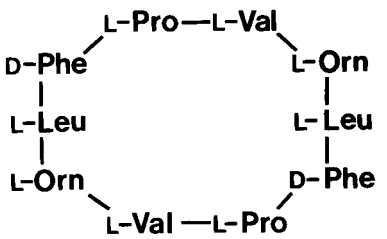
Alamethicin (XII), a cyclic octadecapeptide with weak antibacterial activity appears[31] to form complexes with Na^+, K^+, Rb^+ and Cs^+.

(ii) The membrane-active antibiotics in the second class appear to exert their action by disorganising the membrane structure. Mild action may lead to loss of some low molecular weight cell contents but more active compounds and higher concentrations lead to destruction of the membrane and, consequently, loss of function of the cell. The behaviour of peptides in this class, all cyclic compounds,

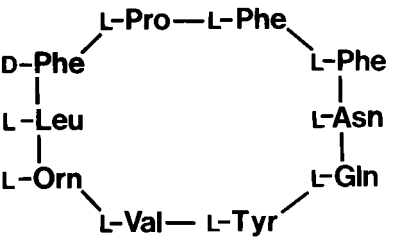


Structure XII:

resembles in many respects that of bactericidal cationic and anionic surface active compounds. It has been suggested[32] that the action of these antibiotics takes place in the stages: (1) adsorption and penetration into the porous cell wall, (2) interaction with lipid-protein complexes, leading to disorganisation of the membrane, (3) leakage of low molecular weight constituents from inside the cell, (4) degradation of proteins and nucleic acids, and (5) lysis by the action of wall-degrading enzymes.



XIII **Gramicidin S**

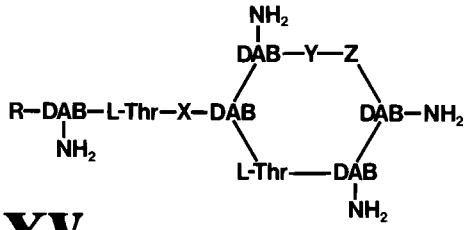


XIV **Tyrocidin A**

Structures XIII and XIV:

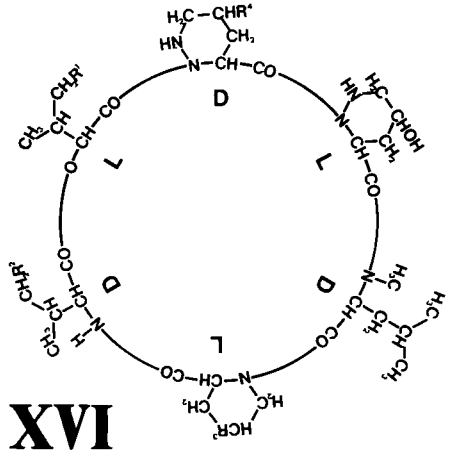
Tyrocidin (XIII) and the closely related gramicidin S (XIV) probably owe their antibacterial activity to such a 'detergent' effect. The open chain analogue of tyrocidin and of the monamycins is inactive[33] while the open chain decapeptide corresponding to gramicidin S has approximately one twelfth of the activity of the natural product.[34]

The three closely related families, polymyxins (XV), colistins and circulins, each consist of a number of molecular species. In the case of the polymyxin B, there is evidence to suggest that initial action on the cell surface of the microorganism involves binding to polyphosphate polymers of the cell wall; this may sensitize the invading organism to the defence mechanisms of the host.[35] Polymyxin B and the closely related colistin, have been used in man, parenterally, but there was evidence of nephrotoxicity. Used orally for treatment of intestinal infections, this toxicity is not manifest since there is poor absorption from the gastrointestinal tract.



XV

Polymyxin	R	X	Y	Z
B_1	MeOct	DAB	D-Phe	L-Leu
B_2	MeHep	DAB	D-Phe	L-Leu
D_1	MeOct	D-Ser	D-Leu	L-Thr
D_2	MeHep	D-Ser	D-Leu	L-Thr



XVI

Monamycin	R^1	R^2	R^3	R^4
A	H	H	Me	H
B_1	H	H	Me	H
B_2	H	Me	H	H
B_3	Me	H	H	H
C	Me	H	Me	H
D_1	Me	H	Me	H
D_2	H	Me	Me	H
E	Me	Me	Me	H
F	Me	Me	Me	H
G_1	H	H	Me	Cl
G_2	H	Me	H	Cl
G_3	Me	H	H	Cl
H_1	Me	H	Me	Cl
H_2	H	Me	Me	Cl
I	Me	Me	Me	Cl

Structures XV and XVI:

The monamycins (XVI), a family of fifteen cyclohexadepsipeptide congeners, are unusual members of this group in that they are also ionophores which form strong complexes with K^+, Rb^+, and Cs^+ but only weakly, if at all, with Li^+ and Na^+ under similar conditions.[36]

Conclusion

To the selection of antibiotics that have been reviewed could be added such compounds as D-cycloserine, the penicillins, the cephalosporins, various epidithio-2,5-diketopiperazines, and others, if the definition of a peptide is stretched to include such derivatives of amino acids. Moreover, the list could be further extended by some other biologically-active peptides of microbiological origin, *e.g.* destruxin A,B (insecticide activity), sporidesmolides (specific mammalian toxicity) and others, as well as various less well-characterised peptide antibiotics. Clearly, the novel features of structure, and their value as biochemical tools, establish the scientific interest of this group of peptides. However, relatively few have found significant practical use in medicine. If compounds used for topical preparations or veterinary applications are excluded, the list narrows to the actinomycins, cephalosporins, penicillins, polymyxins and viomycins.

There is still a need for new antibiotics for human therapy - for improved antiviral and antitumour agents and, in the case of antibacterials, for compounds to combat species less susceptible to existing therapeutic agents and to combat the growing number of resistant strains. Some new antibiotics might be expected from the continued investigations using conventional screening procedures. However, the increasing understanding of the mechanism of action of effective antibiotics and, no less important, factors determining the survival of active compounds *in vivo* and transport to the site of action make it increasingly reasonable to expect to utilise synthesis to produce new or improved antibiotics, by design.

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INTERACTION OF PEPSIN WITH PEPSTATIN

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THE PEPSTATINS, isolated from *Streptomyces*,[1] are very potent inhibitors of pepsin and other acid proteinases.[2] These peptides are characterized by the presence of two residues of 4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA) in the sequence R-Val-Val-AHMHA-Ala-AHMHA, where R is a fatty acid. They bind very strongly to pepsin with dissociation constants of the order of 10^{-10}M,[3,4] giving rise to a red shift in the aromatic UV absorption of the enzyme.

We have used the 292 nm (tryptophan) and 285 nm (tryosine) peaks of the enzyme-inhibitor difference spectra to further study the interaction between pepsin and pepstatin. We found that the difference spectrum is independent of pH in the range 0.5 to 4.5, but above this pH the maxima at 292 and 285 nm decrease (Figure 1), in the absence of measurable pepsin denaturation. This decrease closely parallels the pH-activity curve for peptic proteolysis and the pH dependence of pepsin inactivation by *p*-bromophenacyl bromide,[5] suggesting that pepstatin might bind at or near the pepsin active site.

In order to determine the degree of exposure to the solvent of the aromatic groups responsible for the difference spectrum, solvent pertubation of the pepsin-pepstatin complex by propylene glycol was studied. Pepsin alone presented a difference spectrum with maxima at 285 and 292 nm, which were proportional to the concentration of propylene glycol up to 40% (v/v), without any evident changes in the conformation of pepsin. Admitting that pepsin has 11 exposed tyrosyl and 2.5 exposed tryptophyl residues,[6] differential molar absorptivities of 166 per

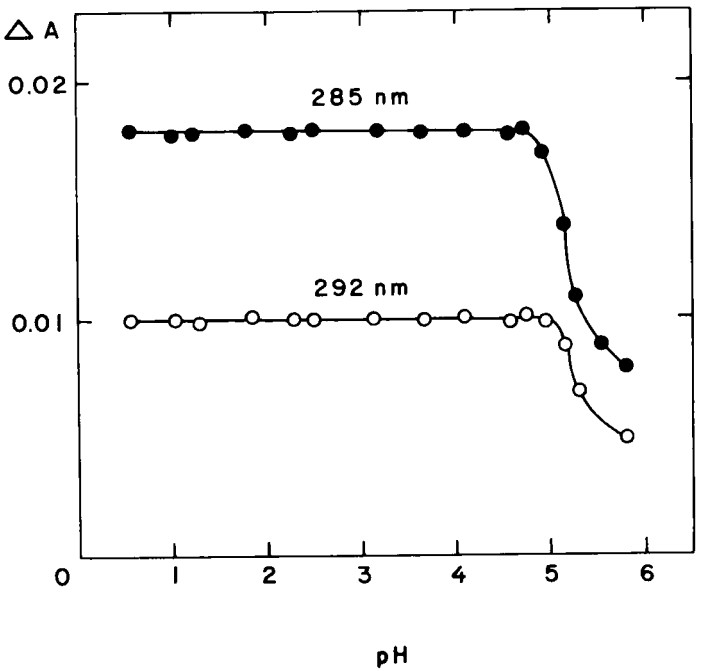


Figure 1: Effect of pH on the differential absorption of a mixture of 3 x 10^{-5}M pepsin and 2 x 10^{-5}M pepstatin.

tyrosyl (at 285 nm) and 420 per tryptophyl (at 292 nm) residue were observed at 30% propylene glycol.

In the presence of 30% propylene glycol the 285 nm absorption due to the pepsin-pepstatin complex was reduced, with $\Delta\xi$ = 250 (Figure 2). This indicates that, upon interaction with pepstatin, 1.5 tyrosyl residues of pepsin are protected from the solvent. An analogous observation at 292 indicated that only 0.3 tryptophyl residues are protected in the pepsin-pepstatin complex.

A better understanding of the pepsin-pepstatin interaction may be obtained by the study of structure-activity relationships. It has been shown[2] that the hydroxyl group of the internal AHMHA residue of pepstatin is essential for activity, while the hydroxyl and carboxyl groups of the C-terminal residue are not. To further investigate the importance of the structure of the C-terminal half of pepstatin for its activity, we have synthesized the two

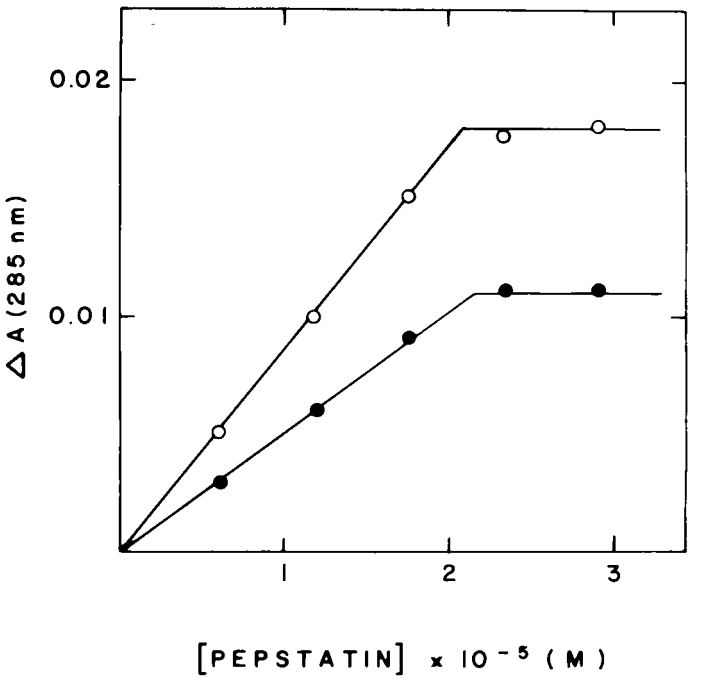
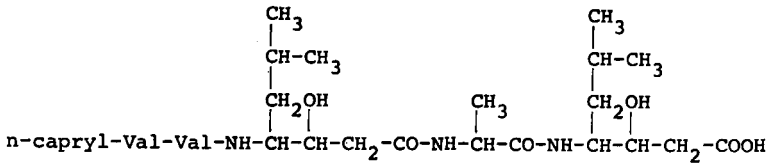
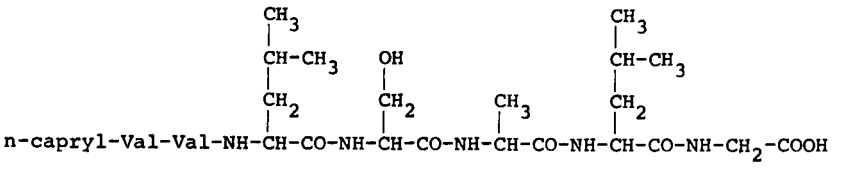


Figure 2: Titration of 2.8 x 10^{-5}M pepsin with pepstatin in the absence (empty circles) and in the presence (filled circles) of 30% propylene glycol.

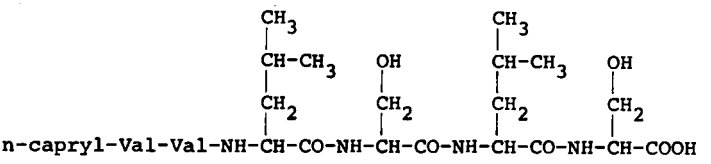
following peptides: *n*-capryl-Val-Val-Leu-Ser-Ala-Leu-Gly (I) and *n*-capryl-Val-Val-Leu-Ser-Leu-Ser (II). These peptides resemble pepstatin G in that they have similar hydrophobic side-chains and have hydroxyl groups, but the relative position of these groups are different (Figure 3). Neither I nor II inhibited peptic proteolysis or produced a difference spectrum when added to pepsin, even at very high molar ratios (10^3-fold) compared with the 1:1 ratio at which pepstatin is effective. This indicates that the position of the hydroxyl group relative to the non-polar side-chains might be important for the interaction of pepstatin with pepsin.



Pepstatin G



I



II

Figure 3: Structures of pepstatin G and two synthetic analogs.

Acknowledgments

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CONFORMATION-DIRECTED PROTEOLYSIS OF KUNITZ BOVINE TRYPSIN INHIBITOR

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THE TRYPSIN INHIBITOR first isolated by Kunitz from bovine pancreas[1] and found later in most tissues of the ox[2] is one of the best characterized trypsin inhibitors since both its covalent[3] and three-dimensional[4] structures have been determined. The single polypeptide chain comprises 58 residues with 3 disulfide bridges and the molecule has a pear-shaped conformation.

It has previously been observed that in the stoichiometric trypsin-inhibitor complex, the ε-amino group of Lys-15, in contrast to those of the three other lysine residues of the inhibitor, is shielded by the enzyme[5] and this result has been confirmed by a crystallographic study of the complex[6]. Furthermore, X-ray data have shown that Lys-15 is located in the exposed apex of the pear-shaped inhibitor molecule[4] and that this apex is inserted in the active center of trypsin[6,7]. Apparently residues 11 to 18 and 34 to 39, which are located in two loops linked by the disulfide bridge Cys-14-Cys-38, are involved in the interaction with trypsin[7]. Actually the disulfide bridge Cys-14-Cys-38, which is readily and selectively reduced when the inhibitor is free[8], is no longer reduced in the trypsin-bound inhibitor[9]. When this disulfide bridge is selectively reduced and carboxamidomethylated, the derivative is still inhibitor of trypsin but no longer of chymotrypsin[8]. Limited proteolysis occurs in each case and the results show the prime importance of the apex conformation in the interaction.

Conformation-Directed Proteolysis of the Selectively Reduced Inhibitor by α-Chymotrypsin

Inhibitor gives with α-chymotrypsin in stoichiometric complex stable around pH 7.0 but dissociated at pH 4.0[10]. No cleavage occurs during the association. By maleylation of the complex, isolation of the maleylated chymotrypsin-bound inhibitor, tryptic hydrolysis of the oxidized derivative and peptide mapping, it can be shown that Lys-15 is fully shielded by chymotrypsin in contrast to the three other lysines in positions 26, 41 and 46 which are maleylated[10] (Figure 1). The contact area of the inhibitor with

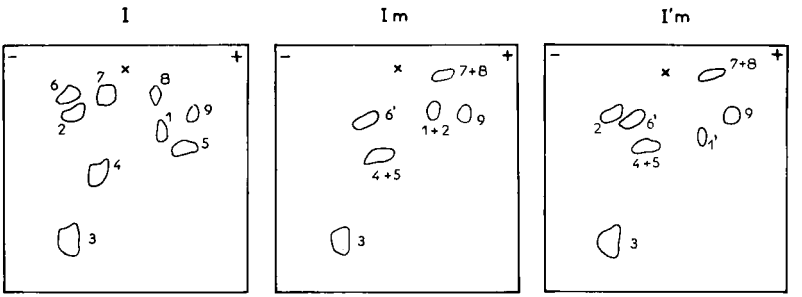

Figure 1: Tryptic peptide maps of the free inhibitor (I), the maleylated inhibitor (Im) and the maleylated chymotrypsin-bound inhibitor (I'm). For Im, the blockage of the four lysines is shown by disappearance of peptide T2 (lysine-15), T4 (lysine-26), T7 (lysine-46) and displacement of peptide T6 (lysine-41). For I'm, the presence of peptide T2 reveals that lysine-15 is free and therefore was shielded by chymotrypsin in the complex.

α-chymotrypsin appears therefore similar to that with trypsin and it can be assumed that the Lys-15 side chain is accommodated in the specificity pocket of chymotrypsin at the cost of lowering its p*K* or burying its positive charge[11].

When the disulfide bridge Cys-14-Cys-38 is selectively reduced by dithiothreitol and carboxamidomethylated[12], the derivative no longer inhibits α-chymotrypsin and becomes a substrate. The derivative was subjected to chymotryptic hydrolysis for 45 min at 37°C with a molar ratio enzyme/substrate 4 : 100. Chymotryptic peptides were separated

by peptide mapping and identified by amino.acid analysis. A hexapeptide Ala-16-Tyr-21 and a decapeptide Gly-36-Phe-45 were present in approximately equal amounts, as well as a large fragment corresponding to the remainder of the molecule. Clearly chymotrypsin has split the derivative at the level of Lys-15, Tyr-21, Tyr-35 and Phe-45. (Figure 2).

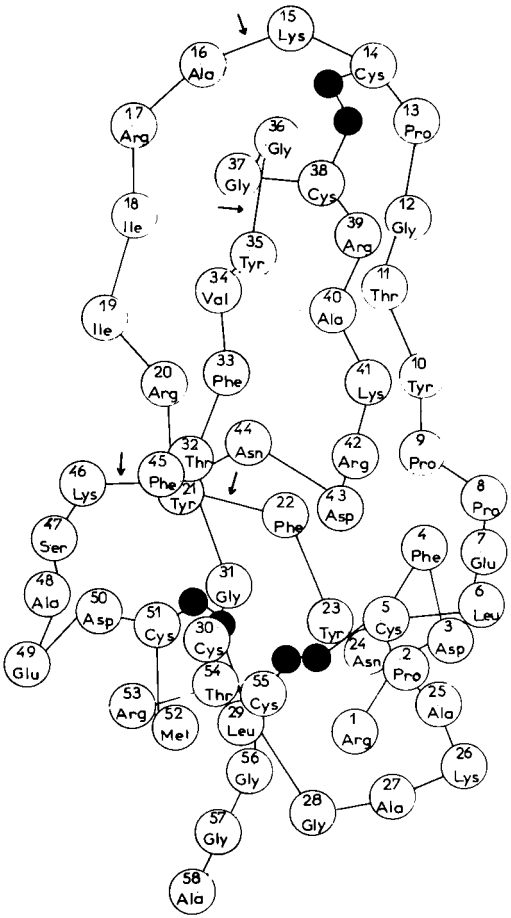

Figure 2: Conformation-directed proteolysis of the selectively reduced inhibitor by chymotrypsin. When the disulfide bridge Cys-14-Cys-38 is selectively reduced and carboxamidomethylated, the inhibitor becomes a substrate for chymotrypsin. Cleavage occurs at the level of the bonds indicated by arrows. The bond Lys-15--Ala-16 is not split by chymotrypsin when fully unfolded inhibitor is subjected to chymotrypsin under the same conditions.

The first cleavage was never observed in the chymotryptic hydrolysis of the fully unfolded inhibitor even after 18 h in contrast to the three others[13]. The selectively reduced inhibitor and native inhibitor have similar circular dichroism spectra and apparently the selective reduction does not generate substantial changes in the native conformation. It can be assumed that the non-specific cleavage by chymotrypsin was directed by the conformation of the inhibitor, and that the specific cleavages occurred subsequently in the disorganized apex.

Conformation-Directed Proteolysis of the Selectively Reduced Guanidinated Inhibitor by Trypsin

When the four lysines of the inhibitor are converted into homoarginine (Har) residues, the guanidinated inhibitor remains fully active against trypsin[14]. However when the disulfide bridge Cys-14-Cys-38 is selectively reduced, the derivative no longer inhibits trypsin. It has been observed that trypsin splits the guanidinated inhibitor fully unfolded either by oxidation or by complete reduction, not only at the level of arginine residues but also with a high yield at the level of homoarginines 26 and 46[15]. However, cleavage at the level of Har-15 is very slow even after 5 h (5-10%) perhaps because of the vicinity of Arg-17, the cleavage of the bond Arg-17--Ile-18 occurring much faster.

When the selectively reduced guanidinated inhibitor is subjected to trypsin for 1 h at 37°C, a new N-terminal residue, alanine, can be detected by Edman degradation suggesting a cleavage either at the level of Har-15 or Arg-39.

After 5 h of tryptic hydrolysis, resulting peptides are isolated by peptide mapping and compared with those obtained in the case of the fully unfolded guanidinated inhibitor. The high yield of the dipeptide H-Ala-Arg-OH (16-17) indicates that the cleavage at the level of Har-15 is almost complete for the selectively reduced derivative, instead of 10-15% for the fully unfolded guanidinated inhibitor. On the other hand the tripeptides H-Ala-Har-Arg-OH (40-42) and H-Ile-Ile-Arg-OH (18-20) are recovered with a high yield showing cleavage at the level of Arg-39, Arg-42, Arg-17 and Arg-20.

These results indicate that the bond Har-15--Ala-16, which is a very poor substrate for trypsin when the guanidinated inhibitor is fully unfolded, becomes the first or one of the first to be cleaved when the guanidinated

inhibitor has a nearly native conformation with residue-15 in a particularly exposed position. On the other hand the selectively reduced non-guanidinated inhibitor blocks temporarily trypsin but is finally split consecutively at the level of Lys-15, Arg-39 and Arg-17[16].

We conclude that the interaction between inhibitor and trypsin or chymotrypsin is mainly determined by a particular fit between two reactive areas of the two molecules rather than by a specific bond between two residues. Apparently the residue in position 15 is conformationally oriented to the specificity pocket of the enzyme. If some flexibility is introduced in the apex by selective reduction of the bridge Cys-14-Cys-38, proteolysis of the peptide bond 15-16 can occur provided that the side-chain of residue-15 can be accommodated in the specificity pocket of the protease. Then the proteolysis goes on for other bonds in the reactive area. So a nonspecific cleavage can be determined by the particular conformation of the polypeptide which interacts with the serine proteases. This conformation-directed proteolysis could give information on the time-dependent changes which occur during the interaction.

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THE DESIGN OF EFFECTIVE RENIN INHIBITORS

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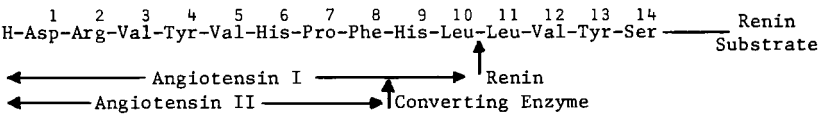
THE AIM OF THIS RESEARCH is to design peptides that are effective *in vivo* inhibitors of the enzyme renin. Renin is a protease which cleaves its natural substrate, an α_2-globulin, between the leucyl residues at position 10 and 11 to generate angiotensin I. In turn, this is converted to the biologically active angiotensin II on passage through the pulmonary circulation. The inhibitors



should bind to renin and prevent formation of angiotensin I. Diminution in the rate of formation of angiotensin I is measured *in vitro* by a radioimmunoassay[1]. The inhibitors are modeled after the amino acid sequence around the cleavage site of the natural substrate[2] to insure that they bind renin. As expected, the peptides are both substrates and competitive inhibitors[3].

The effectiveness of the inhibitors is controlled by both the inhibitory constant (K_I) and the solubility ($I_{saturated}$) of the peptides. The more tightly the

inhibitor binds to renin, the more completely it will prevent formation of angiotensin I. Similarly, the more soluble the renin inhibitor, the greater the amount that can be added to a test preparation. A mathematical formula permitting the expected percent inhibition to be calculated from $I_{saturated}$ and K_I has been developed, which can be applied to graphical evaluation of different peptides as renin inhibitors in terms of the ratio between these two parameters.

The starting point of this investigation was a solid phase synthesis[4] of the known octapeptide sequence from the natural substrate of renin. The octapeptide is both a competitive inhibitor and a substrate for renin[5], but replacement of either of the L-leucyl residues with the D-enantiomorph yields peptides that are not cleaved by renin (Table I). At pH 5.5 the [D-Leu^6]octapeptide binds

Table I

Properties of the Renin Inhibitors at pH 5.5

	K_m, K_I (μM)	% Cleavage
Tetradecapeptide	143	--
His Pro Phe His Leu Leu Val Tyr	39	100
——————D-Leu——————	25	0
——————————D-Leu ———	3	0
——————————————Phe——	6	--

renin an order of magnitude more tightly than the parent octapeptide but above pH 6.0 it becomes relatively insoluble and does not bind to renin. It is inactive at physiological pH. When coupled with CNBr activated Sepharose-4B the [D-Leu^6]octapeptide serves as an excellent ligand for the purification of renin by affinity chromatography[6]. In the search for an effective *in vivo* inhibitor, however, this modification represents a dead end.

Efforts in solubilizing octapeptide inhibitors at physiological pH have followed three main approaches. The first type of modification involves synthesis of analogs in which hydrophilic, isosteric residues are substituted for hydrophobic residues. K_I is, however, more closely linked to hydrophobility than is solubility. In general, replacement of a hydrophobic residue with a hydrophilic residue increases K_I and makes the inhibitor less effective (Table II). However, increased solubility does

Table II

Properties of the Renin Inhibitors at pH 7.5

	K_I (μM)	Solubility (μM)	Solubility / K_I
His-Pro-Phe-His-Leu-Leu-Val-Tyr	--	161	--
——————————Serinol	--	81	--
——————————Thr——	>100	300	<3
Pro————————————	42	324	8
Pro-Pro-Pro————————————	157	374	2
Pro-Pro-Pro-Pro-Pro————————————	140	1328	9
Pro——————————Tyr———	12	303	25
Pro——————————Phe———	4	412	103
Pro——————————Phe-Phe———	1	100	100
Pro-Pro-Pro-Pro-Pro——————————Phe———			

not invariably follow hydrophilic substitution. Because of this and the inverse relationship between hydrophobicity and binding to renin (K_I), solubilization by substitution with hydrophilic isosteric residues was abandoned.

The second approach is based on the observation that reduction of the C-terminal carboxylic acid to an alcohol greatly increases solubility of tetrapeptide renin inhibitors[7]. With the octapeptide inhibitors the desired replacement was effected by addition of serinol to the carboxyl terminus rather than by chemical reduction of an existing peptide. Serine occurs naturally at this position, and its presence or absence does not affect K_m of other renin inhibitors[3]. Addition of serinol to the C-terminus was accomplished in 19% yield by displacing the fully protected octapeptide from the resin with 1 M serinol in dimethylformamide (DMF), or, in 50% yield using hydroxybenzotriazole catalysis. The protected peptide was purified by chromatography on Sephadex LH-20 in DMF which was 1 M in toluene. Evaporation yielded the desired

product which was deprotected and purified in the standard way[5]. However, octapeptidyl-serinol is only one fifth as soluble as the parent octapeptide at physiologic pH (Table II).

A third approach to solubilization involves addition of polyprolyl residues to the N-terminus of the inhibitors. Addition of either one or three prolyl residues doubles solubility at pH 7.4 (Table II). Addition of a pentaprolyl-sequence increases solubility an order of magnitude. K_I of the inhibitors can be decreased by substituting more hydrophobic residues at the cleavage site. Thus K_I's for the substituted peptides are: Pro-octapeptide, 42 μM; Pro-[Tyr^6]octapeptide, 12 μM; Pro-[Phe^6]octapeptide, 3 μM; and Pro-[Phe^5, Phe^6]octapeptide, 1 μM. Ostensibly the last inhibitor is the best, but, as will be shown, a decrease in solubility cancels the advantage of the smaller K_I and the Pro-[Phe^6]octapeptide remains the most effective inhibitor.

Design of an effective renin inhibitor rests on a quantitative understanding of the relationship between the inhibition that would be expected, solubility of the inhibitors, and K_I. From the standard equation for competitive inhibition[5] and definition of % inhibition as $\frac{V_o - V_I}{V_o} \times 100$ we arrive at

$$\% \text{ Inhibition} = \frac{2\, K_m I_{saturated}}{SK_I + K_m K_I + 0.02\, K_m I_{saturated}}$$

where K_m = Michaelis constant for the generation of angiotensin I from natural substrate, K_I = inhibitory constants for the renin inhibitors, S = molar concentration of natural substrate, and $I_{saturated}$ = molar concentration of renin inhibitor in a saturated solution. The volume of saturated inhibitor solution is 2% of the final test mixture.

Figure 1 shows the relationship between the ratio of $I_{saturated}/K_I$ and the % inhibition for the human system where K_m = 0.5 μm and S varies between 0.8 and 5 μM[8]. $I_{saturated}$ to K_I ratios for the various peptides are given in Table II. At present the best inhibitor is the Pro-[Phe^6]octapeptide with a ratio of 103. For the human system inhibition varies between 19-43% depending on the concentration of natural substrate. With the Pro-[Phe^5, Phe^6]octapeptide the ratio is 100. Decreased solubility cancels the gains for decreased K_I. The ratio of $I_{saturated}/K_I$ shows that simply decreasing K_I offers no advantage unless solubility can be maintained.

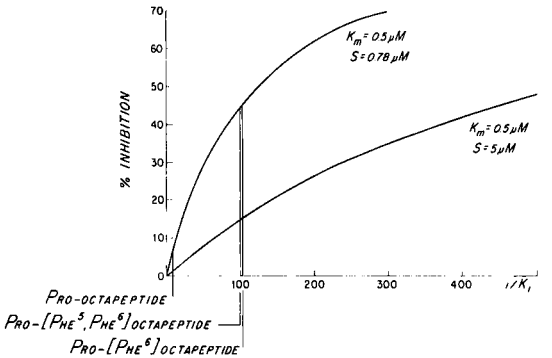


Figure 1: % Inhibition of the formation of angiotensin I from human natural substrate by human renin as a function of $I_{saturated}/K_I$.

Peptides developed in this way offer the potential of acting as *in vivo* inhibitors. The relationships developed previously between % Inhibition, $I_{saturated}$, and K_I for the *in vitro* system are also applicable to *in vivo* testing, however, various catabolic processes such as rate of enzymatic degradation, rate of escape into extravascular spaces, and rate of renal excretion are not taken into account. Experiments designed to check our hypotheses are now in progress.

To date, the development of specific inhibitors has progressed from peptides that are weakly active at acidic pH to those capable of the complete inhibition of renin in human plasma under physiologic conditions. Design changes based on an understanding of the relationship between K_I, solubility, and hydrophobicity have in large part permitted this improvement. Extention of the inhibitors from *in vitro* to *in vivo* systems is now underway. This transition may require that additional factors be considered. These, however, like previous problems, should be subject to the rational design of peptides.

Acknowledgments

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by a Hypertension-SCOR Grant from the NHLI #14150 and by the Danish Heart Association and the Danish State Medical Research Council.

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CONFORMATION AND BIOLOGICAL ACTIVITIES OF THE TOXIC CYCLOPEPTIDES OF AMANITA PHALLOIDES

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THE GREEN BULBOUS MUSHROOM produces 2 families of toxins, the phallotoxins and the amatoxins[1]. In both types of cyclic peptides, the side chains of tryptophan and cysteine are bridged.

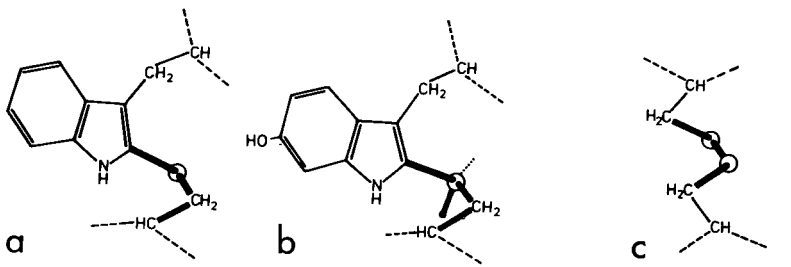

Figure 1: Chirality of the tryptophan-cysteine-moiety in the phallotoxins (a) and in the amatoxins (b); disulfide bridge (c) for comparison.

The resulting α-indolyl-thioether possesses a helical chirality similar to that of disulfide bridges (Figure 1c). This makes the aromatic part an inherently dissymmetric chromophore. In the case of the amatoxins (Figure 1b), there is also a centre of tetrahedral chirality at the sulfoxide. Especially in the phallotoxins the chromophore is rigidly fixed over the peptide backbone and may

serve as a sensitive probe for conformational changes in the neighbouring peptide region. Conformational changes were produced by distinct chemical modifications at the side chains and could be correlated to the biological activity of the toxins.

For many years the biological activity was defined solely in terms of the *in vivo* toxicity of both toxins in the white mouse. Today we are able to differentiate biological activity and to study it on 3 levels. *In vitro*

Table I

Biological activities of Amanita phalloides toxins

	Phallotoxins	*Amatoxins*
in vivo	*Toxicity:* 2.0 mg/kg white mouse(LD_{50})	*Toxicity:* 0.35 mg/kg white mouse(LD_{50})
in cells	Protrusions and bulges in *liver cells*	Growth inhibition of EB-virus transformed human *Lymphocytes*
in vitro	Irreversible polymerisation of G-*actin* to filaments	Inhibition of *RNA-polymerase II* (block of protein synthesis)

assays (i) quantitate the molecular mechanisms of action, which are known for both types of toxin. The phallotoxins bind to actin and polymerize this protein irreversibly into filaments. The amatoxins inhibit the nuclear enzyme RNA-polymerase II (or B) and block the protein synthesis. The *in vivo* toxicity assays (ii) have already been mentioned. (iii) A third assay, established by us, measures cytotoxic effects on isolated cells or cells in culture.

We observed that for each of the toxins biological activity was associated with a unique conformation of the cyclic peptide[2]. In Figure 2 the solid line represents the CD-curve of all toxic amanitins, α-, β- and γ-amanitin, including toxic derivatives of the naturally occurring compounds as methylated α-amanitin (compound A). After oxidative degradation of the side-chain by periodate, the resulting aldehyde (B) had a changed conformation

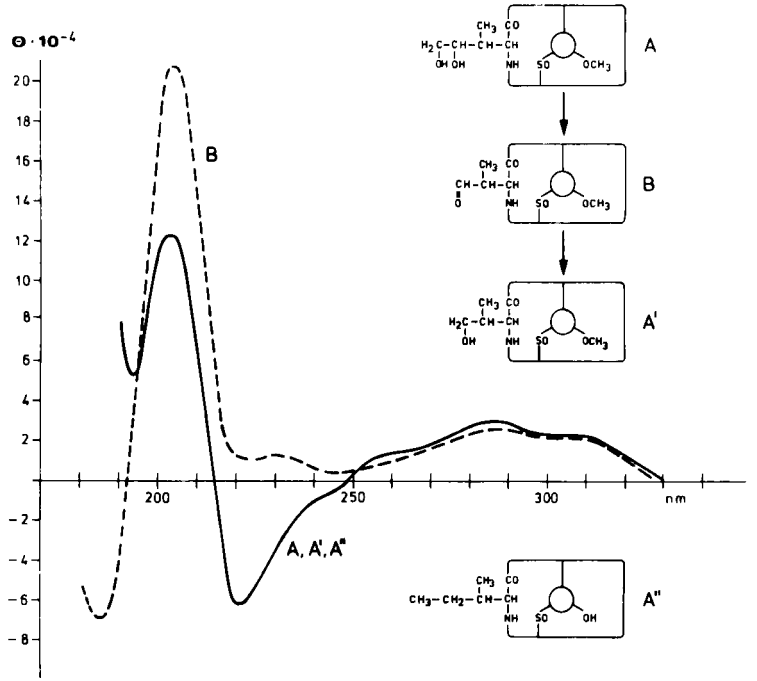

Figure 2: CD-curves of the biologically active conformation. (A, A', A") and a biologically inactive conformation (B) of amanitins. (A): methyl-α-amanitin, (B): methyl-aldoamanitin, (A'): methyl-demethyl-γ-amanitin, (A"): amanullin.

(Figure 2, broken line) and had lost its toxicity. Hydrogenation restored the native conformation (solid line) and the biological activity (A'). A direct proof of the conformational change in compound B was obtained recently by radioimmunoassay.[3] For a 50% substitution of labelled amanitin from amanitin-specific antibodies, a 120 times higher concentration of methylaldoamanitin (B) than of α-amanitin was needed. Another nontoxic compound is the naturally occuring amanullin (A"); however it exhibits the native CD curve and inhibits RNA-polymerase II. The present explanation for nontoxicity of amanullin is that the lipophilic peptide is sequestered by unknown lipids in the body before reaching its target. The nontoxicity is not caused by a hindered permeation into cells, because amanullin inhibited the growth of Epstein-Barr virus-transformed

lymphocytes nearly as effectively as γ-amanitin, the most potent toxin. The toxicity towards isolated cells concomitant with nontoxicity *in vivo*, gives hope for the use of amanullin and similar compounds to surpress selectively the proliferation of tumor cells *in vivo*.

A strong correlation between a typical ORD curve and biological activity was also established for the phallotoxins.[4,5] About 15 toxic phallopeptides exhibit a characteristic positive Cotton-effect in the range of 250 nm and a series of smaller positive Cotton effects of differing strength in the range of 300 nm. By extensive synthetic work[5,6] about 7 analogs of the phallotoxins with modifications in all sidechains were obtained. The correlation of chemical modification, conformation, and biological activity in these compounds permitted us to distinguish between those substitutions which had changed the conformation and hereby destroyed the biological activity, and others, which had not affected the conformation, but had likewise abolished biological activity. This analysis revealed three functional groups of the peptide, which represent the active site, see Figure 3. The first group

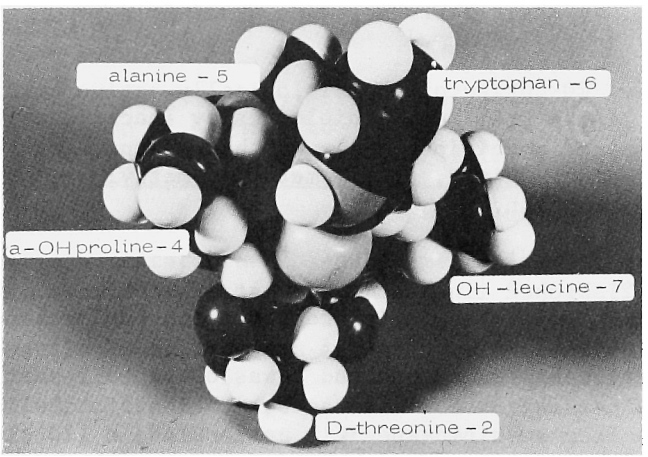

Figure 3: Atomic model of the conformation of phalloin as suggested by Patel *et al.*[7]

essential for binding to the actin receptor is the OH-group in the allo-position of proline. The isomeric compound with OH in the position of natural hydroxyproline is nontoxic because of a changed conformation. If the OH-group

is omitted as in the proline analog, toxicity is likewise lost, however, the conformation is identical to that of the toxic compounds. We deduce from this, that the OH-group in the allo-position of the proline residue is essential for binding to the protein. The second side chain identified by a similar argumentation is the methyl group of the alanine residue adjacent to tryptophan. The third group, finally, which effects binding to the protein is the tryptophan thioether group. There are some chemical arguments for this [4,8,9]. A direct proof was obtained by uv difference spectra[10] and by CD spectra, both indicating changes around the 300 nm absorption band, which can only originate from the thioether part of the toxin. This can be seen from the two CD curves in Figure 4 A, where the

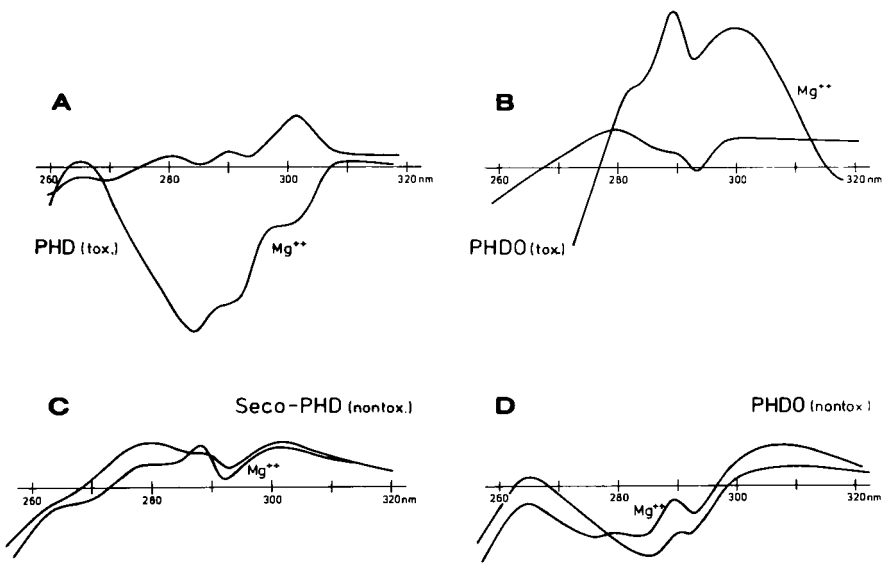

Figure 4: CD-curves of mixtures of G-actin with various phallotoxins before and after addition of Mg^{++} ions.

addition of Mg^{++} ions, which are essential for binding, to a mixture of actin and phalloidin causes a change of sign in the Cotton effect at the longest wave length, which must be attributed to the thioether part. Generally, the binding process causes large changes in the CD curve; another example of this is the toxic diastereomer of the

sulfoxides (4 B). No binding, and consequently only small changes in the CD were observed for nontoxic compounds like secophalloidin (4 C) and the nontoxic sulfoxide (4 D).

The consequences of phalloidin binding to actin as elucidated by Wieland and coworkers are summarized in Figure 5. Starting either from the monomeric G-actin or

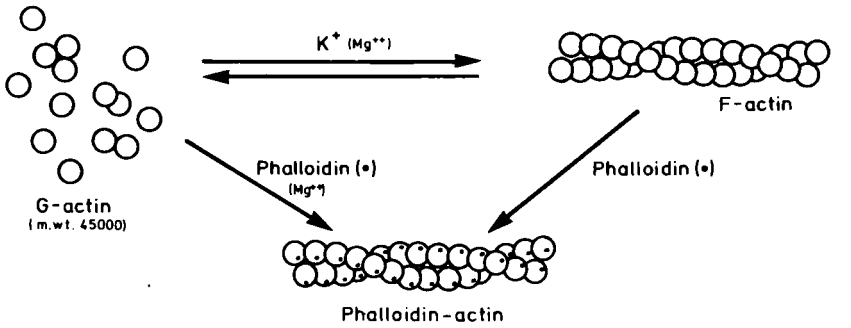

Figure 5: Phalloidin-induced filaments of actin.

the filamentous F-actin, the interaction with phallotoxins produces in both cases a filamentous form of actin with properties different from normal F-actin: for example, the phalloidin induced filaments can no more be dissociated by 0.6 *M* KI[11]: they withstand denaturation by heat or EDTA better than F-actin does; they exhibit no ATPase activity in ultrasonic treatment[12] and, they have lost the inhibition capacity for DNAse I, (Schäfer *et al.*, unpublished) which recently was described for F-actin by Lazarides and Lindberg.[13]

Phalloidin induces changes not only in muscle actin, but also in the actin associated with the plasma membranes of cells. Isolated liver cells, which are treated with phalloidin in μM concentration, produce characteristic protrusions and bulges.[14,15] The mechanism by which these protrusions develop and the function of actin in liver cells are not yet understood. We hope that phalloidin may help to elucidate this problem.

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SYNTHETIC AND CONFORMATIONAL STUDIES OF THE NEUROTOXINS AND CYTOTOXINS OF SNAKE VENOM

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THE TOXIC COMPONENTS of snake venom, viz., neurotoxins and cytotoxins, are currently being extensively investigated. Particularly this pertains to the neurotoxins which are capable of highly specific interaction with the cholinergic receptor of the postsynaptic membrane, the effect of the cytotoxins being less specific and confined to impairing of the membrane integrity[1,2]. The present study is part of the analytical, physicochemical and synthetic investigations into toxic proteins that are being carried out in our Institute.

With the aid of the CD technique a comparative determination has been made of the conformational stability of different toxins isolated from the venom of the Middle Asian cobra *Naja naja oxiana*: namely, neurotoxin I (NT I, 73 amino acid residues, 5 disulfide bonds), neurotoxin II (NT II), and cytotoxins (CT) I and II (each with 60-61 residues, 4 disulfide bonds). The amino acid sequence of the first three was previously established in this laboratory[3-5] and that of CT II is nearing completion.

The CD spectra in the 185-290 nm region of NT II do not change appreciably over the pH range of 2 to 12, whereas those of NT I and of both cytotoxins undergo considerable modification in the regions of both the amide band and the aromatic chromophore (Figure 1). Thus with increase in pH the cytotoxin spectra display a bathochromic shift and a broadening of the 225 nm peak, effects ascribed to titration of the tyrosine residues. The

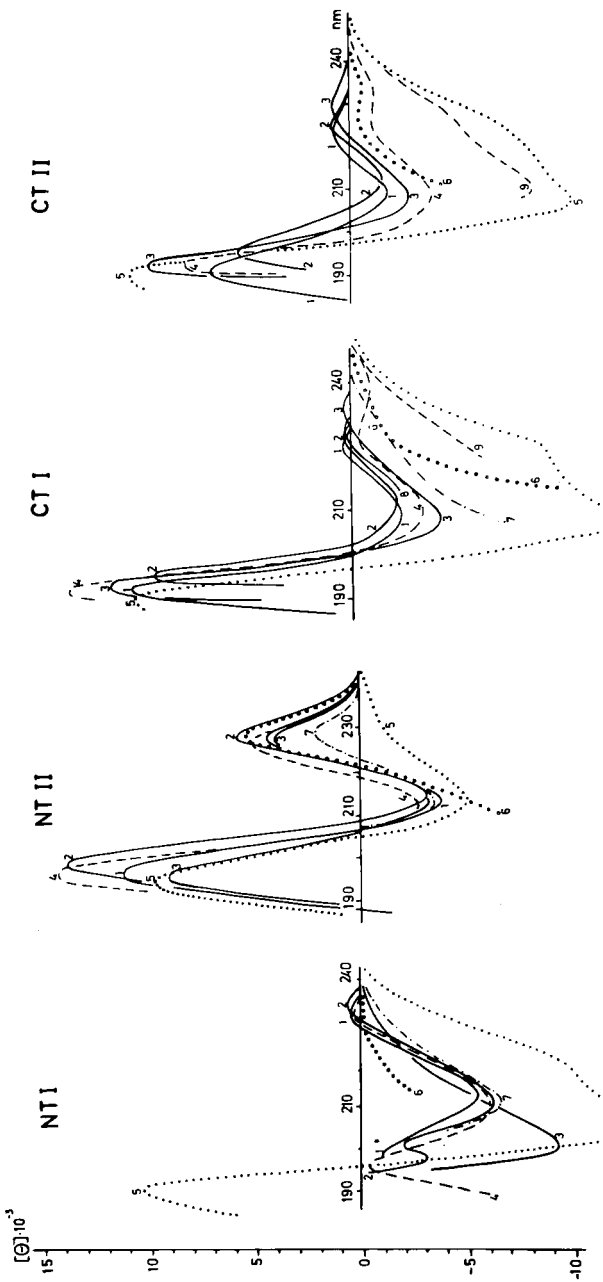

Figure 1: CD spectra of *N. naja oxiana* toxins.

greater rigidity of NT II is evidenced by the CD spectra taken at different temperatures and also in the presence of denaturing agents. Its much lower flexibility can also be seen from a comparison of the CD spectra of this and the other toxins in aqueous-organic media or in pure organic solvents. In the case of neurotoxin I and the two cytotoxins the use of trifluoroethanol as solvent yields very similar CD curves, a sign of the onset of structural ordering in this medium. These results do not confirm the earlier suggestion of higher conformational stability of long toxins compared with short toxins[1]. The CD spectra revealed that all toxins have more or less stable conformations but with some differences in spatial organizations between the long and short chain neurotoxins and the cytotoxins.

The functionally important Tyr and Trp residues were probed by spectrophotometric titration and UV perturbation spectrometry and the His residues by NMR-^{1}H. The results (Figure 2 and Table I) show that Tyr-24 is buried in

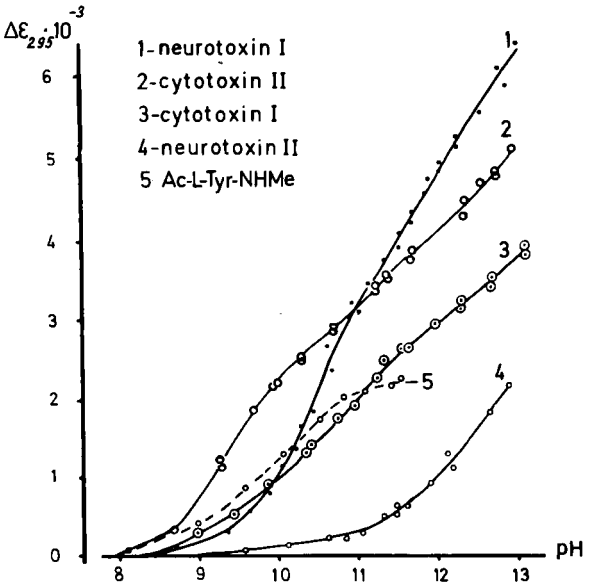

Figure 2: Spectrophotometric titration of tyrosine residues in the *N. naja oxiana* neurotoxins and cytotoxins.

Table I

Data on the aromatic residues
of the *N. naja oxiana* neurotoxins and cytotoxins

Compound	*Number of Residues*			*pK*		*Number of accessible residues*			
				Spectrophotometric titration	*NMR-^{1}H titration*	*ethylene glycol*		*ethylene glycol and 8 M urea*	
	Tyr	*Trp*	*His*	*Tyr*	*His*	*Tyr*	*Trp*	*Tyr*	*Trp*
NT I	3	2	1	10.8; 11.5; 11.5	5.01	2.0	1.3	3.0	1.8
NT II	1	2	2	>12	5.99; 5.12	0.3	1.1	1.1	1.5
CT I	2	-	1	11; >12	6.02	1.5	-	1.2	-
CT II	3	-	-	9.8; >12: >12	-	1.8	-	1.6	-

neurotoxin II, and that at least one of the tyrosyl residues is not accessible in the other three toxins. No tryptophan residues are present in cytotoxins; in neurotoxins they are partially exposed. Our data agree well with those for the chemical modification of Tyr and Trp in related toxins. The rather low pK values for His residues as found by NMR titration (Figure 3 and Table II) indicate

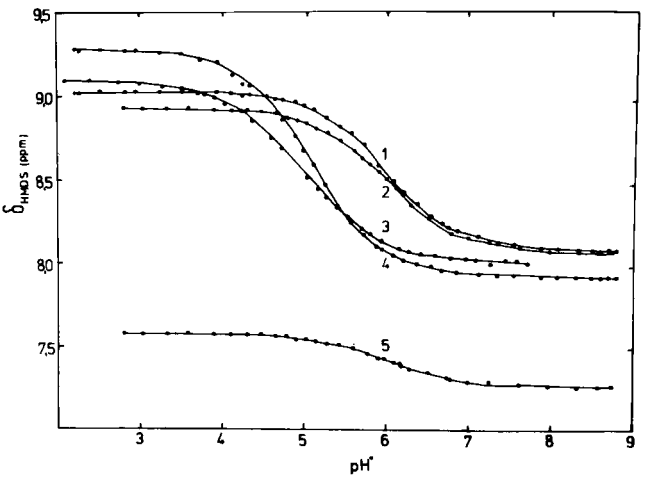

Figure 3: NMR-^{1}H titration curves for the histidine C 2 and C 4 protons of the *N. naja oxiana* neurotoxins and cytotoxin (see also Table II). Concentration, ~$7 \cdot 10^{-3}$M in D_2O containing 0.3M NaCl. Data obtained at 100 MHz in Fourier transform mode.

Table II

NMR titration data for the histidine residues of the neurotoxins I and II, and cytotoxin I

			Chemical Shift, (ppm)				*Hill*
N	*Signal*	*Compound*	δH^+	δH°	$\Delta\delta H^+,H^\circ$	*pK*	*Coefficient*
1	C-2H His-4*	NT II	9.03	8.07	0.95	5.99	0.99
2	C-2H His-31	CT I	8.93	8.06	0.87	6.01	0.95
3	C-2H His-67	NT I	9.10	8.00	1.10	5.01	0.86
4	C-2H His-31	NT II	9.28	7.92	1.36	5.12	0.97
5	C-4H His-67	CT I	7.58	7.27	0.31	6.03	0.91

* Assignment of the His signals in neurotoxin II were made by comparison with the spectrum of a toxin in which the His-4 residue had been selectively oxidized in the presence of methylene blue.

a possible presence of charged arginine and lysine groups close to the histidine residues.

Possible stereochemical arrangements of the polypeptide chains in neurotoxins I and II and cytotoxin I were assessed by the method of Chou and Fasman[6]. The analysis of related toxins (sequences 1-26 in [1]) permitted elucidation of the common structural units (α-helical, β-conformation or β-turn). NT II was found to have a structure with a NH_2-terminal 1-7 α-helical region, β-pleated sheets at 13-17 and 53-57 and a number of β-turns (21-24, 31-34, 38-41, 46-49, 57-60). The latter are also encountered in CT I (14-17, 17-20, 35-38, 42-45, 55-58, 57-60) and in NT I (4-7, 16-19, 23-26, 32-35, 34-37, 62-65 and 65-68). Their folding is less clear, as these toxins have sequences which are likely to be organized as a β-structure but also have a high probability of forming α-helices. This may explain the ability of neurotoxin I and cytotoxins to produce α-helical structures in trifluoroethanol. In sum, the different types of spatial structures displayed by the toxins investigated is in good accord with the above mentioned differences in their CD curves.

Figure 4 shows an outline of some of the structural units of the neurotoxin II backbone which follow from our data and are in accord with computations by the method of Kabat and Wu[7], but differ from the model of Ryden *et al.*[8] The validity of our model and the principles governing the conformations of long chain toxins and of cytotoxins are subjects of continuing studies.

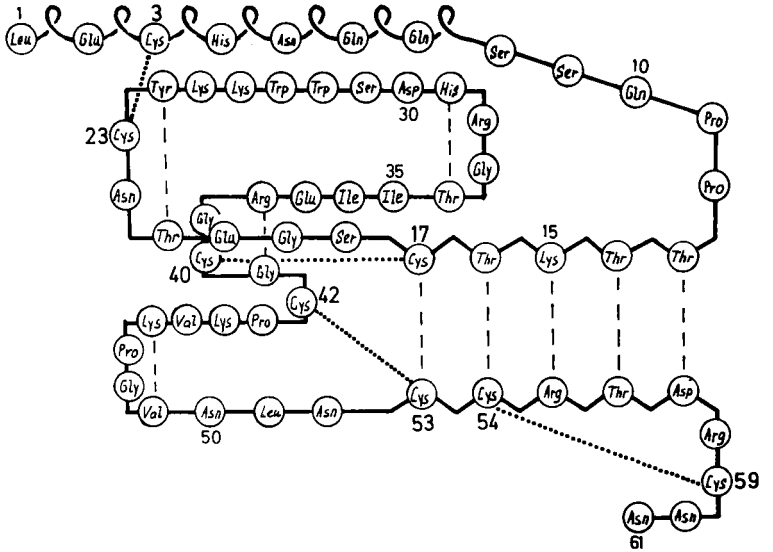

Figure 4: Scheme for polypeptide backbone folding in neurotoxin II.

Concurrently we are engaged in the synthesis of α-bungarotoxin to obtain a long chain neurotoxin and also its fragments and specifically labelled analogs required for further spectral study. For the synthesis we used a classical route employing a maximum number of hydrophobic groupings to protect the polyfunctional amino acids, thereby improving the solubility of the protected peptides in organic solvents and minimizing side reactions. We plan to prepare 11 protected fragments preferably with COOH-terminal proline and glycine residues (Figure 5). Condensation of these fragments will be followed by deprotection and oxidative disulfide bond formation. For synthesis and condensation of the fragments the dicyclohexylcarbodiimide/hydroxybenzotriazole (DCC/HOBT), activated ester and mixed anhydride techniques are being used. All fragments have been synthesized. Condensation of fragments 21-27 and 28-37 by the azide method gave heptadecapeptide 21-37; coupling of peptides 54-64 and 65-74 by DCC/HOBT gave an intermediate which was condensed with the hexadecapeptide 38-53 to yield the protected peptide 38-74, which corresponds to the COOH-terminal part of the toxin molecule. Synthesis of the remaining part is in progress.

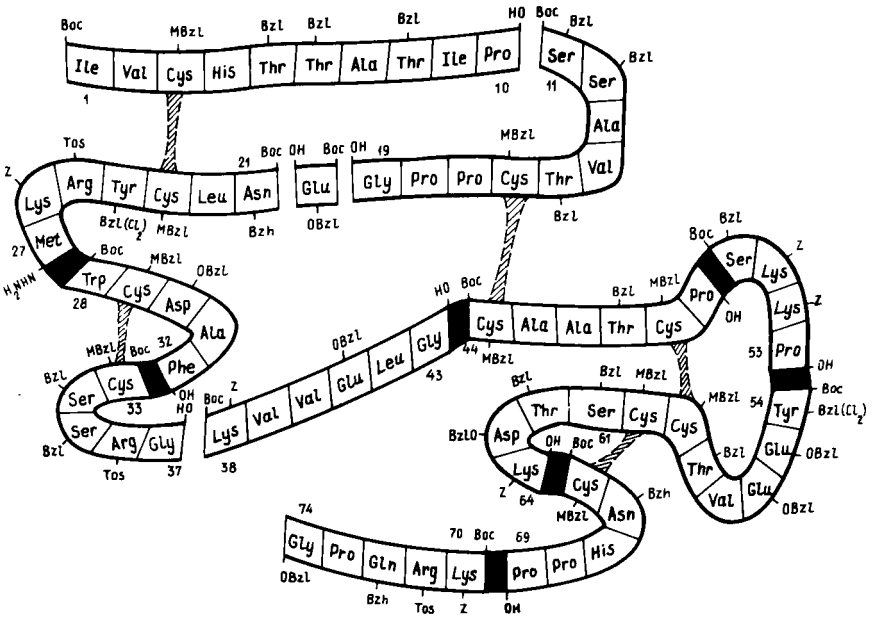

Figure 5: Scheme for the synthesis of α-bungarotoxin.

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SYNTHESIS OF CYCLIC TETRAPEPTIDES RELATED TO TENTOXIN

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TENTOXIN (1), a cyclic tetrapeptide produced by the phytopathogenic fungus *Alternaria tenuis*, causes chlorosis when applied to germinating seed of certain plants.[1] Tentoxin was assigned structure 1 from nmr, and mass spectrometic data and from chemical degradations.[2]

In order to study the relationship between structure, including conformation, and biological activity, the synthesis of tentoxin and several analogs was carried out. Linear tetrapeptides *4*, *5*, and *7* were synthesized by stepwise addition of Boc-amino acids to H-Gly-OEt and DCC in $CHCl_3$, and also by solid phase synthesis, followed by methonolysis.

Boc-MeAla-Leu-Phe(β-S-Bzl)-Gly-OMe (*2*)
Boc-MeAla-Leu-ΔPhe-Gly-OMe (*3*)
Boc-MeAla-Leu-MeΔPhe-Gly-OMe (*4*)
L-MeAla-L-Leu-L-MePhe-Gly-OTCP (*5*)
D-MeAla-L-Leu-L-MePhe-Gly-OTCP (*7*)

Tetrapeptide *3* was prepared by $NaIO_4$ oxidation of *2* to the sulfoxide followed by pyrolysis.[3,4] Methylation of the (Z)-isomer of *3* using K_2CO_3/CH_3I in DMF gave the corresponding N-methyl peptide.[3] Each of the linear tetrapeptides was converted to the trichlorophenylester and, after removal of Boc protecting group, cyclized (at c $10^{-4}M$) in refluxing pyridine. Tentoxin 1 was obtained in 17% yield, L-MePhe dehydrotentoxin (DHT) *6* in 0.3% (an average of ten cyclization experiments), and D-MeAla-L-MePhe DHT *8* in 10% yield. These cyclizations were run under identical

conditions. No attempt has been made to optimize yields. D-MePhe DHT *9* was prepared by catalytic hydrogenation using Pd/C[2].

The nmr studies of Dale and Titlestad[5] have shown that in solution many cyclic tetrapeptides, especially those containing alternating N-methylated residues, adopt conformation *10*. The close similarity between the chemical shift data (Table 1) obtained for tentoxin *1* and its

Table I

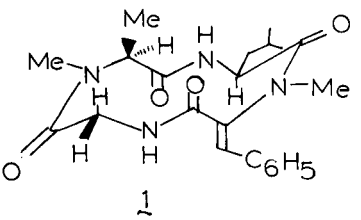
Chemical Shift Assignments for Cyclic Tetrapeptides

Compound	*Leu*			*Ala*		*Gly*	*MeΔPhe or MePhe*		
	δH	α-H	β-H	$N-CH_3$	α-H	α-H	α-H	$N-CH_3$	β-H
1	0.53 0.63	4.18	1.53	2.80	4.37	5.21 (d,d; J=15,9) 3.57 (d, J=15)	---	3.18	7.75
6	0.78 0.85	4.68	1.56	2.83	4.29	4.95 (J=15,10) 3.58 (J=15)	4.60	2.89	---
8	0.83 0.90	4.53	1.51	3.00	4.09	4.80 (J=15,10) 3.55 (J=15)	4.60	3.05	---
9	0.83 0.90	4.05	1.20	2.67	5.21	4.21 (J=17,9) 3.57 (J=17,6)	5.65	2.81	---

The spectra were obtained with Bruker-HX90E spectrometer in deuteriochloroform. The chemical shifts are expressed in ppm relative to internal TMS.

analogs *6*, *8*, *9* and those of Dale and Titlestad for the simpler systems suggested that the basic ring geometry of *1*, *6*, *8* and *9* would be similar to that shown for *10*. Thus in solution tentoxin *1* and D-MePhe DHT *9* adopt the conformations shown.[2]

The chemical shift data (Table I) for analog *6* and *8* are consistent with the conformations shown for these compounds. Thus the glycine protons are in the 8-position. The chemical shift of the α-proton of Ala is consistent with a proton in the 11-position. Similarily, the α-proton of MePhe can be assigned to the 5-position. Generally the α-protons of MePhe residues are deshielded (about 0.3 ppm) by the phenyl ring. The α-proton of Leu can be assigned to position 2 where it is shielded by the transannular aromatic system. The nmr results indicate that the conformations of analogs *6* and *8* are very similar to that of tentoxin in that the ring geometry and placement of side chains about the peptide ring system are similar.



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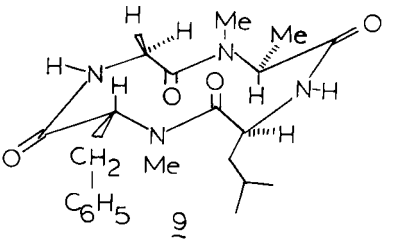

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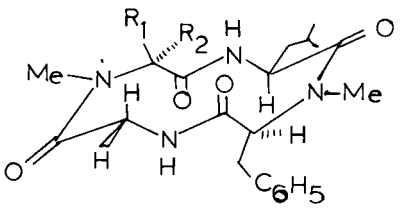
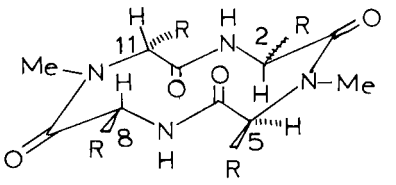


6 $R_1 = CH_3$ $R_2 = H$

8 $R_1 = H$ $R_2 = CH_3$



10

All derivatives of tentoxin were applied to germinating lettuce seeds to determine if they would cause chlorosis. Synthetic tentoxin possessed full activity but analogs *6*, *8* and *9* did not cause chlorosis even at high concentrations. The low activity of *6* is particularly interesting since its conformation is very close to that of tentoxin except for the orientation of the phenyl group with respect to the peptide ring system. These results establish that the double bond in tentoxin is essential for high biological activity but do not establish the function of the double bond.

During the synthesis of tentoxin it was noted that portions of the nmr and cd spectra of the methylated linear peptide *4* were similar to those of tentoxin (*1*) and significantly different from those of the unmethylated linear peptide *3*. (Table II and Figure 1). These results led to the suggestion that linear tetrapeptide *4* exists in a folded conformation containing a *cis* amide bond between Leu and MeΔPhe and not in the trans conformer *4a*.[3] In addition, the conformations of tripeptides *11* and *12* appear to be similar to *4*. Shielding of the α- and δ-leucyl protons in the nmr spectra and the positive elipticity in the cd spectra at 280 nm are similar to *4* and consistent with a *cis*-amide bond between Leu- and MeΔPhe. However, the nmr of the dipeptide ester *13* indicates that this compound is a mixture of *cis* and *trans* conformers. The cd spectrum of

Table II

Chemical Shift Assignments
for Linear Dehydrophenylalanyl Peptides

Compound	Leu			Ala		Gly		MeΔPhe	
	δH	α-H	β-H	N-CH_3	α-H	α-H	CO_2CH_3	N-CH_3	β-H
3	0.91	4.4	1.27	2.77	4.50	4.05 (d, J=6)	3.71	---	---
4	0.54 0.60	4.18	1.26	2.70	4.66	4.14 (d, J=6)	3.71	3.21	7.74
11	0.55 0.56	4.25 (d,d, J=6)	---	---	---	4.15 (d, J=6)	---	3.20	7.70
12	0.55 (d, J=6)	4.2	---	---	---	4.2 (d, J=6)	---	3.2	7.70
13	0.60	4.38	---	---	---	---	3.84	3.10	7.78
cis	0.53	(d,d, J=6)							
trans	0.90 0.80 (d,d, J=6)	4.95	---	---	---	---	3.86	3.12	7.73

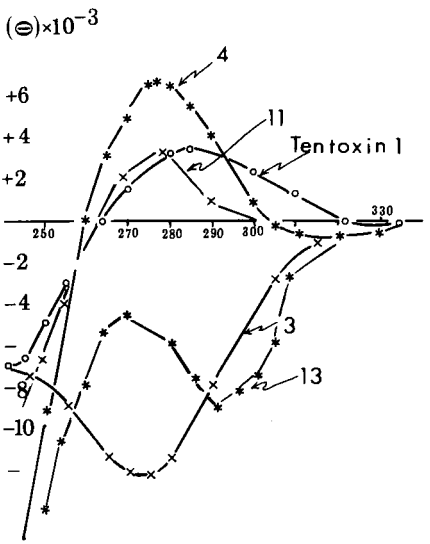
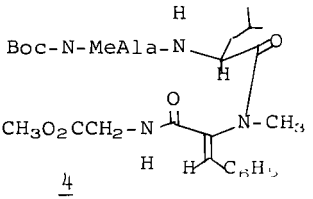


Figure 1: Partial circular dichroic spectra of dehydrophenylalanyl peptides. (1) Tentoxin: (2) Boc-MeAla-Leu-(Z)ΔPhe-Gly-OMe; (4) Boc-MeAla-Leu(Z)MeΔPhe-Gly-OMe; (11) Boc-Leu-(Z)MeΔPhe-Gly-OMe; (13) Boc-Leu-(Z)MeΔPhe-OMe. [Symbol (Z) designates the configuration of the olefin (zusammen); IUPAC rules for nomenclature of organic compounds[6]].



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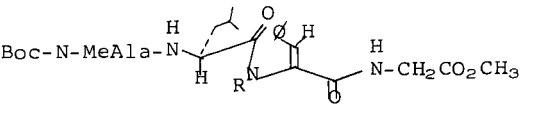

4a

Boc-Leu-Me-(Z)ΔPhe-Gly-OET (*11*)*
H-Leu-Me-(Z)ΔPhe-Gly-OET (*12*)
Boc-Leu-Me-(Z)ΔPhe-OMe (*13*)

13 is that expected for 1:1 mixture of *cis* and *trans* conformers. These results suggest that the amide derivative of MeΔPhe stabilizes the *cis* conformer.

The cyclization studies indicate that linear peptide *4* cyclizes in unusually high yield, compared with linear peptides *5* and *7* when subjected to identical cyclization conditions. The low yield of cyclic peptide *6* from *5* is not surprising; the type of configurational sequence found in *5* has been shown to give only low yields of product[7] due to a severe steric hinderance which develops between the methyl group of alanine and glycine during cyclization. Peptide *8* is formed in higher yield from *7* probably because this steric interaction does not develop when D-MeAla is used. It would appear that tentoxin *1* is formed in unusually high yields from *4* in spite of the presence of the L-MeAla residue. It is possible that the folded conformation of *4* persists during the cyclization reaction and thus facilitates ring closure by placing the mutually reactive ends of the tetrapeptide in close proximity to each other.

Acknowledgment

This work was supported by a grant from the National Institutes of General Medical Sciences.

* Symbol (Z) designates the configuration of the olefin (zusammen): IUPAC rules for nomenclature of organic compounds[6].

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SOLID PHASE SYNTHESIS OF AN OCTADECAPEPTIDE WITH FULL APAMIN ACTIVITY

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APAMIN IS A NEUROTOXIC POLYPEPTIDE isolated from bee venom[1]. Its toxicity (LD_{50} for a 20 g mouse: about 60 µg) is low compared to toxicity of scorpion neurotoxins (LD_{50} as low as 0.2 µg) or snake neurotoxins (LD_{50} as low as 1 µg). Its mode of action is also peculiar: snake and scorpion neurotoxins have a peripheral action (on the acetylcholine receptor for the snake toxins and on the membranes of excitable cells for the scorpion toxins) but apamin passes the blood-brain barrier. It acts specifically at the level of the synaptic mechanisms in the spinal cord. Vincent *et al.* even showed a high specificity of the dorsal and lumbar spinal cord to bind radioactive (and biologically active) apamin[2].

Apamin is the smallest neurotoxic peptide known so far. It is 18 amino acids long, cross-linked by two disulfide bridges. Apamin has been synthesized by the solid phase procedure of Merrifield[3], according to the sequence

H-Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH_2

established by two independent groups[4-6].

N^{α}-Boc-N^{im}-Tos-L-histidine was coupled to a benzyhydrylamine derivative of Kel-F-g-styrene resin[7,8] using dicyclohexylcarbodiimide. Synthesis was carried out in the reaction vessel of a Beckman Model 990 Peptide Synthesizer following the general procedures described by Tregear *et al.* for the synthesis of the biologically active N-terminal peptide of

human parathyroid hormone[9]. We added, however, an automated second coupling step in the later parts of the synthesis. Effectiveness of incorporation of each amino acid was tested by the qualitative fluorescamine procedure[10]. In case of a positive test the coupling reaction was repeated after a new neutralization step with triethylamine in methylene chloride.

The hydroxyl group of threonine was protected as benzyl ether; the side chain carboxyl group of glutamic acid was protected as its benzyl ester and the imidazole nitrogen of histidine and the guanidine function of arginine were protected by the tosyl group. The benzyloxycarbonyl group was used to block the ε-amino function of lysine and the t-butylmercapto group to protect the sulfhydryl function of cysteine[11]. Asparagine and glutamine were coupled as their *p*-nitrophenyl esters. Automated Edman degradation on a peptide-resin sample[12] after coupling of the cysteine, indicated an excellent yield of incorporation of every amino acid: approximately 99.3% average yield over 15 steps (taking into account only N^{α}-unblocked peptides).

Cleavage from the resin and removal of the side chain protecting groups by hydrogen fluoride in the presence of anisole yielded the crude peptide (approximately 50% based on N^{α}-Boc-N^{im}-Tos-L-histidine incorporated on the resin). Due to partial instability of S-t-butylmercapto-L-cysteine in HF the crude peptide was mainly composed of polymers after random formation of disulfide bonds. After reduction of the crude peptide with tributylphosphine following the procedure described by Rüegg and Rudinger[13] and reoxydation in 0.1 M tris-HCl pH 8.0, the peptide was concentrated at the top of a carboxymethylcellulose (Whatman CM-52) column and elution was achieved with a conductivity gradient of ammonium acetate. The column was monitored by UV absorbance at 240 nm and the main peak, eluting at a conductivity of 16 milli-Siemens, was lyophilized and applied on a Bio-Gel P_4 gel filtration column in 0.1 M acetic acid. Synthetic apamin eluted as a clean symetrical peak following a low amount of a high molecular weight material. Synthetic apamin was found to be homogeneous as assessed by high voltage paper electrophoresis, by amino acid analyses after acid hydrolysis and total enzymatic digestion. Edman degradation showed all amino acids at the expected step up to arginine at position 13 and no detectable pre-appearance of leucine at step 9. The position of the disulfide bridges was checked by electrophoretic analysis of the tryptic and chymotryptic digests of the synthetic peptide compared to natural apamin

(following the procedures used by Callewaert *et al.*[6] to assess the positions of the disulfide bonds in natural apamin). There was no detectable appearance of a peptide due to disulfide bridges other than between Cys^{1}-Cys^{11} and Cys^{3}-Cys^{15}. All these tests showed a high degree of purity of the synthetic peptide and its chemical identity with natural apamin. The synthetic apamin was found to be equipotent with natural apamin in the toxicity test on mice. The envenoming symptoms were also identical.

This synthesis allows us to start a structure-function relationship study on apamin for a better understanding of the structural or chemical requirements for its neurotoxic activity.

Acknowledgments

The graft copolymer resin was kindly provided by Dr. G. Tregear from the University of Melbourne, Parkville, Australia. Natural apamin was given generously by Prof. M. Lazdunski and his coworkers from the University of Nice, France.

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SECTION IX

ANALYTICAL AND ISOLATION PROCEDURES

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND DROPLET COUNTERCURRENT CHROMATOGRAPHY IN THE PEPTIDE LABORATORY

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) and droplet countercurrent chromatography (DCC) are two new analytical techniques which are useful in the purification and characterization of peptides. DCC is a purification procedure based on the partition of a solute between two immiscible solvents; HPLC is primarily an analytical procedure, but preparative HPLC is increasing in use.

High Performance Liquid Chromatography

Within the last ten years there have been significant advances in column chromatographic separations that have given rise to the names high speed liquid chromatography, high pressure liquid chromatography, or the more general term, high performance liquid chromatography. The advances in column chromatographic analysis are due to improved column packings and designs and are based on principles established in gas chromatography (GC). HPLC features low dead volume, narrow-bore columns, usually 2-5 mm in diameter, 25 cm to 2 m long, uniform packings, usually 5-60 μ in diameter, and operating pressures in the range of 30-400 atm. With the development of suitable instruments, the speed and resolving power of HPLC are now comparable to GC. These two efficacious procedures are complementary as HPLC is more suited to the analysis of nonvolatile, thermally-labile substances and GC to volatile, thermally-stable compounds.

HPLC separations have been achieved by each of the four major separation mechanisms including ion exchange, molecular exclusion, adsorption and partition.[1,2] The classic Moore and Stein procedure for amino acid analysis with little modification has evolved into an HPLC method. Analyses are now performed in 90 minutes using uniform, sulfonated polystyrene beads about 5 μ in size and operating pressures up to 200 atm. Faster and more efficient analysis would be possible if the porous resin beads were replaced with packings consisting of a thin layer of ion exchange resin coated or covalently bonded to a high surface area, solid-core support less than 10 μ in diameter. Unfortunately, such packings today have small capacities, and the more popular pellicular anion exchangers are much less stable than porous polystyrene beads.

High performance molecular exclusion chromatography has been very useful in the analysis of organic-soluble polymers, but of little value for polar substances, because the packings used (*e.g.*, polystyrene beads) are incompatible with the polar solvents necessary to dissolve peptides and similar water-soluble substances. Packings such as the Sephadexes or Bio-Gels which are used with aqueous solvents deform at the pressures required for HPLC. Several laboratories are attempting to solve this problem. The aim is to find rigid, nonadsorbing packings, compatible with aqueous solvents and able to fractionate polar substances over a wide molecular weight range. Unimpressive results have been obtained so far with controlled pore glass supports containing covalently bonded carbohydrate.

High performance liquid-solid chromatography or adsorption chromatography is commonly practiced with uniform, small size silica or alumina. Thin-layer chromatographic (TLC) separations are often achieved by adsorption as well as by partition chromatography and it is often possible to use TLC data to develop an HPLC method which offers greater speed, resolving power, capacity and ease of quantitation. However, TLC could be the method of choice when very large numbers of samples are analyzed. Problems can arise in liquid-solid chromatography when gradient elutions are employed, especially when the gradient increases in polarity. Precise equilibration with nonpolar starting solvent prior to the next analysis is often difficult to achieve. When equilibration is incomplete, retention times are not reproduced, an unacceptable circumstance in repetitive assays where identification is based on retention time. Perhaps re-equilibration is most effectively achieved by reversal of the gradient.

The greatest successes with HPLC have been in the areas of liquid-solid and liquid-liquid partition chromatography. Originally, liquid-liquid chromatography was practiced with supports *coated* with the immobile phase. Today, the best supports are a uniform 5 to 10 μ in size and the partitioning phase is covalently *bonded*. Packings with bonded phases are more stable and easier to use. However, the separation mechanism with these supports is rarely partition alone; it is usually a combination of partition and adsorption. The best bonded phases contain a C_{18}-hydrocarbon or an aliphatic ether. These are reversed-phase packings since the organic phase is the immobile phase. Columns prepared with these packings have been used for the analysis of PTH amino acids formed in the Edman procedure for the determination of amino acid sequences of polypeptides.[3] Most of the PTH's soluble in the organic phase in Edman's procedure are separated in less than 40 minutes (Figure 1). Those not separated on

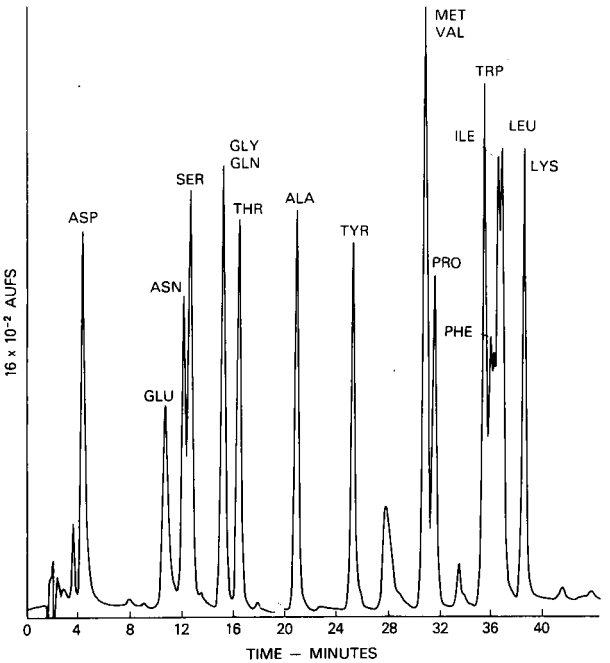

Figure 1: Separation of amino acid phenylthiohydantoins on a 25 cm x 2 mm i.d. Zorbax-ODS column. Solvent system: Solvent A, sodium acetate, 0.01 M, pH 5.0, acetonitrile (95:5). Solvent B, acetonitrile. Solvent B was added at the rate of 1%/min. Linear gradient. Pressure 2000 psi. Flow: 0.5 ml/min. Temp: 50°. Sample size ∿1 μg of each derivative.

Zorbax-ODS (purchased from the Instruments Products Div., DuPont Co., Wilmington, Delaware), *i.e.*, Met and Val, are separated on a DuPont Permaphase ETH column (Figure 2). These columns may be developed isocratically to give complementary results. The more polar PTH's are separated on Zorbax-ODS and the less polar PTH's on Permaphase-ETH using 0.01 M sodium acetate, pH 5.0, acetonitrile (1:1). If the columns are used simultaneously, most of the PTH's can be identified in less than 20 minutes. However, Trp and Lys

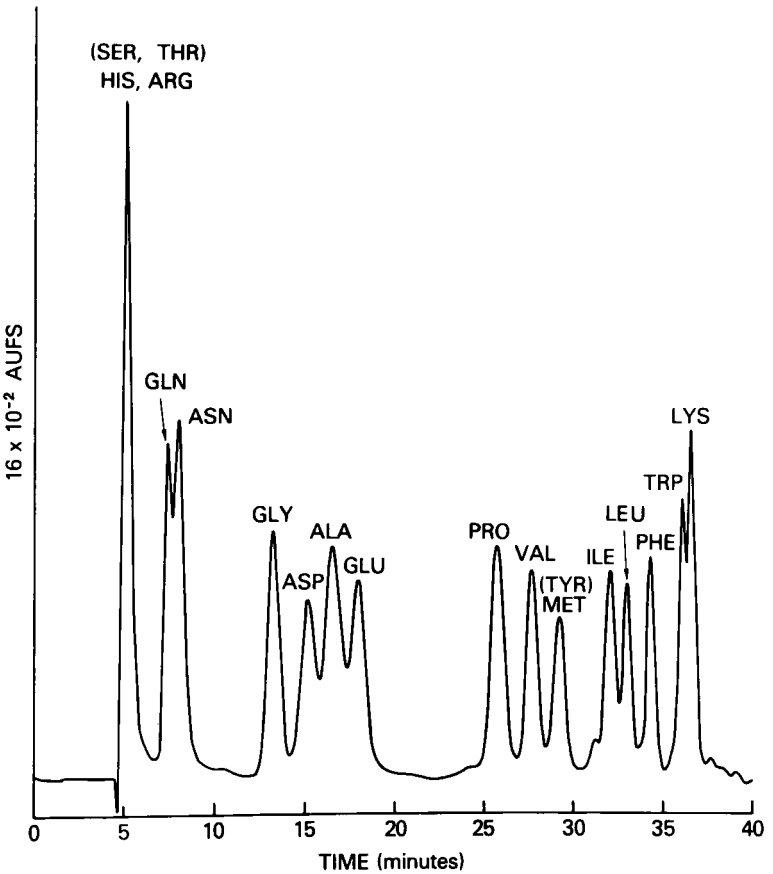

Figure 2: Separation of amino acid PTH's on a 2 m x 2 mm Permaphase-ETH column. Solvent system: Solvent A, sodium acetate, 0.01 M, pH 4.5. Solvent B, acetonitrile. Solvent B was added at the rate of 2%/min. Concave, exponential gradient. Pressure: 1500 psi. Flow: 1 ml/min. Temp: ambient. Sample size ∿1 μg of each derivative.

co-elute in 35 minutes on the ETH column with this solvent. Fortunately, the need for their separation is infrequent since Trp occurs rarely in polypeptides. The amino acids soluble in the aqueous phase in Edman's procedure Arg-PTH and His-PTH are easily separated in less than 15 minutes by either column. Sodium acetate, 0.01 M, pH 7.5, may be used with the ETH column and sodium acetate, acetonitrile (1:1) with the ODS column. Samples of PTH's obtained from automated Edman degradations using either the Beckman Sequencer or an instrument employing Laursen's solid phase method[4] are readily analyzed. Samples obtained from the solid phase method are especially free of background peaks. PTH amino acids also have been separated on silica columns.[5] It appears from these interesting preliminary data that the best results are obtained by separate analysis of the polar and nonpolar derivatives with two different solvent systems. Using two columns, 16 derivatives could be identified in less than 10 minutes. More needs to be learned about silica columns as several investigators have noted difficulties in eluting His-PTH and Arg-PTH and in reproducing retention times of the other derivatives.

In addition to the PTH's other common amino acid derivatives may be effectively analyzed by HPLC. Notable examples are the dansyl and 2,4-dinitrophenyl amino acid derivatives (DNP's). The DNP's like the PTH's give sharp symmetrical peaks on Zorbax-ODS and may be detected with comparable sensitivity (20 pmoles). All the DNP's have been eluted from this column but not all have been resolved to date. The successful HPLC analysis of DNP's may refocus attention on their utility in end-group analysis. The dansyl derivatives have been preferred because they are detected by highly sensitive fluorescent methods. However, the HPLC analysis of DNP's offers comparable sensitivity. Furthermore, the DNP method may be preferred for end-group analysis because dansyl chloride does not react with sterically hindered N-terminal amino acids in proteins and Ser-PTH is unstable and sometimes lost.

HPLC has a few shortcomings at present. Only a few types of reversed-phase packings are available, and there are no generally useful ion exchange or molecular exclusion packings for polar substances. Investigators are dependent on a limited number of manufacturers who usually have different priorities. The use of small size particles in the 5-10 μ range requires special packing techniques not fully understood or easily practiced by most investigators.

In need of study is the HPLC separation of polypeptides and derivatives which can be dissolved in organic solvents.

Such peptides could be separated in milligram quantities in minutes using efficient silica columns packed with uniform 5-10 μ particles. The separation mechanism probably will be a combination of adsorption and partition. Adsorption would be significantly less on reversed-phase columns of the type used for the amino acid PTH's and DNP's. Not to be overlooked is the potential effectiveness of liquid-liquid partition chromatography employing coated rather than bonded phases. High capacity, minimal adsorption, and a variety of coatings are attractions of this approach.

The major advantages of HPLC are the high resolving power, speed and sensitivity. High sensitivity is presently achievable only with substances which may be detected spectrophotometrically or fluorometrically. Another advantage of HPLC is that it is nondestructive. We developed the HPLC method because it is 10 to 100 times more sensitive than our GC method and we wanted to collect the samples for further study.

Droplet Countercurrent Chromatography

Countercurrent chromatography (CC) is a term coined by Y. Ito and R. Bowman, of the National Heart and Lung Institute (NIH) to describe an all-liquid separation technique based on the partition of a solute between two immiscible solvents.[6] Although it resembles countercurrent distribution (CCD), CC differs from this classic technique because CC is a continuous, nonequilibrium process. Tanimura *et al.*[7] developed a variation of CC named droplet countercurrent chromatography (Figure 3). In DCC the solute partitions between droplets of moving phase passing through a column of stationary liquid phase. The droplets may be either heavier or lighter than the stationary phase. When heavier, they are formed at the top of the column; when lighter, at the bottom. The columns (glass, Teflon, metal, ceramic, etc.) are mounted vertically or at an angle and filled with stationary phase. Immiscible moving phase previously equilibrated and degassed is then introduced at a suitable flow rate and through an appropriate tip (*i.e.*, suitable diameter transfer tubing) so that a steady stream of droplets forms. When they reach the end of the column, the droplets enter the narrow-bore, transfer tubing which is connected to the next column of stationary liquid phase where the process is repeated. It should be noted that only the moving phase passes through the narrow-bore transfer tubing, while the stationary phase remains in the column. A series of as many as 300 columns may be used. Considerable turbulence is

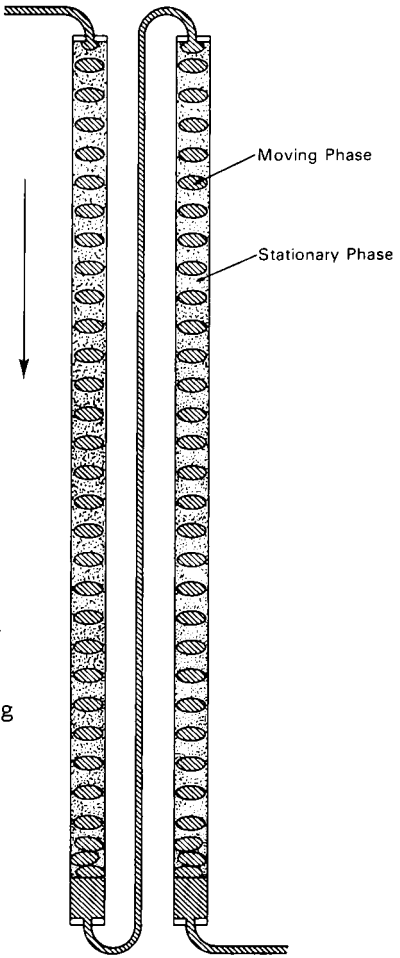

Figure 3: Schematic diagram of droplet countercurrent chromatography. Immiscible moving phase is introduced at the top when heavier, as shown, or through the bottom when lighter. Droplets coalesce at the opposite end and enter the transfer tubing as a solid plug of moving phase. Stationary phase remains in the column.

observed within the droplets as they traverse the column of stationary liquid. This results in efficient partitioning of solute between the two phases. Samples may be introduced dissolved in stationary phase, moving phase, or a mixture of both phases. The stream of moving phase at the outlet is passed through a suitable detector and collector.

The first unit was constructed of glass columns connected with Teflon tubing.[7] Another very useful unit has been constructed entirely of standard wall Teflon TFE spaghetti tubing.* Columns, approximately 20 cm long, are

*Teflon TFE spaghetti tubing, standard wall, may be purchased from the Penntube Plastics Co., Holley Street and Madison Ave., Clifton Heights, Pa. 19018 or from Read Plastics, Inc., 12331 Wilkins Ave., Rockville, Md. 20852.

made with AWG 14 (1.7 mm i.d.) tubing and they are connected with AWG 24 (0.56 mm i.d.) transfer tubing approximately 24 cm long. The connections are made with a 1.5-cm length of coupler made with AWG 18 (1.1 mm i.d.) tubing (Figure 4). Before inserting the transfer tubing with coupler into the column, about 1 mm is cut off the end of the coupler because droplets may not form if the transfer tubing is not fully inserted into the coupler.

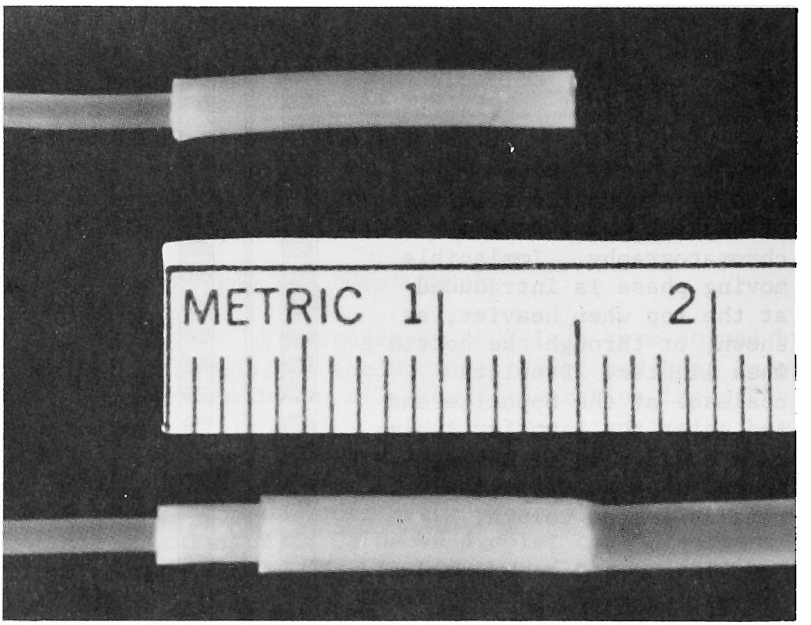

Figure 4: Connection of transfer and column tubing. The approximate lengths of the column, transfer, and coupler tubings are 20, 24 and 1.5 cm, respectively.

Aqueous phase is always the moving phase with Teflon columns. With glass columns, either phase may be the moving phase. However, the glass columns first must be siliconized so that the aqueous phase will form droplets. A number of variables have been tested with a unit consisting of 100 Teflon columns having a capacity of 55 ml (Figure 5). Resolution is not significantly affected by varying the sample load volumes from 0.1 to 5.0 ml and flow rates of moving phase from 1.1 to 4.4 ml/hr. Above 4.4 ml/hr, droplets may

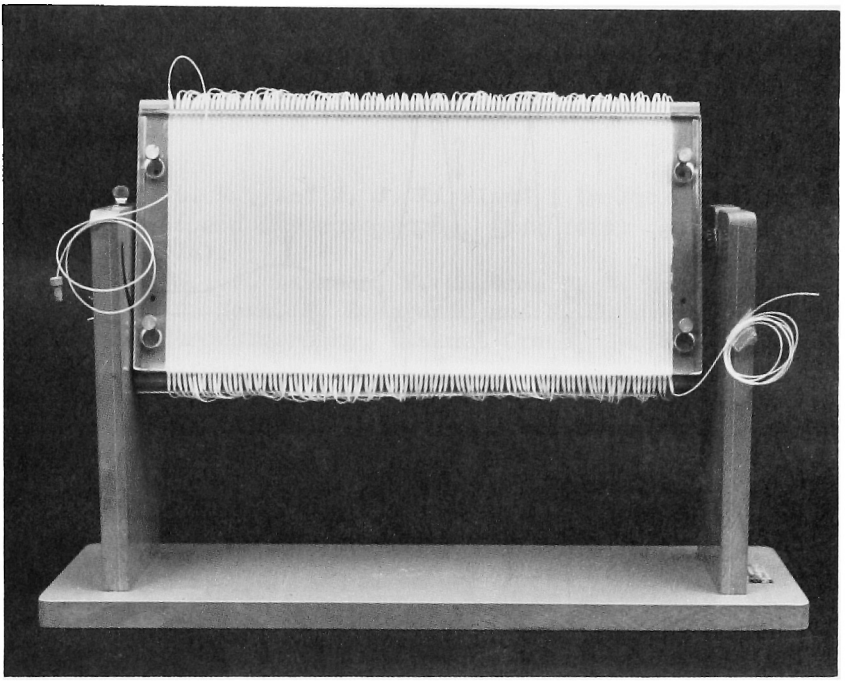

Figure 5: DCC unit consisting of 100 teflon columns connected in series with transfer tubing and placed in channels made in a polyvinyl chloride plate and held in place with a lucite cover. The frame is constructed to allow tilting of the plate when desired to slow the movement of droplets through the column.

coalesce to form plugs which displace stationary phase. A nonpulsating pump is required. The potential of using gas to pressurize the solvent reservoir has not been explored. As little as 1 μg of peptide has been quantitatively recovered since there is no solid support to adsorb sample. In other applications, several hundred mg of sample have been fractionated in a single run. Analyses are usually completed in 8 to 70 hours with excellent reproducibility. Elution volumes may be calculated if the partition coefficient and volume of stationary phase are known, as the apparent partition coefficient is equal to the volume of the stationary phase divided by the elution volume.

Like CCD, DCC separates substances which partition between two immiscible solvents (Figure 6). The technique

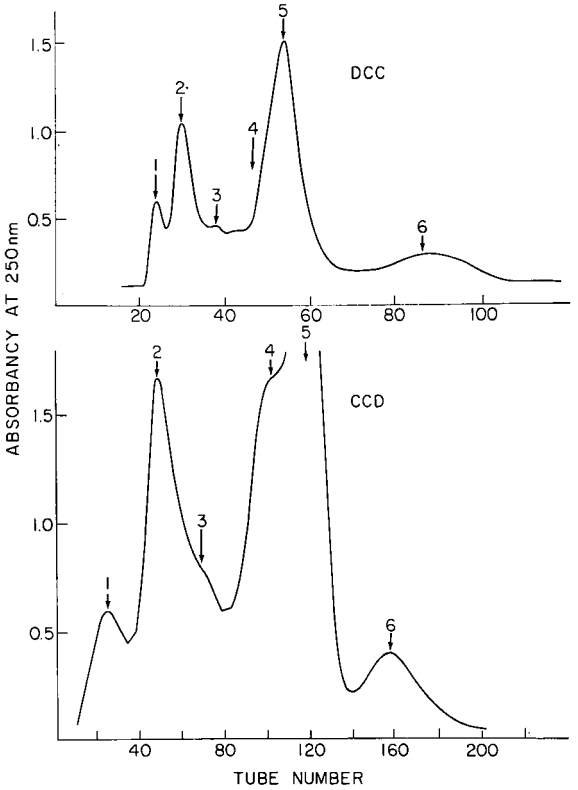

Figure 6: A comparison of droplet countercurrent chromatography and countercurrent distribution. The DCC unit consisted of 200 columns. Three-hundred transfers were performed in the CCD separation. Solvent systems for both methods: n-butanol, acetic acid, water (3:1:4). Sample: BrCN-treated NISIN. For DCC 20 mg was dissolved in 1.5 ml of a 1:1 mixture of aqueous and organic phases. Flow rate 4.3 ml/hr. Fraction vol. 1.45 ml.

lacks the capacity of CCD. Presently the largest unit has a 500-ml capacity of stationary phase and may be loaded with as much as 20 ml of sample. Such loading volumes could contain a gram of sample.

Features of DCC are: 1) no losses of peptide due to adsorption; 2) no emulsion formation since there is no air-solvent interface; 3) compatibility with many of the solvent systems used in CCD; 4) compact, low cost apparatus, easily

built by the investigator, simple to use, and operates unattended, and 5) applicability to peptides not adequately separated by methods based on charge or size.

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COLORIMETRIC AMINO ACID ANALYSIS USING FLUORESCAMINE

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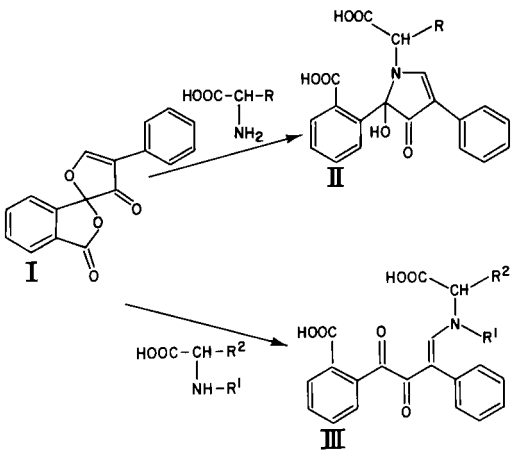
FLUORESCAMINE[1] (I in Scheme) is a nonfluorescent reagent which reacts almost instantaneously with primary amines at room temperature (pH 8-9) to produce fluorescent pyrrolinones (II) which have been utilized for highly sensitive analyses of primary amino acids,[2] peptides,[3] and proteins,[4] and for other analytical applications.[5,6] The pyrrolinones have absorption maxima at 380-410 nm (ε 6,000-7,000) which permit their colorimetric determination.[7] Secondary amino acids also react directly with fluorescamine forming nonfluorescent aminoenone chromophores (III) which show absorption maxima at 310-330 nm (ε 16,000-18,000) and single



inflections at 340-360 nm (ε 9,000-12,000).[8] Since II and III have nearly equal absorption at about 380 nm, this wavelength may be used for simultaneous measurement of both chromophores. A procedure was therefore developed for quantitative colorimetric analysis of primary and secondary amino acids at the nanomole level using one fixed wavelength (380 nm).

A single column analyzer was constructed in which standard citrate buffers were used for elution and borate buffer (pH 9.7, 0.1 *M*) for adjustment of eluant pH (8.5-9.0). Fluorescamine (60 mg/l in acetone) was introduced into the stream and the eluant was then monitored with a colorimeter. It was very advantageous to have the colorimeter in series with a fluorometer. The DuPont Model 836 Fluorescence/Absorbance Photometer is ideally suited. It utilizes a single light source for simultaneous fluorescence and absorbance measurements in a single flow cell. Simultaneous colorimetric and fluorometric analysis enabled immediate distinction between primary and secondary amino acids since fluorometric measurements reveal primary amino acids exclusively. Chromatography of a mixture containing 100 nanomoles of amino acids is shown in Figure 1. Peptides with N-terminal primary and secondary amino acids have also been distinguished by this procedure.

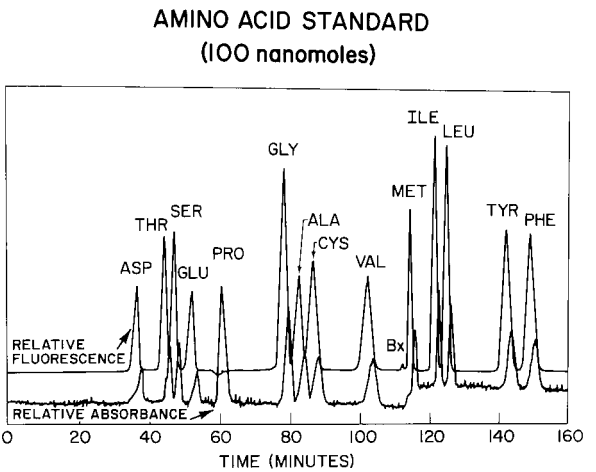


Figure 1: Chromatographic separation of a standard amino acid mixture (100 nmoles of each) on a 50 x 0.28 cm column of Durrum DC-4A resin at 59.5°. Simultaneous fluorometric (upper) and colorimetric (lower) analysis using the DuPont Model 836 Fluorescence/Absorbance Photometer (325-385 nm). Bx, buffer change.

Integration of recorded colorimetric peak area at 380 nm provided operational color values which ranged from 0.11 for ammonia to 1.93 for tryptophan, relative to leucine (Table I).

Table I

Operational Color Values of Standard Amino Acids

Asp	0.36	Ala	0.84	Phe	1.41
Thr	0.81	Cys/2	1.20	Lys	1.49
Ser	0.87	Val	1.00	His	0.98
Glu	0.57	Met	0.86	NH_3	0.11
Pro	1.23	Ile	1.10	Trp	1.93
Gly	1.31	Leu	1.00	Arg	0.93
		Tyr	1.46		

Relative absorbance was linear over a large concentration range. The amino acids were detectable to at least 10.0 nmole, and gave a linear response up to 1000 nmole or more. Fluorometric analysis with fluorescamine permits the detection of at least 100 pmoles with a linear response to about 10.0 nmoles.[2] Thus, the colorimetric methodology complements the fluorometric procedure and enables amino acid analysis with fluorescamine over a broad concentration range.

The sensitivity of the colorimetric fluorescamine analysis was compared to the fluorometric procedure by measuring the detection limits for off-column injection of glycine using the DuPont Model 836 Fluorescence/Absorbance Photometer (Figure 2). In this system the sensitivity of fluorometric analysis with fluorescamine (5 pmole limit) was 200 times greater than that determined by colorimetry (1.0 nmole).

An important advantage of the colorimetric fluorescamine procedure is the capability of analyzing secondary amino acids, including proline analogs, sarcosine, and other N^{α}-methylamino acids. In these special cases, for the exclusive detection of secondary amino acids, measurements are best carried out at 350 nm for maximum sensitivity. These amino acids have very low color values in the standard ninhydrin amino acid analysis and require special elongated coils[9] to obtain maximum color development and in fluorometric fluorescamine analysis oxidation by N-chlorosuccinimide is required for detection.[10] The resolution and colorimetric fluorescamine detection for a mixture of proline analogs is shown in Figure 3.

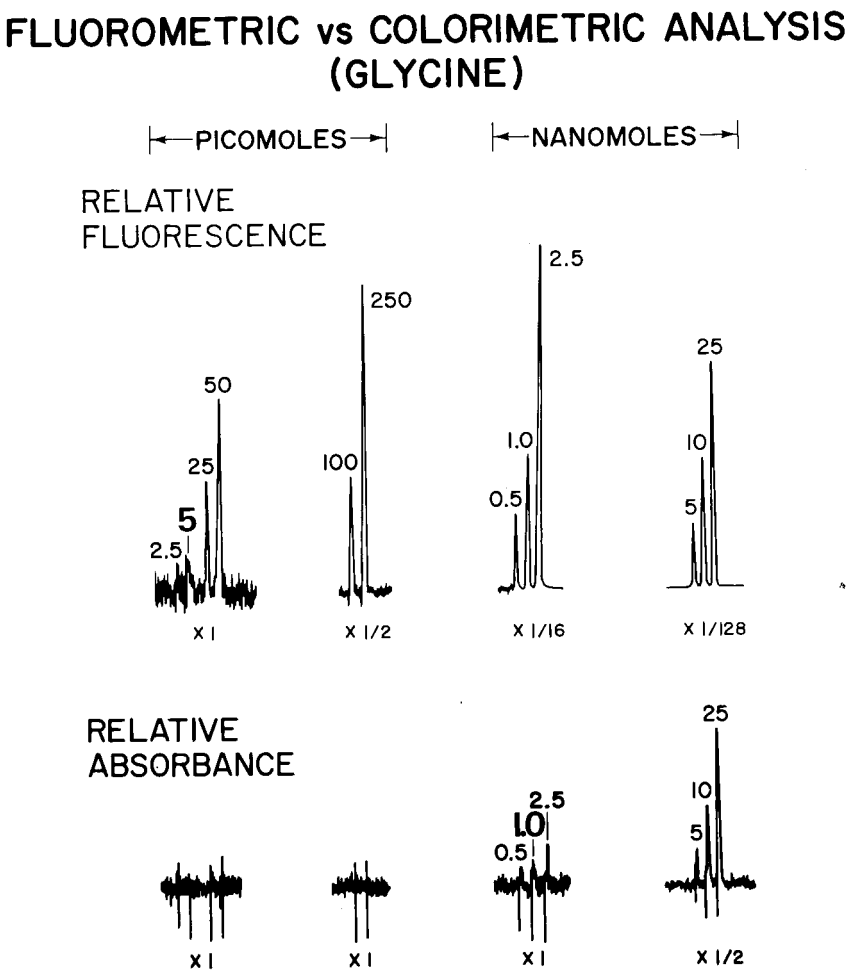


Figure 2: Fluorescamine detection of glycine (off-column) Simultaneous fluorometric (upper) and colorimetric (lower) analysis using DuPont Model 836 Fluorescence/Absorbance Photometer (325-385 nm).

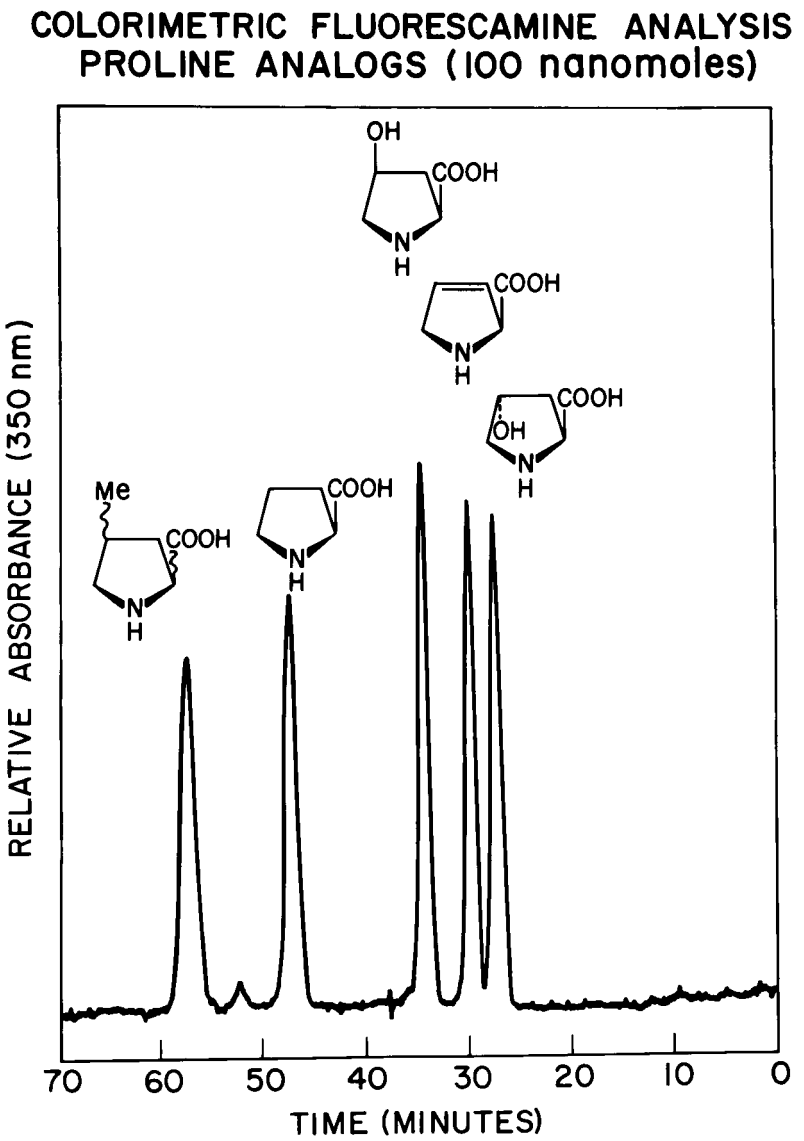


Figure 3: Chromatographic separation on a 50 x 0.28 cm column of Durrum DC-4A resin at 59.5° of a mixture of 100 nmoles each of (left to right) DL-*cis*,*trans*-4-methylproline, L-proline, L-*allo*hydroxyproline, L-3,4-dehydroproline and L-*trans*-4-hydroxyproline. Colorimetric fluorescamine analysis at 350 nm using a Schoeffel SF 70 Spectroflow monitor. Elution was carried out with 0.2 *M* citrate buffer (pH 3.28).

The novel colorimetric procedure has certain advantages over the conventional ninhydrin methodology[11] in spite of somewhat lower sensitivity for primary amino acid detection.* The instantaneous reaction of fluorescamine with primary and secondary amino acids at room temperature circumvents the long reaction coil and elevated temperature requirements of ninhydrin. In addition, measurements may now be carried out at a single wavelength for all the amino acids commonly occurring in protein hydrolysates. The analysis of hydrolysates of the actinomycins, which contain both primary and secondary amino acids, clearly demonstrates the utility of the new method.[12] The chromatogram from a hydrolysate of a representative analog, actinomycin C_2, is shown in Figure 4.

Acknowledgments

The authors thank Mrs. Karin Manhart for valuable technical assistance. Helpful discussions with Dr. Johannes Meienhofer are gratefully acknowledged.

*The sensitivity of the ninhydrin method is estimated to be three times greater than that of the fluorescamine colorimetric procedure based on ε_{570} 20,000 for diketohydrindylidine-diketohydrindamine anion and ε_{380} 6,000-7,000 for pyrrolinones (II).

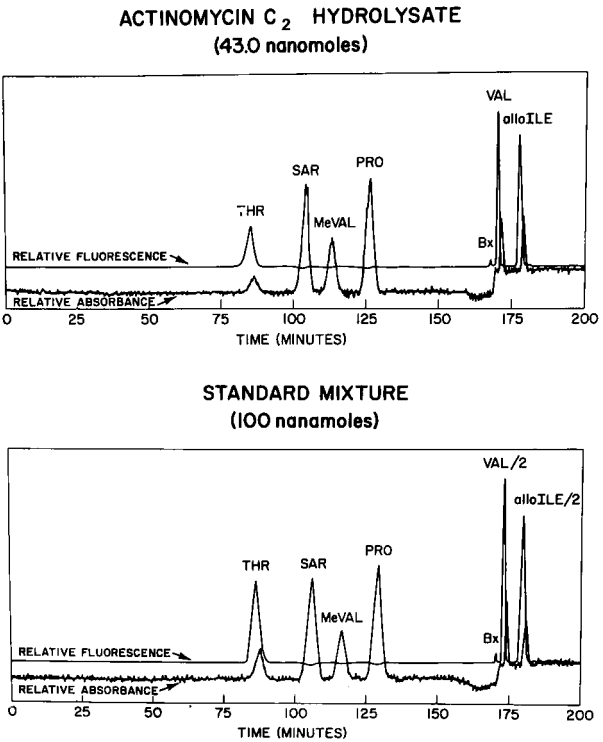


Figure 4: Chromatographic separation on a 50 x 0.28 cm column of Durrum DC-4A resin at 59.5°. Top: Actinomycin C_2 hydrolysate (43.0 nmoles). Bottom: Standard of the six amino acids in Actinomycin C_2 (100 nmoles of each). In each case, simultaneous fluorometric (upper) and colorimetric (lower) analysis was carried out using the DuPont Model 836 Fluorescence/Absorbance Photometer (325-385 nm). Bx, buffer change.

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ADVANCES IN ULTRASENSITIVE SEQUENCE ANALYSIS OF PEPTIDES

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THE FIRST QUESTION TO CONSIDER IS: what at the present time can be regarded as an "ultrasensitive" sequence analysis? In practice, the amount of material required for a complete sequence analysis is very much influenced by the characteristics of the particular peptide under examination. However, improvements in sequencing sensitivity over the past several decades can be broadly related to the increased sensitivity of two ancillary techniques; *i.e.*, amino acid analysis and N-terminal end group analysis. The advent of micro-column amino acid analyzers (such as the Beckman 121 M or Durrum D 500) has made reasonably accurate compositional studies of peptides feasible at the 10^{-9} molar level. The most sensitive end group methods based on ^{14}C dansyl,[1] ^{35}S phenylisothiocyanate[2] or sulphoindonyl[3] techniques now require only picomole (10^{-12} *M*) quantities. For the purposes of this paper, therefore, I shall regard as "ultrasensitive" those approaches which are applicable or potentially applicable to amounts of peptide in the range 10^{-9} - 10^{-12}*M*.

Automated Sequence Analysis: The Edman-Begg Sequenator

Standard methods based on the use of the spinning cup sequenator allow prolonged degradations (20-60 cycles) on polypeptides in amounts of 200-500 nanomoles. Over the past several years with colleagues at the Endocrine Unit, Massachusetts General Hospital, Boston, I have attempted to increase the sensitivity of this procedure. Initial experiments (Figure 1) showed that degradation of progressively

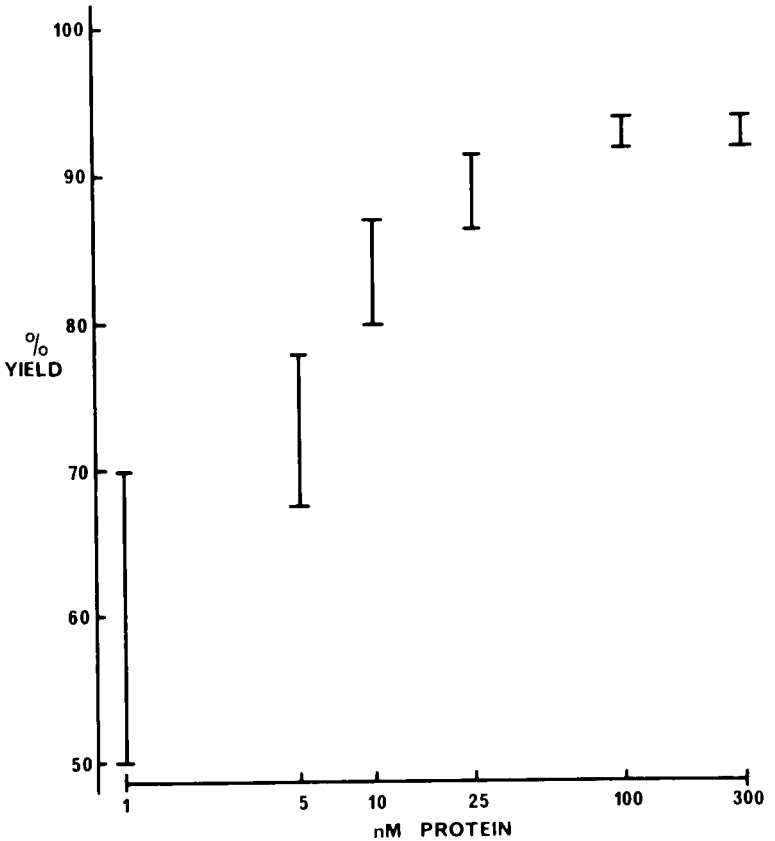

Figure 1: Varying amounts (1-300 n*M*) of sperm whale apomyoglobin were sequenced for 12 cycles in the Beckman Model 890 Sequenator. Repetitive yields of the phenylthiohydantoin derivatives obtained are shown to decrease with decreasing mass of sample analyzed.

smaller quantities of a standard protein (sperm whale apomyoglobin) led to a rapid fall-off in repetitive yield. This effect was particularly evident when amounts of less than 25 nanomoles were analyzed. Table I lists possible explanations. It became clear that there were two factors to consider. First, both the sample being degraded and the cleaved thiazolinone derivative had to be protected from chemical and mechanical losses. Secondly, a more sensitive system was required for identification of the resultant phenylthiohydantoin derivatives.

Table I

High Sensitivity Sequence Analysis

Limiting Factors

1.	Loss of Polypeptide in Sequenator
	Mechanical
	Extractive
	Chemical Side Reactions
2.	Loss of Cleaved Derivative (ATZ,PTH)
	Incomplete Extraction
	Adsorption in Outflow Tract
	Chemical Side Reactions
	Handling Losses
3.	Limits of Detection System

The technique of "protected Edman degradation" was devised to deal with the first problem.[5] Degradation of small quantities (0.5-0.25 nanomoles) of peptide is carried out in the presence of a synthetic poly(α) amino acid carrier. The increased mass of peptide in the sequenator cup appears to minimize mechanical "washout" of the unknown sample. In addition, it is likely though we have not yet formally shown it, that the carrier also acts as a chemical "sink," in absorbing trace amounts of impurities in the reagents and oxidants (including "leak-in" of atmospheric oxygen) which might otherwise irreversibly block the degradation.

The carrier molecule must be soluble in the coupling buffer and in the fluoroacid used for cleavage; it should be composed of non-natural α-amino acids. These will generate thiazolinone derivatives which will act as carrier for the thiazolinones from the sample until they are lodged in the fraction collector. The carrier (succinylated polyornithine) suggested by Silver and Hood,[6] who have independently developed a similar method, does not provide protection against either chemical losses or losses of the cleaved thiazolinone since it is not itself degraded. We found that polyhydroxyproline, which would be suitable for degradations on most natural proteins (obviously excluding those derived from collagen) acted as an effective carrier. However, the phenylthiohydantoin (PTH) of this amino acid interfered with thin layer chromatographic identification of other residues.

A repeating copolymer of norleucine and arginine was then synthesized by solid phase methods (the t-Boc derivative of homoarginine was not available) and the usefulness of the procedure was demonstrated, as reported previously.

Figure 2 shows a more recent degradation carried out on 800 picomoles of apomyoglobin. The PTH derivatives were identified at high sensitivity through the use of high specific activity ^{35}S-labeled phenylisothiocyanate as the coupling agent, using detailed procedures described elsewhere.[7] In the absence of carrier only the first two residues could be identified. With the addition of 100 nanomoles of the poly (Nle-Arg) copolymer, the sequence could be readily determined for 17 cycles. Quantitation was achieved at most cycles by thin layer chromatography, autoradiography, elution of radioactive spots and scintillation counting.

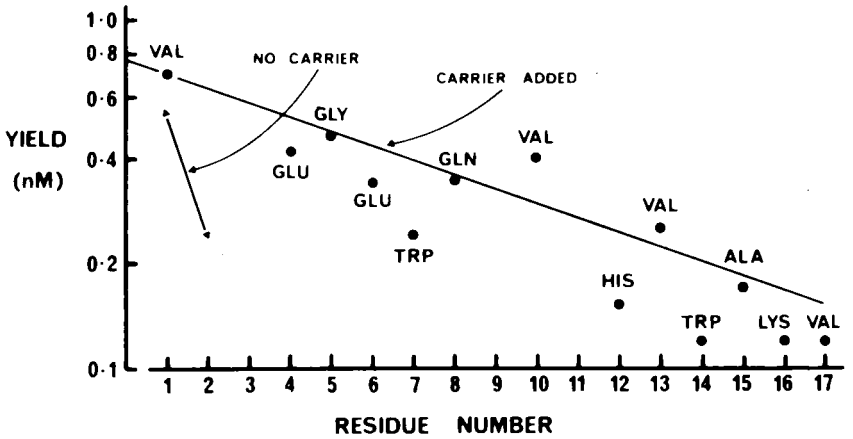

Figure 2: Sperm whale apomyoglobin (800 p*M*) was sequenced in the Beckman 890 Sequenator in the presence (●) and absence (▲) of synthetic poly(norleucine-arginine) copolymer (100 n*M*).

This experiment demonstrates the efficacy of the combined carrier-radioisotope approach, since it represents a three hundred-fold increase in sensitivity over the standard sequenator method. However, there is considerable room for further improvement. With the carrier used so far, there has been a rather sudden fall-off in yields after 20-30 cycles, preventing more extensive degradation. It is likely that this is due to extraction of the carrier itself, which is only 54 residues long. Probably a chain length of

100-120 residues would be optimal. If ^{35}S-phenylisothiocyanate is used at the highest available specific activity (about 200 mCi/m*M*) extrapolation of our present results suggests that a 60 cycle degradation on 1 nanomole of polypeptide should be possible.

Sequence Analysis of Internally Labeled Radioactive Peptides

In the work just described sensitivity was achieved in part through the use of an extrinsic labeling agent (^{35}S-phenylisothiocyanate). However, ultrasensitive analysis based on the Edman procedure can also be achieved where the polypeptide itself is radiolabeled prior to the degradation. Identification then rests on the formation of a radiolabeled phenylthiohydantoin (PTH) amino acid. Nonradioactive phenylisothiocyanate (PITC) is used for the degradation, and any suitable "cold" polypeptide or mixture of polypeptides can be used as carrier. Three variations on this procedure have been examined in our laboratory.

Biosynthetic Labeling

This approach may be illustrated by experiments which led to the sequence analysis of several parathyroid hormone-related peptides. Fresh bovine parathyroid glands or human parathyroid adenomata removed at operation were incubated in 100 mg amounts with mixtures of ^{3}H or ^{14}C amino acids for 1 hour at 37°C in a modified and supplemented Earle's balanced salt solution.[8] The radiolabeled human and bovine parathyroid hormones and their biosynthetic precursors (proparathyroid hormones) were subsequently isolated from the tissue by chromatographic and electrophoretic procedures. Aliquots of 5-10 picomoles of internally labeled peptide were mixed with 2 mg of apomyoglobin as carrier and sequenced in the Beckman Model 890 C sequenator for 10-30 cycles. Aliquots of the chlorobutane solutions obtained at each cycle were counted directly in Liquifluor (NEN) in a Packard dual channel spectrometer using settings which allowed simultaneous counting of ^{14}C and ^{3}H. Samples, to which cold PTH amino acids were added as carrier, were then chromatographed on thin layer plates using previously described systems. Radioactive spots were eluted and counted.

Using this approach the N-terminal sequence of bovine proparathyroid hormone, previously established by other methods, was confirmed and the corresponding sequence of human proparathyroid hormone established.[8] In other experi-

ments[9] the identity of a number of disputed residue positions in human parathyroid hormone[10,11] was established clearly. The results support our previously proposed sequence for the N-terminal 37 residues of this peptide[10] and indicate that the structure proposed by Brewer and associates[11] is incorrect. The method was particularly helpful in deciding between the alternatives of glutamine (Brewer *et al.*) and glutamic acid (Niall *et al.*) for residue 23 since it helps to eliminate uncertainties which could result from deamidation of glutamine side-chains during isolation or Edman degradation. Since the ^{14}C or ^{3}H label cannot be affected by deamidation, the radioactivity found at a particular cycle indicates unequivocally which residue was originally inserted by the biosynthetic apparatus. The logic and details of this approach have been described by Keutmann *et al.*[9]

In another application[12] in collaboration with Drs. Kemper and Rich of MIT, we determined the N-terminal sequence (25 residues) of preproparathyroid hormone, a further biosynthetic precursor of parathyroid hormone isolated from an *in vitro* wheat germ system.[13]

Huang *et al.*[14] and McKean *et al.*[15] using essentially similar biosynthetic sequencing approaches have independently obtained comparable results in terms of sensitivity. Only a few picomoles are required for the degradation, which is approximately 10,000 times more sensitive than the standard sequenator method.[16] Since many peptides of biological interst can be produced from *in vitro* tissue or cell culture systems, the method ought to have very wide applicability.

Exchange Labeling

Several procedures are available for exchange labeling of peptides with ^{3}H. We have carried out some preliminary experiments involving exchange tritiation of apomyoglobin through incubation in anhydrous tritiated trifluoroacetic acid (obtained from New England Nuclear). Significant labeling was obtained without internal peptide bond cleavage occurring to more than a very minor extent. Amino terminal degradation in the sequenator of a small amount of the labeled material showed that all residues examined were labeled, as judged by the production of tritiated PTH derivatives. However, there were very large variations in specific activity from one residue to another. In particular, the tryptophans present at positions 7 and 14 from the amino terminus were very heavily labeled (50-100 times other

residues). Tryptophan is known to be unstable in anhydrous fluoroacids, but it is not known whether this is relevant to our observations. Because of the unevenness of labeling and the uncertainty of back-exchange during the conditions of Edman degradation, much more work needs to be done before one could envisage this approach as generally useful. A recently described procedure for tritium labeling of peptides by microwave discharge activation of tritium gas appears to be well worth further investigation as the basis for a possible ultrasensitive sequencing technique.[17]

Selected Labeling of Specific Amino Acids

Chemical derivatization of peptides under controlled conditions can be used to introduce radioactive substituents into particular amino acid side-chains. The labeled peptide can then be subjected either to direct Edman degradation or to peptide mapping with localization of labeled peptides by autoradiography. At least 9 of the 20 naturally-occuring amino acids could in theory be labeled to high specific activity by this approach (Table II). Of the amino acids

Table II

Selected Labeling

Residue	*Labeling Method*
TYR	Iodination ^{125}I, ^{131}I
HIS	Iodination ^{125}I, ^{131}I
CYS	Alkylation ^{14}C, neutral pH
MET	Alkylation ^{14}C, acid pH
TRP	DNPS - chloride ^{14}C
ASP,GLU	Carbodiimide ^{14}C
SER	Phosphorylation ^{32}P (?)
LYS	Succinylation* ^{14}C
*	Blocks N-terminus
Procedure	Label (1 p*M* - 1 n*M*)
	Polyamino acid carrier if necessary
	Sequenator run and/or peptide map

listed, tyrosine, histidine, cyst(e)ine, methionine and lysine have been successfully labeled by the procedures listed. In addition, aspartic and glutamic acid side chains have been labeled using a water soluble carbodiimide reagent for the addition of ^{14}C labeled glycinamide. Several methods specifically label tryptophan residues; besides the nitrophenyl sulfenyl chloride reagent listed in Table II, one could perhaps use, in radioactive form, the recently described 2-3 dioxo-5 indoline sulfonic acid, which has an additional advantage of being water soluble.[18] Whether phosphorylation of serine by enzymatic or chemical means can be achieved is perhaps doubtful. In addition, serine-O-phosphate is rather unstable to Edman degradation. However, it is included here for completeness.

Not all the procedures listed have been as yet applied to peptides at the subnanomole level. I suggest that the use of carefully selected synthetic poly(α) amino acid carriers (which would necessarily lack groups susceptible to the particular derivatizing radioactive reagent being used) may greatly facilitate the chemical and mechanical manipulations required, and allow this degree of scaling down.

The rationale of selected labeling would be to allow a process of "sequenator mapping" to be carried out on trace quantities of polypeptide. Identification of the appropriate radioactively labeled PTH derivative allows the sequence position of the specifically labeled residue to be established. If applied to several different residues, the cumulative information would provide partial sequence data, would allow comparisons between series of related homologous polypeptides and could be used in planning more definitive sequence strategy. In addition, the labeled peptides may be useful for metabolic studies. In work which for reasons of space cannot be described in detail here, we have used the iodination procedure (with ^{125}I) to localize sequence positions of Tyr and His in human, bovine and porcine parathyroid homrones on picomole amounts of material.[10,19] Waterson and Konigsberg have most effectively used succinylation of lysines with ^{14}C succinic anhydride and alkylation of methionines with ^{14}C iodoacetate in peptide mapping studies of *E. coli* leucyl-t RNA synthetase. Peptide maps carried out on only 1 μg of protein gave interpretable results.[20]

Alternate Approaches

The radiolabeling and carrier approaches I have described are, I believe, the most useful available techniques for sequence analysis of medium to large peptides. Though they are also applicable to sequencing of small peptides by Edman degradation, loss of material in the solvent extraction steps remains a limiting factor. At present the microdansyl method probably remains the most effective (and certainly the most widely used) approach for sequencing nanomole quantities of small peptides. However, solvent extractions are still necessary, limiting its use in practice below about 1 nanomole level, though the derivatives *per se* can be detected at much lower levels. It has additional disadvantages since quantitation is not easy or accurate, and amide assignments cannot be made. The solid phase degradation approach[21] has made very substantial progress over the last few years. Again, the use of synthetic carrier peptides should facilitate chemical manipulations during the coupling of peptides to solid support and allow much smaller quantities of peptide to be analyzed.

Sequence analysis is also limited by the ability to isolate peptides in trace amounts. At the picomole level contamination from operator or reagents is a real problem. "Aseptic" no-touch techniques and rigorous reagent purification are essential. Use of fluorescamine[22] and phthalaldehyde[23] for location of peptides during isolation has proved very useful since both are extraordinarily sensitive. However, there is still need for a truly non-destructive method for localization of picomole amounts of peptides in situations where radioactive approaches are not applicable. A highly sensitive C terminal sequencing procedure would also be most helpful; we are currently trying to develop an isotope dilution method based on the 1-acyl-2 thiohydantoin technique of Stark.[24] Other advances, which are not reviewed here, include sequencing by mass spectrometry and the use of high pressure liquid chromatography for identification of PTH amino acids. These topics are dealt with elsewhere in this volume.

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THE USE OF DIPEPTIDYL AMINOPEPTIDASE (DAP) AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) IN POLYPEPTIDE SEQUENCING

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A PROMISING ENZYMATIC METHOD for sequential analysis of polypeptides with potential advantages of simplicity, rapidity and sensitivity has recently appeared.[1-3] The method employs an enzymatic approach wherein the polypeptide and its single Edman degraded congener are separately digested with DAP to yield two sets of dipeptide fragments. The sequential order follows directly from the determination of the dipeptides present in both mixtures. In earlier work[1] the identification of the dipeptides formed from digestion was a major analytical problem which restricted its utility. Another restriction was the inability of DAP-I to digest a polypeptide when ARG or LYS appeared at the amino terminus or when PRO appeared one or two residues distal to the amino terminus.

As a possible solution to the problem posed by dipeptide identification, the GC-MS technique was investigated because it provided a high potential for simplicity, speed and sensitivity. To render the dipeptides volatile for their subsequent identification by GC-MS, two derivatives were considered, the trimethylsilyl (Tms) and the N-trifluoroacetylamide methyl esters (TFAOMe). The Tms derivative proved to be superior, having the advantages of: 1) speed and ease of preparation; 2) higher sensitivity in the GC and MS; and 3) distinction of ASP and GLU from ASN and GLN, respectively. The Tms derivative is easily hydrolyzed, but this presents no problem, as there is never a need for isolation from the silylation media, which protects against hydrolysis. In practice, both the digestion and formation of the Tms

derivative are carried out in the same vial; enzyme removal is unnecessary. The Tms derivatives are prepared by heating the dipeptide mixture in a screw cap vial with 1:1 BSTFA (N,O-Bis(trimethylsilyl trifluoroacetamide)-CH_3CN at 140° for 10 minutes.

The gas chromatography column routinely used is a 2 mm x 0.6 m column packed with acid-base washed and siliconized 100-120 mesh chromosorb W, coated with 1% OV-1. All Tms derivatives which eluted gave sharp, symmetrical peaks. However, some dipeptides containing ASN, GLN and HIS, and all those containing ARG did not elute. Identification of such dipeptides requires direct MS probe introduction. A gas chromatogram obtained from DAP digestion of TYR-THR-MET-SER-SER-GLN-LEU-THR-LEU-PRO-THR-VAL (unknown #13) is shown below:

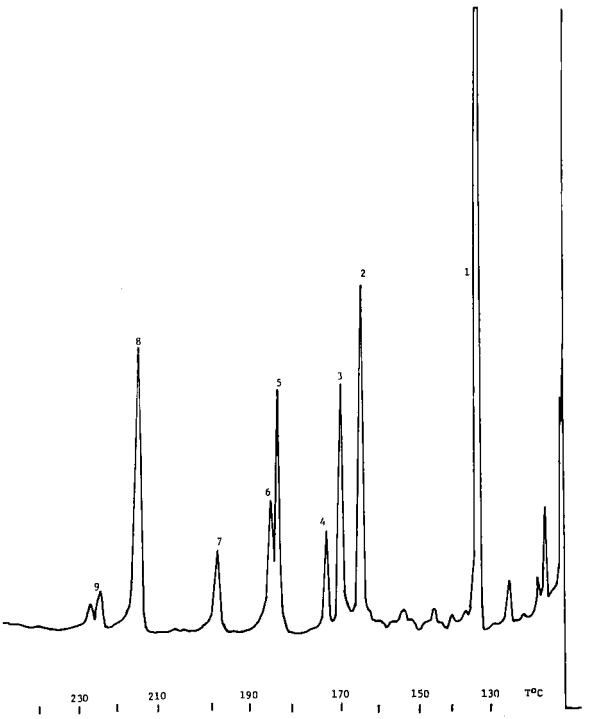

Figure 1: Gas chromatogram obtained from DAP-I/IV digestion of 20 nm UNK #13 for 1 hr, Tms derivitization: peak #: 1) $[HO(CH_2)_2S]_2$; 2) LEU-PRO, THR-VAL; 3) LEU-THR; 4) background; 5) background; 6) MET-SER; 7) SER-GLN; 8) TYR-THR; 9) background.

The GC-MS was carried out on an LKB model 9000 spectrometer. At present, over 150 out of a possible 400 dipeptides have been encountered and characterized. This range has provided several representatives of dipeptides containing the common 20 amino acids in either the amino or carboxyl terminal position of the dipeptide. The usual MS ion mass peaks used in identification of the Tms dipeptide are those resulting from: 1) the amino terminal portion of the dipeptide following central CH-CO bond scission, and 2) the intact Tms dipeptide molecule following loss of the one Tms methyl group. A typical mass spectra is shown below:

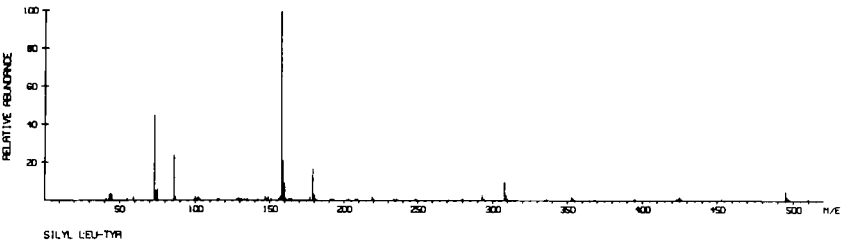

Figure 2: Mass spectrum of Tms LEU-TYR. Ions at m/e 158 due to amino terminal LEU, and at m/e 495 due to the intact molecule minus Tms CH_3. The ratio of peak height at m/e 41 and 43 allows LEU/ILE distinction.

The ion mass peak due to central CH-CO bond scission is absent with some peptides with an amino terminal GLY and with all (except PRO) dipeptides containing amino terminal ASP. Both these phenomena are due to cyclization reactions. The following trimethylsilylations are observed in the MS: 1) carboxyl, hydroxyl groups: silylated; 2) α-amino group: silylated once, except GLY which is usually silylated twice; 3) ε-amino group: usually silylated twice; 4) indolyl, imidazoyl NH: silylated; 5) guanido group: usually silylated three times; 6) primary amide NH_2: silylated once; 7) secondary amide NH: not silylated.

The resistance to digestion by DAP-I posed by amino terminal ARG or LYS was overcome by several changes in the previously reported digestion conditions.[1] Thus, by increases in pH and activator (Cl^-, $HO(CH_2)_2SH$) concentration, the polypeptides ARG-LEU-LEU-GLN-GLY-LEU-VAL and LYS-PHE-ILE-GLY-LEU-MET were successfully digested. These changes had the added benefit of increased dipeptide yields.

Conditions presently employed use pH 7, 1% NaCl and 5µl mercaptoethanol per 0.5 ml buffer (0.05 M $(CH_3CH_2)_3N \cdot CH_3CO_2H$).

The inability to digest peptides containing proline was partially surmounted by the application of another enzyme, DAP-IV.[4,5] Our studies indicated that this enzyme has broad activity toward cleavage of the peptide bond involving the carboxyl group of proline, but was inactive toward peptide bonds involving the imino group of proline. Before DAP-IV could be successfully used, GC background-forming material and previously unrecognized proteases, chiefly dipeptidases, had to be removed. This was accomplished by further column purification (Sephadex G-200, CM-cellulose) and with metal chelators (EDTA, 1,10-phenanthroline).

The DAP/GC-MS method was tested with 13 polypeptide knowns (Table I). In all cases the expected dipeptides were observed in the GC-MS (or direct probe MS if necessary). No particular problems in identification were encountered when Tms dipeptides were not resolved in the GC or from background peaks. Some of the polypeptide knowns were digested before and after a single Edman degradation. The longest series of digestion steps occurred with oxidized insulin β chains, where, using a combination of DAP-I and DAP-IV, the total 30-residue chain was digested.

Table I

Known Peptides Digested with DAP

1. Carboxymethylated Insulin A-chain (before and after single Edman)
2. Oxidized Insulin B-chain (before and after single Edman)
3. ACTH
4. Angiotensin II
5. Asp-Ile-Asn-Val-Lys
6. Glu-Ala-Thr-His-Lys
7. Thr-Ser-Thr-Ser-Pro-Ile-Val-Lys (before and after single Edman)
8. Ile-Asp-Gly-Ser-Glu-Arg
9. Asp-Glu-Tyr-Glu-Arg
10. Ser-Phe-Asn-Arg
11. Lys-Phe-Ile-Gly-Leu-Met
12. Phe-Leu-Asn-Asn-Phe-Tyr-Pro-Lys
13. His-Asn-Ser-Tyr-Thr-Cys-$CH_2CH_2NH_2$

A more valid test of the method was provided by the analysis of 13 polypeptide unknowns, all of which were correctly sequenced (Table II).

Table II

Polypeptide Unknowns Sequenced

1.	Asp-Ser-Thr-Tyr-Ser-Met-Ser-Ser-Thr-Leu-Thr-Leu-Thr-Lys
2.	Phe-Ser-Gly-Ser-Gly-Ser-Gly-Thr-Asp-Phe-Thr-Leu-Gln-Ile-Ser-Arg
3.	Phe-Ser-Gly-Ser-Gly-Ser-Gly-Lys
4.	Thr-Phe-Gly-Gly-Gly-Thr-Lys
5.	Val-Lys-Ala-Gly-Asp-Val-Gly-Val-Tyr-Cys-CH_2-CH_2-NH_2
6.	Ala-Ser-Gly-Val-Ser-Asn-Arg
7.	Ser-Gly-Ala-Gly-Ala-Gly
8.	Arg-Leu-Leu-Gln-Gly-Leu-Val
9.	His-Ser-Gln-Gly-Thr-Phe
10.	Trp-Met-Asp-Phe
11.	Phe-Asp-Ala-Ser-Val
12.	Phe-Val-Gln-Trp-Leu-Met-Asn-Thr
13.	Tyr-Thr-Met-Ser-Ser-Gln-Leu-Thr-Leu-Pro-Thr-Val

The arginine-containing dipeptides from DAP digestion of unknowns 2, 6 and 8 required direct MS probe introduction for identification, as did the Gln-X dipeptides resulting from unknowns 2, 8, 9, 12 and 13. Peptides containing sensitive amino acids such as SER, TRP, GLN or ASN presented no problems. With unknown 13, the dipeptides resulting from the digestion of as little as one nanomole of polypeptide were detectable. As indicated earlier, polypeptides containing more than one proline may not be totally digested by presently available DAP's, *e.g.*, bradykinin, ARG-PRO-PRO-GLY-PHE-SER-PRO-PHE-ARG. What is needed is an enzyme capable of cleaving peptide bonds involving the imino group of proline. Encouraging results have been obtained with a preparation from hog kidneys. This material hydrolyzes the model polypeptide β-naphthylamides of VAL-ALA-PRO-ALA, GLY-PRO-PRO-ALA, PRO-ALA-PRO-PRO and GLY-PRO-PRO-PRO, as judged by the fluorescence. Digestion by endopeptidase activity was ruled out by the lack of β-naphthylamine fluorescence when the β-naphthylamides of Z-PRO-ALA or Z-GLY-GLY-PRO-ALA were employed.

In summary, a sensitive (2-20 nmoles) and rapid (6 unknowns containing 7-10 residues sequenced in 2 days) method for sequencing polypeptides using DAP's I and IV and GC-MS analysis has been developed and successfully applied to a number of unknowns. Digestion and derivitization are conducted in the same vial; enzyme removal is unnecessary. Over 150 dipeptides containing all the common 20 amino acids in both positions have been identified.

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CHARACTERIZATION OF PUTATIVE CYCLIC TETRA-L-ALANINE

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SOME TIME AFTER THE INTRODUCTION of high-molecular-weight synthetic polypeptides as protein models, several groups of investigators recognized that oligopeptides offer special advantages for such studies. The work of Craig's group on the optical activity of cyclic oligopeptide antibiotics[1] stimulated us to view diketopiperazines as peptides of highly constrained structure with potentially interesting ORD and CD spectra.[2,3] It appeared in these studies that certain diketopiperazines showed an optical activity qualitatively resembling that of α-helical polypeptides. One component of L-Ala diketopiperazine preparation was tentatively identified as cyclic L-Ala tetrapeptide,[3] but our subsequent attempts to prepare samples of this compound have proved unsuccessful. The report of Dale and Titlestad[4] on the preparation of a series of cyclic oligopeptides, including tetra-L-alanine, was therefore of great interest to us. These investigators very kindly provided us with a 7 mg sample of their material, with a notation by Dr. Titlestad that the preparative yield was very poor, and that the configuration of the product was not clear. We here report studies on the nature and optical activity of this sample.

Results

The putative cyclic peptide was insoluble in water but completely soluble in trifluoroethanol. A stock solution

of the peptide in trifluoroethanol was used for all experiments. Amino acid analysis (Spinco model 120 Analyzer) on a hydrolyzed sample of the putative cyclic peptide revealed the only ninhydrin-positive material to be alanine. No di, tri or tetra peptides of alanine, or any other amino acid, were detected. The cyclic peptide, which was initially insoluble in the 6.0 *N* HCL, was completely soluble after heating at 110°.

To test the putative cyclic peptide for free amino groups, a sample of the tetrapeptide was dansylated[5] along with samples of the trifluoroethanol solvent and standard alanine. After the dansylation, all samples were hydrolyzed in 6.0 *N* HCl at 110°C for 22 hours. Polyamide chromatography[6] in the benzene:acetic acid = 9:1 solvent system identified the only dansylated product of the putative cyclic peptide as dansyl sulfonic acid. No dansylalanine could be detected. Under the same experimental conditions, samples of standard alanine were dansylated to yield a modified amino acid that co-chromatographed with authentic dansyl-alanine. This test confirms the absence of free amino groups and suggests that the peptide is either amino-blocked or cyclic.

Since the peptide was synthesized as the benzyloxycarbonyl (Z) tetrapeptide, it was possible that removal of the Z group and subsequent cyclization was incomplete. This would result in a peptide with a Z-modified amino group. In order to examine this possibility, UV spectra were determined for the putative cyclic peptide and for authentic N-Z-L-alanine. These spectra are shown in Figure 1. The putative cyclic peptide failed to show any aromatic absorption in the 260 nm region of the spectra. From this test, it is evident that all Z functional groups have been removed (as little as 10% Z would have been detected in our measurements). Since there were no other amino blocking groups used in the synthesis, this result indicated that the peptide was cyclic.

The putative cyclic peptide was examined by mass spectrometry (Figure 2). Except for a trace component at m/e = 328, no components higher than the cluster at m/e = 284 were shown. A m/e of 284 corresponds to that for a cyclic tetrapeptide of alanine. The fragmentation pattern is also consistent with that of tetraalanine. No substantial amounts of fragments derivable from Z are seen in the spectrum. From this mass spectrum, it is apparent that the product is a tetramer and does not contain higher polymer. The results also confirm the absence of Z-containing peptide in appreciable concentration.

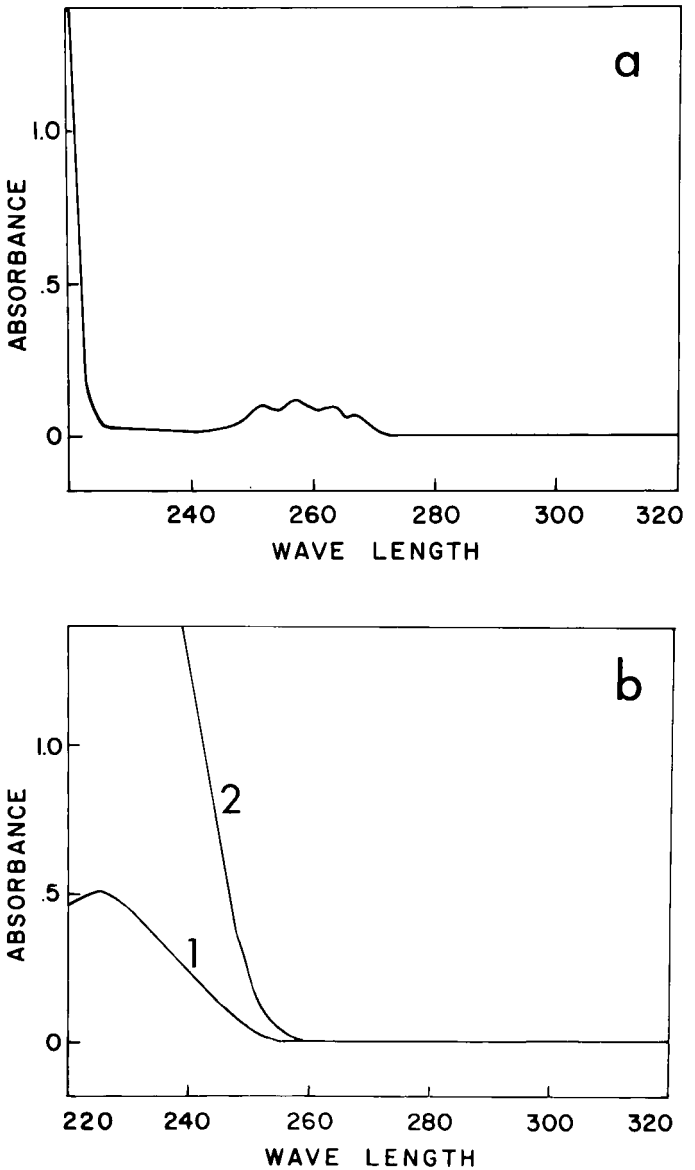

Figure 1: Ultraviolet spectra of a) 5 x 10^{-4} *M* N-Z-L-alanine in ethanol, 1.0 cm light path; b) putative cyclic tetraalanine 4.7 x 10^{-4} *M* in trifluoroethanol: 2.00 mm light path (curve 1); 10.00 mm light path (curve 2).

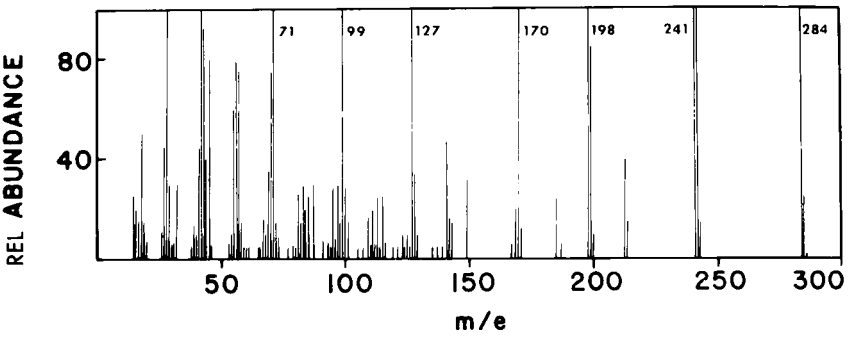

Figure 2: Mass spectrum of putative cyclic tetra L-alanine.

To test the configuration of the alanine residues of the putative cyclic tetrapeptide, samples of the peptide along with samples of authentic L-alanine were subjected to acid hydrolysis. The circular dichroic spectrum of the acid hydrolysis products is seen in Figure 3. As can be seen from this figure, the value of $[\theta]_{210}$ for the cyclic peptide is half of that found for standard L-alanine. The

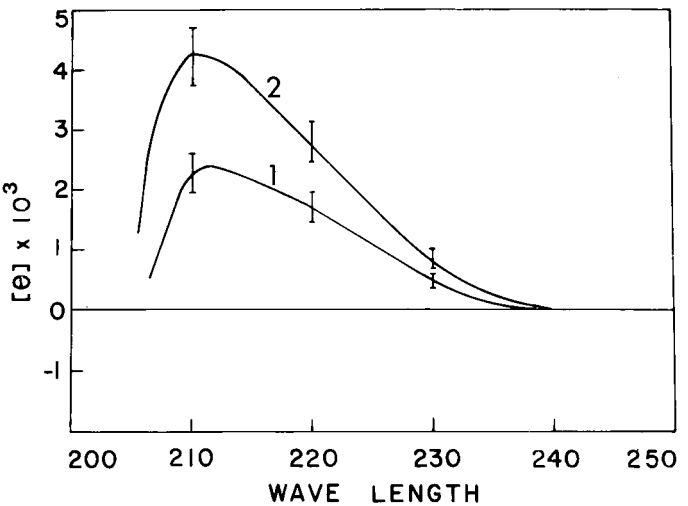

Figure 3: Circular dichroism of acid hydrolysis products of the putative cyclic peptide (curve 1) and standard alanine (curve 2). The spectra were determined in 1.0 *N* HCL, in a 10.0 mm path cell, using a calibrated Jasco-Durrum J-10 instrument. The molar ellipticity has units of Deg. cm^2 $decimole^{-1}$. The wavelength is recorded in nanometers.

possibility of racemization during acid hydrolysis was examined by determining the circular dichroism of authentic L-alanine not subjected to the conditions of acid hydrolysis. This spectrum was within experimental error of that shown for the "hydrolyzed" authentic L-alanine in Figure 3. These results therefore imply ∿25% D-alanine content in the tetrapeptide sample.

The CD spectrum of putative cyclic tetra-L-alanine (TFE solvent) showed a nearly symmetrical negative band (minimum at 232 nm, a slight shoulder visibile ∿213 nm). The amplitude of the ellipticity at 40° was ∿15% lower than at 25°. The ORD (TFE solvent) showed a negative Cotton effect minimum at 243 nm, zero crossing at ∿232 nm, and a positive plateau from 220 nm to 205 nm, where S/N had decreased to 3.

Discussion

The solubility and results of the mass spectra, dansylation and UV spectra suggests that the putative cyclic peptide is indeed a cyclic tetrapeptide of alanine. However, the stereochemistry of the alanine residues is in doubt. The observation of half the theoretical molar ellipticity for L-alanine in the hydrolyzed tetrapeptide suggests a stoichiometric ratio of 3 L residues to one D residue per tetrapeptide. The data do not distinguish between a homogeneous product with 3 to 1 stoichiometry and a heterogeneous product with an average D residue content of ∿25%.

The synthetic pathway described by Dale and Titlestad shows no obvious steps where racemization would have taken place. If any step allowed complete racemization all optical activity would have been lost. Since this was not found we must assume that in their synthetic system, racemization was limited. A low yield of D-Ala in the tetrapeptide before or during cyclization may have been responsible for the reported low yield of cyclic tetrapeptide. This argument implies that the cyclization L_4^- tetraalanine is more difficult than the cyclization of L_3^-, D_1^- tetraalanine. This idea appears to be experimentally testable, but we are not presently in a position to do so.

Comment on the CD spectrum and the ORD cannot be useful until the covalent structure of the cyclic tetrapeptide is clarified.

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METHODOLOGY AND APPLICATION OF BIOSPECIFIC AFFINITY CHROMATOGRAPHY

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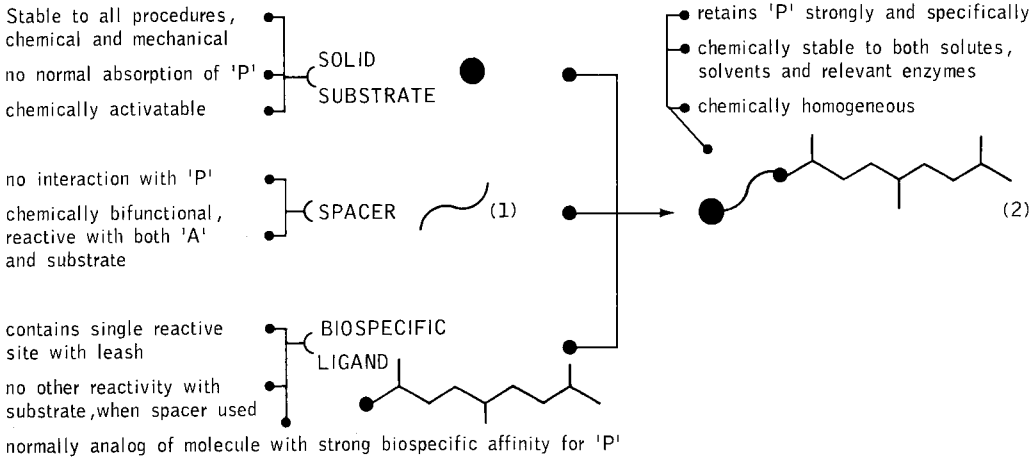
THE USE OF THE BIOSPECIFIC AFFINITY of a hapten-antibody complex represented the initial applications of this area of technique.[1] The method of activating agarose by reaction with cyanogen bromide[2,8] and the use of spacers[3] may be regarded as major experimental advances in the preparation of biospecific affinity systems, which led to the explosion of the area in the last five years. The use of different support systems, their chemical derivatization, and various techniques of ligand attachment have been substantially brought together in "Methods in Enzymology" 34.[4] We shall review those details pertinent to the use of affinity chromatography in the peptide field, and refer the reader to the above volume for more general summaries.

The "Ideal" Experiment

Let us assume that we wish to isolate a component (P) that binds to a certain peptide (A). An application of affinity chromatography might proceed as shown in Figure 1.

The criteria for systemizing the support-spacer-ligand complex are similar or identical to those for immobilized enzymes[5] and hormones.[6,7] Most of the considerations for the column or batch procedure are those standard to chromatography. More detailed experimental considerations quickly lead to a number of major and minor difficulties.



(1) Possibly redundant

(2) More than one leash/ligand may be present per substrate. No significance in relative scale of three parts.

Figure 1: Scheme and conditions for a generalized preparation of a biospecific affinity complex of 'A' to isolate 'P.'

The Suitable Support

Agarose has clearly been employed most frequently in this technique.[9] Polyacrylamide beads have been somewhat passed over because of a lower exclusion limit, about 400,000 daltons, while controlled pore glass beads, though successfully applied, *e.g.*,[10,11] are often felt to have poorly defined residual surface charge properties. Advantages of these two alternatives to agarose (particularly mechanical stability and resistance to organic solvents) may make their consideration worthwhile. Properties of the support will dictate some of the desired characteristics of the final absorbent, in particular pore size, and available derivatizable surface.

Leakage from the Support Matrix

In applications of affinity chromatography to the purification of estrogen receptors, early investigations revealed significant problems of reproducibility and apparent total loss of binding material. Utilizing the high specificity and binding constant of the putative receptor population, it was possible to demonstrate that the column was "leaking" the ligand into solution at a fairly constant rate.[25,26] The stability of cyclic AMP-sepharose complexes was found to be similarly imperfect.[24,28] The problem of leakage from non-agarose supports has not been investigated in the same detail. Agarose consists of alternating (1→3) linked β-D-galactopyranose and (1→4) linked 3,6-anhydro-α-L-galactopyranose residues (Figure 2). Model studies[27] and general considerations of the reagents[23] suggest that the activation of agarose proceeds through the isocyanate, and may form either an inert carbamate, or a reactive imidocarbonate. From the agarose structure one would expect that

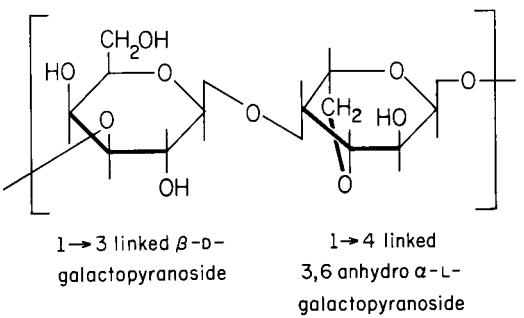


Figure 2: Agarose structure.

the imidocarbonate may be formed either intra-residually between C4 and C6 in the β-D-galactopyranoside, or between inter-chain hydoxyls that are particularly close. From the chemistry of the breakdown properties, the effect of aminolysis with RNH_2 seems most likely to form a substituted isourea, possibly in equilibrium with a two-point attached species (Figure 3). Tesser *et al.*[24] found the reaction products illustrated from hydrolysis, ammoniacal hydrolysis and basic hydrolysis, that are consistent with the proposed structure. Wilchek *et al.*[75] have observed that a soluble super-active form of insulin can be released from insulin-agarose by aminolytic cleavage leading to N_1-N_2-disubstituted guanidines. There is no evidence indicating that bonds formed in this way by cyanogen bromide and agarose can be other than intrinsically unstable above pH 5. A maximum figure for the release of ligand at pH 8.0 might be 0.1% per diem, with many cases leaking more slowly.[29]

How does this interfere with the chromatography? If one applies 10^{-13} moles of a "receptor" to a column containing 10^{-8} moles of ligand, then at 0.1% release per diem, a molar excess of ligand to receptor is released in 3 hours. If the free ligand is more effectively bound to its receptor than the matrix-ligand, the free will interfere with absorption. The release of ligand can substantially confuse subsequent binding assays. Objections may be raised to the use

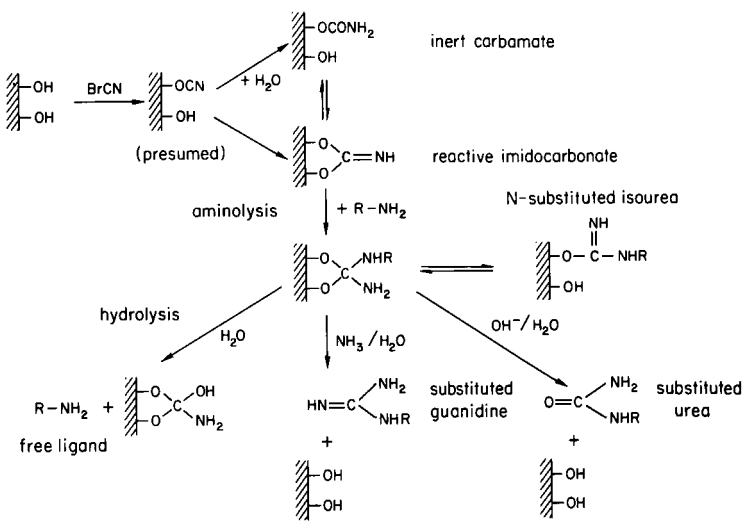


Figure 3: Activation, reaction and cleavage mechanisms from agarose.

of such matrix-ligand complexes non-chromatographically. The application of immobilized hormones to test for efficacy without cell penetration has received such criticism among others.[60,63] An ingenious reduction of this problem is afforded by the use of multipoint attachment with poly amino acids.[17,19] Cross linking in this fashion can afford greater mechanical strength, at the cost of lowering the upper limit of molecular exclusion.

The Spacer Group

The original purpose of introducing a "leash," the spacer group, centered around the desirability either of forming a new reactive group, other than the one from the "activation" of the support, and the probable increase in efficacy in binding due to the removal of the attached ligand from the immediate vicinity of the support where it might either be sterically hindered from its binding reaction, or its properties more directly modified by the proximity of the matrix by, for example, hydrogen bonding. Numerous examples of the employment of alkyl amino groups illustrate this technique and realize those objectives. However, it has recently become obvious that such spacers may not be acting passively. O'Carra *et al.*[12] has shown that the purification of β-galactosidase can be performed by the spacer groups themselves without the use of the ligand β-thiogalactoside, and similar observations on L-histidinol-phosphate aminotransferase[13] confirm previous suggestions[14,15,21] that alkyl groups play a role in the separation through hydrophobic interactions. More hydrophilic spacers may be employed. One approach is the use of hydroxyalkyl amines[16,30] or polyaminoacids or denatured proteins[17-19] with lysine side-chains containing the second reactive group which have other advantages as we have seen. Activation of agarose by bisoxiranes, instead of cyanogen bromide followed by coupling, introduces an hydrophilic spacer.[20]

The Attached Ligand

If the material to be attached is a large natural product, *e.g.*, bovine serum albumin, then very little real choice is available in the particular derivative used--the principal objective of attaching such a material is to retain the activity of binding sites already present in the molecule, distorting the normal structure, in the case of BSA predominantly globular, as little as possible. Coupling

reactions are tested for interference with binding. The reactive group (*e.g.*, amino or carboxyl or thiol) with statistically few members interfering with binding is to be preferred.

When dealing with very small ligands, *e.g.*, analogs of NAD or cyclic AMP, exact sites of attachment becomes very important, and may usually be guessed from the efficacy of analogs substituted with bulky groups at potential sites of attachment.

An important consideration in the choice of ligand is the application, and whether a particularly high degree of specificity is desirable in the choice of ligand. For example, in the purification of acetylcholine receptor, very substantial amounts of acetylcholinesterase are present. There is a substantial overlap of binding specificity between these two, and many nicotinic effectors or inhibitors. As has been experimentally demonstrated[51] columns may be selected to bind both or either separately, with a high degree of specificity.

Smaller peptides present a combination of both problems. These are relatively few reactive side chains, and the blocking of one can readily reduce activity. The availability of data on synthetic analogs is clearly the key to the resolution of the problem. In the cases of, for example, angiotensin and ACTH, enough is known about sequential substitution to allow a rational choice of "ideal" attachment site. The chemistry for attachment now is required. In Figure 4 some strategies for attachment are outlined. Studies

* 1 Direct reaction of unmodified peptide
ex: Cuatrecasas 1973 (22)

* 2 Reaction of specifically protected peptide
a- modified after synthesis or preparation
b- synthesised and specifically partially deprotected

* 3 Introduction of reactive group in synthetic analog
ex: Möschler & Schwyzer, 1974 (50)

Figure 4: Some strategies of attachment of peptides to matrix.

of insulin by Cuatrecasas[22] provide the clearest case of strategy 1. Only lysine B29 and the α-amino groups are potentially reactive with an activated ester derivative of agarose. Suitable control of pH is presumed to restrict attachment to the group of appropriate pK.

Möschler and Schwyzer[50] have described the introduction of an additional reactive group, cysteinyl thiol. Boc-L-Cys(Am) angiotensin was synthesized from the coupling of the free angiotensin II (AII) and the protected cysteine hydroxysuccinimide ester and the thiol specifically deprotected by mercuric acetate. The free thiol may be linked to a maleimide coupled to agarose. Fauchère *et al.*[49] have avoided the problem of leakage from agarose by synthesizing a maleimide-acrylamide matrix. Cys-A II and Boc-Cys-$(ACTH_{5-24})(OBu^t)$ $(BOC)_4$ $-OBu^t$ were attached, with subsequent deprotection on the support, without apparent loss of structure. This has suggested the following completely general approach.

Application of the Methods of Solid Phase Peptide Synthesis to Affinity Chromatography

The commonality of an insoluble support to both solid phase peptide synthesis and affinity chromatography leads one to consider the possibility of applying the methods of solid phase synthesis to the preparation of affinity columns. Ideally one would like to synthesize a fully protected peptide on an agarose support, deprotect the peptide and use the resulting derivatized support for affinity chromatography. Some attempts have been made to use carbohydrate supports in solid phase peptide synthesis. The model peptide Leu-Ala-Gly-Val was synthesized on Sephadex LH-20 by Merrifield.[46] Vlasov and Bilibin have reported the binding of Boc-Gly to Sephadex LH-20. Subsequent deprotection with TFA, coupling with active esters and cleavage of hydrolysis yielded a pure pentapeptide.[47] Recently bradykinin was synthesized on a similar support by DCC coupling.[48] Sephadex G-25 has also been used as a support. Until agarose has been tested and proven useful as a solid phase peptide synthesis resin, a less direct approach for the production of affinity columns seems to be indicated. A protected peptide can be made on a standard solid phase synthesis resin, cleaved from the resin, selectively deprotected, coupled to derivatized agarose and totally deprotected (Table I).

The above mentioned method introduces at least one additional level of protection and as such substantially complicates the choice of protecting groups. In fact were

Table I

Strategies for the Use of Protecting Group Combinations for a Solid Phase Peptide Synthesis Aided Approach to the Preparation of Affinity Column Materials

Amino Acid	Protecting groups			Methods of deprotection			Method of cleavage from resin	Method of attachment to support	References
	Side chain selected	Other side chains	N - α -	N - α -	Selected	Other			
Lys	Boc	Z,(2,4Cl_2Z) N-Bzl Formyl O-Bzl, Nitro Tos	Bpoc Boc	0.5%TFA /DCM(1) 50%TFA /DCM	50%TFA /DCM	Catalytic hydrog.	Trans-esterification	DCCI coupling to COOH derivitized agarose	31-44
Glu, Asp	t-Bu	Z,(2,4Cl_2Z) N-Bzl, Formyl O-Bzl, Nitro Tos	Bpoc Boc	0.5%TFA /DCM 50%TFA /DCM	50%TFA /DCM	Catalytic hydrog.	(1) Hydrozinolysis (2) Mild oxidation	DCCI coupling to NH_2-derivitized agarose. (4)	31-44 76-79
Cys	Sam (2)	Boc,t-Bu Formyl	Bpoc Boc	0.5%TFA /DCM 50%TFA /DCM	$Hg(OAc)_2$	0.1M HCl /formic acid	(1) Hydrozinolysis (2) Mild oxidation	Coupling to N-maleimide-agarose analog.	31-45 49-51 76-80
Cys	Set (3)	Z,(2,4Cl_2Z) N-Bzl,Formyl O-Bzl,Nitro Tos	Bpoc Boc	0.5%TFA /DCM 50%TFA /DOC	Mercapto- ethanol	Catalytic hydrog.	(1) Hydrozinolysis (2) Mild oxidation	Reaction with FDNP derivitized agarose (5)	31-44 52-53 55-57 76-80
His	Boc	Z,(2,4 Cl_2Z) N-Bzl, Formyl O-Bzl, Nitro Tos	Bpoc Boc	0.5%TFA /DCM 50%TFA /DCM	50%TFA /DCM	Catalytic hydrog.	(1) Hydrozinolysis (2) Mild oxidation	Reaction with FDNP derivitized agarose	34,35 51,52 55,59 62,64 76-79

Notes for Table I

1. Dichloromethane - DCM

2. S-acetamino-methyl - SAM

3. S-ethyl thiol - SEt

4. Alternatively, a spacer may be introduced into the peptide by, for example, deprotecting the -COOH, coupling to 1,4-diamino butane (1-Boc), and proceeding as in the lysine case.

5. 1-(agarose amino), 2-fluoro, 3,6-dinitrobenzene-FDNP

6. Merrifield (unpublished) has observed that catalytic hydrogenation is apparently an ineffective method of deprotection for peptides in the solid phase (polystyrene). Fauchère *et al.*[49] have used 0.1 *M* HCl/formic acid for the deprotection of their ACTH analog.

7. Although many individual steps are well established, the overall applications are NOT tested. The presentation is designed to illustrate the strategy.

it not for the recent development of extremely acid labile groups, *e.g.*, Bpoc, the method would be at best extremely limited. With these protecting groups, however, virtually any peptide can in principle be bound to agarose. C- and N-terminal derivatization is also a trivial problem. N-terminal derivatization can be done during the initial solid-phase synthesis and by varying the cleavage conditions, esterification or amidation of terminal amino acids becomes simpler. Examples of various synthetic strategies are given in Table I.

The reactions outlined are listed to illustrate the overall strategies and there may often be cases of simple and improved replacement (*e.g.*, the coupling of an amino group through an N-hydroxysuccinimide ester of agarose by the method of Cuatrecasas and Parikh).[74]

Other Methodological Considerations

The processes of chromatography or batch use of the support-ligand require almost identical conditions to those used in conventional chromatography. The amount of ligand must be sufficient to bind, in its modified form, all the specific material passed over it. Insufficient ligand on the matrix will allow passage of "P," while excess may greatly exaggerate problems of leakage and nonspecific binding. Elution must be sufficient to remove nonspecific material first, yet not be so great as to carry away substantial quantities of "P" by dilution. The problem of several types of "P" may be investigated by the use of gradient or stepwise elution with analogs of "A". The central problem of predicting the behavior of any column[59] lies in the measurement of the equilibrium constant between "P" and the matrix-ligand, which can be quite different from the soluble ligand equilibrium constant.[68]

Applications

Two classes of application will be considered: The use of affinity chromatography to purify natural and synthetic peptides and the use of peptides as ligands in the preparation of specific binding proteins.

Purification of Peptides

One useful application has been reported below the level of peptides--the resolution of DL-tryptophan on BSA-agarose.[64] The same support has been used for purifica-

tion of ferriporphorin c-peptide.[67] With the advent of automated sequenators and amino acid analyzers, a rate-limiting step in sequencing is commonly the isolation of peptides. How can a site for binding be introduced into peptides which may be completely uncharacterized? By suitable side chain modification, a more general chemical "handle" can be put on the peptide, and the handle may be retrieved by one of two methods. The handle may resemble a hapten, and the peptide can then be absorbed to a specific antibody-matrix absorbent. An ingenious alternative is to use a handle with affinity not only for a binding protein, but for a site on the original protein that was labeled viz. an affinity or site specific reagent (Figure 5). After conversion of the affinity labeled protein to peptides, by methods which do not cleave peptide-modifying reagent bonds, small peptides containing the affinity label will probably be sufficiently conformationally free that the affinity group can again bind to the original native protein. The antibody approach has been applied to the isolation of

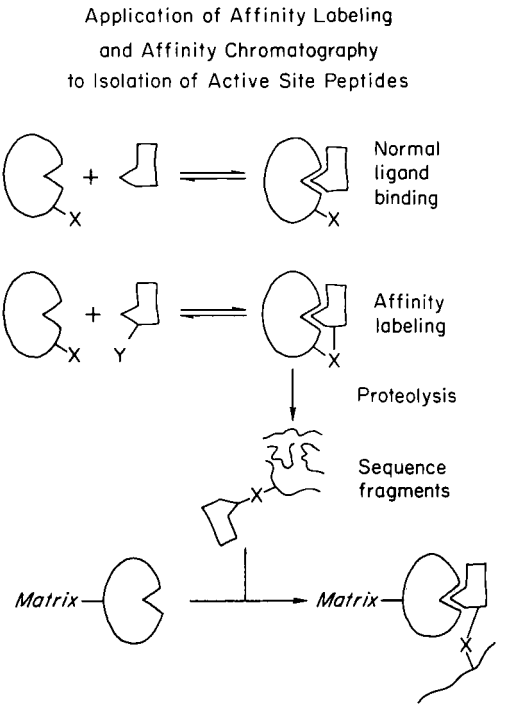


Figure 5: Application of affinity labeling and affinity chromatography to isolation of active site peptides.

specific peptides by modifying cysteinyl, methionyl, tryptophanyl, and tyrosyl peptides.[66,67] Ribonuclease, staphylococcal nuclease, anti-DNP antibodies and dinitrophenol-binding mouse myeloma protein have been reacted with affinity labels and peptides purified by affinity chromatography on the original native proteins, or close relatives.[67] These methods rely on the specificity of a modifying reagent and are thus inapplicable to the purification of synthetic peptides.

Chaiken[69] has demonstrated that purification of synthetic peptides may be conveniently done in the case of peptides overlapping a protein sequence that can be enzymically removed from that protein, *e.g.*, RNase S. This biospecific affinity chromatography then works through normal protein purification systems, specificity residing in the original conjunction of peptide and protein. The use of substrate analogs as ligands to purify synthetic enzymes has been applied.[70]

The methods of purification of binding proteins for peptide hormones and their identification as receptors by various criteria[71] was demonstrated by Cuatrecasas.[22,72]

Briefly, a purified plasma membrane preparation is extracted in a nondenaturing detergent, commonly non-ionic polyoxyethylene alkyls like Triton X-100. Specific peptide binding is removed by the solubilization from the membrane particles into the extract. An assay of soluble peptide-receptor complex is necessary, and should show approximate linearity of peptide bound to volume of extract added, and incorporate some nonspecific control more demanding than the dilution of the radioactive peptide with unlabeled peptide. There should be no cross reaction with relatively closely related but endocrinologically irrelevant peptides, *e.g.*, using oligo-lysine to block nonspecific ACTH binding.

Cuatrecasas was able to purify a preparation of insulin receptor up to 8000-fold by affinity chromatography. Obviously purification procedures of the power and specificity of affinity chromatography are required for purifications of solubilized receptors of this kind. As indicated by Catt,[71] only thousands of such molecules exist per cell, as little as 1 in 10^7 of the protein (by weight). Purifications of 100,000 to 1 million-fold are therefore required. It is not surprising that the isolation of receptors for acetylcholine from tissues of electric fish has proceeded much more rapidly and with generally more satisfactory characterization of the purified products[73] considering the amounts available in the comparable cases.

A factor of 100 to 1000 in material available and in purification leads quickly to the situation where radically new methods for the handling and characterization of the products are required.

An original apparent utility of affinity chromatography --a one-step purification--has not yet been realized in applications of this kind. Therefore, major advances in the purification of receptors for peptide hormones will probably depend on the development of more sensitive methods in protein chemistry and competitive binding assays, as well as in affinity chromatography. Many other recent advances in receptor studies have used approaches formulated in pharmacology, and the characterization of purified peptide hormone receptors will presumably benefit from biochemical criteria. In particular, number and size of peptide chain and identification and role(s) of subunits will be of great interest.

Acknowledgments

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QUANTITATIVE AFFINITY CHROMATOGRAPHY AND ITS APPLICATION TO STUDIES OF LIGAND BINDING BY SEMISYNTHETIC RIBONUCLEASE-S' ANALOGUES

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WHEREAS THE ORIGINAL DEVELOPMENT of affinity chromatography of proteins on insoluble matrices containing bound ligand has involved predominantly purification,[1] the establishment of this technique as a method for measuring quantitative parameters of protein-ligand interactions has been quite recent.[2,3] In our own laboratory[2,4] we have observed that competitive elution of a protein on inhibitor ligand - Sepharose by soluble competitive inhibitor or substrate can be implemented to measure the equilibrium constants for the binding of the protein with both soluble and matrix-bound ligands. This capability has suggested, among other features, that the technique could be applied in cases for which ligand-binding parameters are difficult or inconvenient to obtain by other procedures. Our protein chemical studies on semisynthetic ribonuclease-S' analogues[5-7] occasionally have been beset by such difficulties, especially for enzymatically inactive analogues.[6] In view of the importance of measuring binding parameters in such cases, we investigated the application of competitive elution ("quantitative affinity chromatography")[2] to study ligand binding for native ribonuclease-A, native ribonuclease-S, and semisynthetic ribonuclease-S'.

Competitive Elution Experiments for Ribonuclease A

Uridine 5'-(Sepharose-4-aminophenyl phosphoryl)-2'(3')-phosphate (Sepharose-APpUp) has been described previously[8]

as an affinity matrix capable of binding ribonuclease-A and thereby aiding in its purification. In spite of more recent findings[9,10] that suggest a certain amount of non-biospecific binding of ribonuclease to this matrix, we decided to use this documented species for our initial quantitative studies. We prepared Sepharose-APpUp with a procedure similar to that used previously,[8] by coupling 5'-aminophenyl phosphoryl-uridine-2'(3')-phosphate (generously supplied by Ash Stevens, Inc.) to Sepharose 4B (Pharmacia) which was previously activated by cyanogen bromide. The concentration of ligand sites capable of binding protein, determined as capacity for ribonuclease-A, was $1.8 \times 10^{-4} M$. This concentration is smaller than that of total coupled nucleotide, $1.2 \times 10^{-3} M$ (as determined by phosphate analysis).[11,12] The fact that the concentration of total ligand is considerably greater than that of accessible ligand is similar to the situation found previously for the staphylococcal nuclease-nucleotide-Sepharose system.[4]

As found in the initial ribonuclease affinity chromatography experiments,[8] we observed effective ribonuclease-A binding to Sepharose-APpUp at 0.02 and 0.1 *M* ammonium acetate, pH 5.2 and room temperature, and have carried out several experiments under these conditions. However, in view of the small but finite non-biospecific retardation of the inactive fragment ribonuclease-S-(21-124)[13] which we found even at 0.1 *M* buffer conditions, we have carried out more complete analysis at 0.4 *M* ammonium acetate, pH 5.2 and room temperature, where no retardation of ribonuclease-S-(21-124) is observed. At this higher ionic strength, the apparent non-biospecific interactions of ribonuclease with the affinity matrix appear to be largely eliminated.[14]

Figure 1 shows elution profiles of ribonuclease-A on Sepharose-APpUp in 0.4 *M* ammonium acetate, pH 5.2, containing various concentrations of the competitive ribonuclease inhibitor, 2'-cytidine monophosphate (2'-CMP). As expected, elution volume (V), defined as the volume at the peak fraction, decreases with increasing 2'-CMP concentration. We previously have derived the expression,

$$V = V_o + \frac{(V_o - V_m)\,\dfrac{[\overline{IM}]}{K_{\overline{IM}}}}{\left(1 + \dfrac{[I]}{K_I}\right)}, \qquad (1)$$

to describe the variation of V with the concentration of soluble ligand, [I], for such competitive elution data. Here,

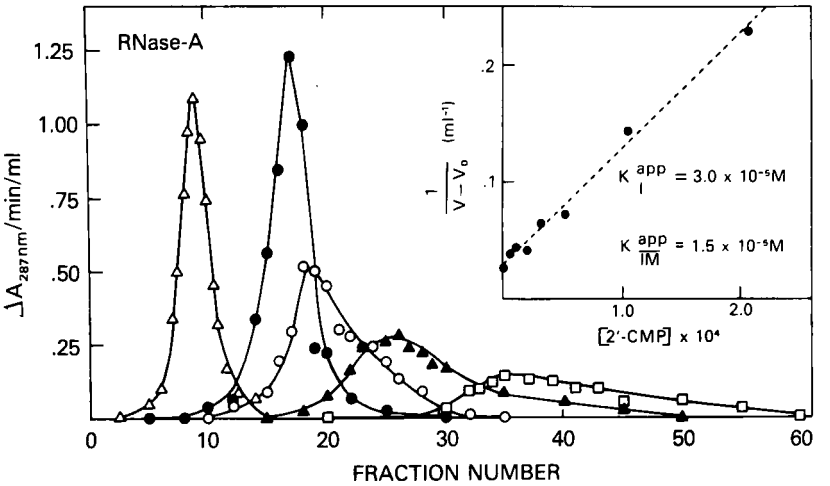

Figure 1: Competitive elution profiles of 2.8 mg bovine pancreatic ribonuclease-A on Sepharose-APpUp (5-ml bed volume) in 0.4 *M* ammonium acetate, pH 5.2 and room temperature, containing various amounts of 2'-CMP as follows: Δ - 2.1 x 10^{-4}*M*; ● - 5.2 x 10^{-5}*M*; ○ - 3.2 x 10^{-5} *M*; ▲ - 2.0 x 10^{-5}*M*; □ - 5.7 x 10^{-6}*M*. Fractions were 20 drops (1.15 ml). $\Delta A_{287\ nm}$/min/ml is enzymic activity measured against cyclic cytidine 2',3'-monophosphate as described previously.[6] Inset: plot of data according to equation (2), with the dotted line representing the best fit calculated by nonlinear least squares using equation (1). The equilibrium constants described by this fit are shown. For data obtained at 1.0 x 10^{-4}, 1.0 x 10^{-5}, and 0 *M* 2'-CMP, the derived points are included in the inset plot but the corresponding elution profiles omitted from the main figure for clarity.

V_o = volume at which protein elutes in the absence of interaction; V_m = void volume (determined by Blue Dextran 2000 elution); $[\overline{IM}]$ = concentration of immobilized ligand (determined as capacity for ribonuclease binding); $K_{\overline{IM}}$ = dissociation constant for interaction of protein with immobilized ligand; and K_I = dissociation constant for soluble ligand-protein interaction. The linear form of equation (1) is given by equation (2):

$$\frac{1}{V-V_o} = \frac{1}{(V_o-V_m)\frac{[\overline{IM}]}{K_{\overline{IM}}}} + \frac{[I]}{K_I\,(V_o-V_m)\frac{[\overline{IM}]}{K_{\overline{IM}}}} \cdot \qquad (2)$$

As shown in the inset to Figure 1, the variation of $1/(V-V_o)$ vs [I] is linear, a behavior predicted by equation (2). Nonlinear least squares fit of the data of Figure 1 to equation (1) using the MLAB computer program at the National Institutes of Health[15] gave values for K_I and $K_{\overline{IM}}$. These values, also given in Figure 1, indicate the bioaffinity nature of ribonuclease-A binding to Sepharose-APpUp (K_I and $K_{\overline{IM}}$ are close to the values expected from our own[14] and previous results[16]) and thus the ability to use competitive elution to measure binding parameters for ribonuclease-inhibitor interactions.

It should be noted that the calculated dissociation constants are expressed as apparent constants. This is done because the calculation of these parameters as described does not take into account the dependence on the amount of protein eluted. We have observed[14] this dependence, in 0.4 *M* buffer, to be slight but finite, and of a nature predicted by the theoretical considerations of Nichol *et al.*[3]

Ribonuclease-S and Semisynthetic Ribonuclease-S' Analogues

The competitive elution procedure was tested for native ribonuclease-S as well as for the fully enzymically active analogue [p-F-Phe^8]semisynthetic ribonuclease-S'.[5] As with ribonuclease-A, there was an inverse variation of net elution volume ($V-V_o$) with concentration of 2'-CMP in the elution buffer for these complexes. Figure 2 shows that the data obtained conform to the linearized expression of equation (2). The dissociation constants calculated, also given in Figure 2, indicate the substantial similarity of parameters for binding of both soluble and matrix-bound ligand to the native and active semisynthetic complexes. These parameters also are comparable to those determined for ribonuclease-A (Figure 1).

Since a major goal of this study was to demonstrate directly the feasibility of using the competitive elution technique to measure ligand binding properties of inactive ribonuclease derivatives, we carried out experiments with the inactive analogue, [4-F-His^{12}]semisynthetic ribonuclease-S'.[6,17] Due to the limiting amounts of this complex

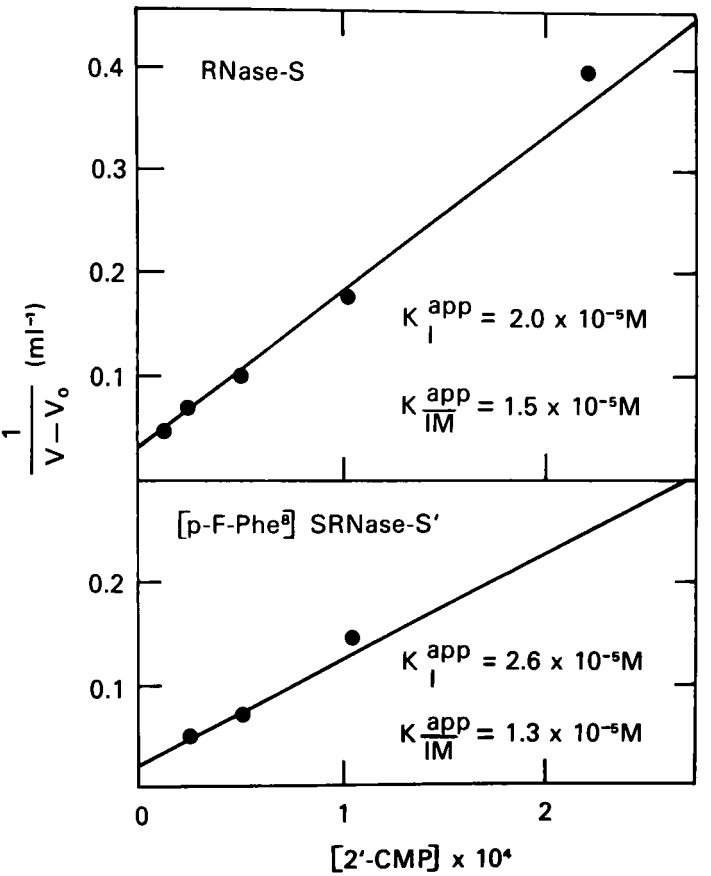

Figure 2: Competitive elution results for ribonuclease-S (RNase-S) and [p-F-Phe^8]semisynthetic ribonuclease-S' ([p-F-Phe^8]SRNase-S') plotted according to equation (2). Elution and column details were as described in the legend of Figure 1. Data were collected in the range of 2.2 x 10^{-4} to 1.2 x 10^{-5} *M* 2'-CMP for RNase-S and 1.0 x 10^{-4} to 2.5 x 10^{-5} *M* 2'-CMP for [p-F-Phe^8]SRNase-S'. The equilibrium constants derived by nonlinear least squares best fit (solid lines) are shown.

available to us at the time, we performed only single elutions, and with 0.7 to 1.0 mg of complex instead of the 2.8 mg used for the other experiments described. As shown in Figure 3A for a preparation of analogue complex containing excess ribonuclease-S-(21-124) fragment, the 4-F-His-containing complex elutes competitively with an elution volume of 0.4 *M* buffer similar to that for ribonuclease-A (also eluted at the lower amount of protein). The elution volume of the analogue complex is clearly distinct from that for ribonuclease-S-(21-124), indicating the specific retardation of the 4-F-His-containing complex. Similar results (Figure 3B) were obtained in a second experiment, carried out for a preparation free of excess ribonuclease-S-(21-124) at a lower ionic strength (under which conditions the differential elution of ribonuclease-A and ribonuclease-S-(21-124) is accentuated due to the stronger binding to ligand-matrix). The results of Figure 3 demonstrate the strong affinity of [4-F-His^{12}]semisynthetic ribonuclease-S' for ligands, a conclusion gratifying in view of our previous difficulty in quantitating this interaction.[6,17]

Conclusions

The results presented demonstrate that the method of quantitative affinity chromatography can be adapted for use in measuring ligand-binding properties of ribonuclease species which bind to Sepharose-APpUp. Some minor non-bioaffinity aspects of protein binding to the affinity matrix have been noted but can be dealt with adequately in order to observe the bioaffinity interaction. The data indicate the future utility of the technique to characterize ligand binding not only for [4-F-His^{12}]semisynthetic ribonuclease-S' but also for other ribonuclease variants. The results also confirm our view of the importance of the competitive elution approach of affinity chromatography for studies of associating systems in general.

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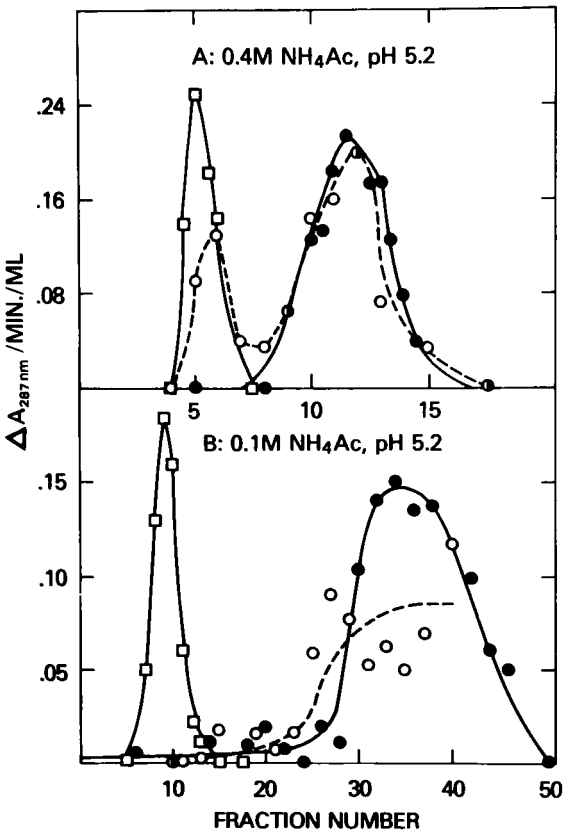

Figure 3: Competitive elution of [4-F-His^{12}]semisynthetic ribonuclease-S' and related species on Sepharose-APpUp with buffers containing 1.0 x 10^{-4} *M* 2'-CMP. Column and elution details were as described in the legend of Figure 1, except as follows. A: □---□, 0.6 mg ribonuclease-S-(21-124); ●---●, 0.7 mg ribonuclease-A; ○---○, 0.7 mg of an approximately 2:1 mixture of [4-F-His^{12}]semisynthetic ribonuclease-S' and ribonuclease-S-(21-124). B: □---□, 0.8 mg ribonuclease-S-(21-124); ●---●, 1 mg ribonuclease-A; ○---○, 1 mg [4-F-His^{12}]semisynthetic ribonuclease-S'. Activity was measured for ribonuclease-S-(21-124) and [4-F-His^{12}]semisynthetic ribonuclease-S' using an assay solution of cyclic cytidine-2', 3'-monophosphate containing an excess of ribonuclease-S-(1-20). For the [4-F-His^{12}] complex elutions, the activity values, which were low presumably due to competition by the [4-F-His^{12}]synthetic-(1-15) present, have been multiplied 8-fold in A and 10-fold in B for clarity.

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ABBREVIATIONS

The abbreviations suggested for amino acids and peptides (J. Biol. Chem. 247, 977, 1972), rules for naming synthetic modifications of natural peptides (ibid., 242, 555, 1967), nomenclature of peptide hormones (ibid., 250, 3215, 1975) and symbols for the description of the conformation of polypeptide chains (ibid., 245, 6489, 1970) were those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature. A number of frequently used abbreviations follow:

A II	Angiotensin II
A II'	Angiotensin II amide ([Asn^1]-Angiotensin II)
Ac	Acetyl
Acm	Acetamidomethyl
AcOH, HOAc	Acetic acid
ACTH	Adrenocorticotropin
Am	Ameletin
AP-M	Aminopeptidase-M
AVP	Arginine-vasopressin
Boc	*t*-Butyloxycarbonyl
Bpoc	2-(*p*-Biphenylyl)isopropyloxycarbonyl
Bu^tOCOCl	Isobutyl chloroformate
BTFA	Boron tristrifluoroacetate
Bu^t	*t*-Butyl
Bzh	Benzyhydryl
Bzl	Benzyl
CCD	Countercurrent Distribution
CD	Circular dichroism
CHO	Formyl
4-CH_3OBzl (MeOBzl)	4-Methyloxybenzyl
CP-A	Carboxypeptidase A
CNS	Central Nervous System
Cys(Cm)	*S*-Carboxymethylcysteine
DCC, DCCI	Dicyclohexylcarbodiimide
DCHA	Dicyclohexylamine
Ddz	α,α-Dimethyl-3,5-dimethoxybenzyloxycarbonyl
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
Dnp	2,4-Dinitrophenyl
Dns	Dansyl, 1-dimethylaminonäphthalene-H-5-sulfonyl
EEDQ	*N*-Ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoline
Et_3N, NEt_3	Triethylamine
EtOH	Ethanol

FDNB	1-Fluoro-2,4-dinitrobenzene
FSH	Follicle-stimulating hormone
<Glu	Pyroglutamic acid, pyrrolid-2-one-5-carboxylic acid
GnRH	Gonadotropin releasing hormone (LHRH)
HBT,HOBt	1-Hydroxybenzotriazole
HGH	Human growth hormone
HOAc, AcOH	Acetic Acid
HONSu, HOSu	*N*-Hydroxysuccinimide
HPT	Hexamethyl phosphoric acid triamide
Hyp	Hydroxyproline
IR	Infrared spectroscopy
i.v.	Intravenous (injection)
LAP	Leucine aminopeptidase
LH	Luteinizing hormone
LVP	Lysine-vasopressin
MA	Carbonic acid mixed anhydride (method)
Mbh	4,4'-Dimethyloxybenzyhydryl
Me, OMe	methyl, methyl ester
MeOBzl, 4-CH_3OBzl	*p*-Methyloxybenzyl
MeOH	Methanol
MRF	Melanotropin releasing factor
MIF, MRIF	Melanotropin-release inhibiting factor
MSH	Melanocyte-stimulating hormone, melanotropin
NHH	Neurohypophyseal hormone
N^{π}-MeHis	*pros* Methylhistidine (*N* nearer to C^{β})
N^{τ}-MeHis	*tele* Methylhistidine (*N* away from C^{β})
NMR, nmr	Nuclear magnetic resonance spectroscopy
Nps	*o*-Nitrophenylsulfenyl
OBt	1-Hydroxybenzotriazole ester
OBzl	Benzyl ester
OBu^t	*t*-Butyl ester
OEt	Ethyl ester
OMe	Methyl ester
ONb	*p*-Nitrobenzyl ester
ONo	*o*-Nitrophenyl ester
ONp	*p*-Nitrophenyl ester
ONSu	*N*-Hydroxysuccinimide ester
OPcp	Pentachlorophenyl ester
OPfp	Pentafluorophenyl ester
ORD	Optical rotatory dispersion spectroscopy
Pht	Phthalyl
PMR	Proton magnetic resonance spectroscopy
RNase S	Ribonuclease S
SPS, SPPS	Solid phase peptide synthesis

TFA	Trifluoroacetic acid
Tfa	Trifluoroacetyl
THF	Tetrahydrofuran
Tlc, tlc	Thin-layer chromatography
Tos	Tosyl,*p*-toluenesulfonyl
TRH, TRF	Thyrotropin-releasing hormone
Trt	Trityl, triphenylmethyl
TSH	Thyroid-stimulating hormone, Thyrotropin
UV	Ultraviolet
Z	Benzyloxycarbonyl

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