Progress in Peptide Research

Volume II

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Gordon and Breach
New York • London • Paris

GORDON AND BREACH, SCIENCE PUBLISHERS, INC.
440 Park Avenue South
New York, N.Y. 10016

Editorial office for the United Kingdom

GORDON AND BREACH, SCIENCE PUBLISHERS, Ltd.
12 Bloomsbury Way
London W.C.1

Editorial office for France

GORDON & BREACH
7-9 rue Emile Dubois
Paris 14e

Library of Congress catalog number 76-153298. ISBN 0 677 13610 2.

Printed in the United States of America.

PREFACE

The proceedings of the 2nd American Peptide Symposium are presented in this volume. The meeting, held at the Cleveland Clinic, August 17-19, 1970, was attended by more than 200 chemists with a mutual interest in all aspects of the physical, chemical and biological behavior of peptides. American chemists were joined by colleagues from Austria, Canada, Denmark, England, France, Germany, Italy, Israel, Japan and the Netherlands, thus attesting to the international scope of the meeting. The present volume is a permanent record of world-wide research activities in peptide chemistry for 1970.

Since arbitrary limits were not placed on subject matter beforehand, the symposium program, and the papers published here, reflect current international trends in peptide chemistry. Fifteen papers dealt with aspects of solid phase synthesis: the development of new supports, means of monitoring reactions, descriptions of new equipment, and applications of the technique to the synthesis of biologically active peptides. Methods of analyzing peptides for sequence, purity, or racemization were described in nine papers on automated Edman techniques, applications of mass spectrometry, and gas or ion exchange chromatography. Four papers on experimental or theoretical approaches to the study of peptide conformation were also given. As might be expected, interest in peptides displaying biological activity continues to run high. Nineteen papers reported synthesis, characterization or structure-activity studies of corticotropin and melanotropin analogues, thyrotropin releasing factor, neurohypophyseal peptides, calcitonins, angiotensins, enzyme inhibitors, bradykinin potentiators, caerulein peptides, scotophobin, mellitin and neocarzinostatin. Certain specialized problems in peptide chemistry were treated in studies on the mixed anhydride procedure, on preparation of bis-cystinyl peptides, on specific N^{ε}-acylation techniques and on reactions of peptides containing α-β unsaturated amino acids.

The caliber and quantity of papers as well as the number of participants at the meeting seem to indicate that the American symposium performs a useful service to peptide chemists. Accordingly, plans are being layed for a third symposium to be held in early summer of 1972.

Saul Lande

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* Contents are arranged according to the program of the Symposium

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New Solid Supports for the Solid Phase Synthesis of Peptides

E. Bayer, E. Breitmaier, G. Jung and W. Parr

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Houston, Texas

Manuscript not received

A POLYMERIC REAGENT FOR PEPTIDE SYNTHESIS: O-HYDROXYNITROPHENYL DERIVATIVE OF POLYSTYRENE

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It is known that the stepwise coupling of amino acids (eq 1) may be accelerated to completion by the use of excess

$$R\text{-}CO\text{-}X + NH_2\text{-}R' \longrightarrow R\text{-}CO\text{-}NH\text{-}R' + HX \qquad (1)$$

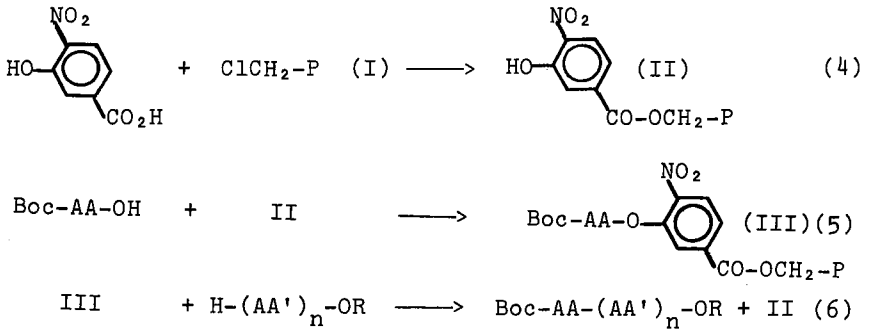
molar quantities of either R-CO-X or R'-NH_2. The desired product may be isolated by filtration or centrifugation if either reagent is an insoluble polymer (eqs 2,3). Reaction 2 corresponds to the well known Merrifield solid phase method.[1]

$$R\text{-}CO\text{-}X \text{ (excess)} + NH_2\text{-}R'\text{-}P \longrightarrow R\text{-}CO\text{-}NH\text{-}R'\text{-}P + HX \qquad (2)$$

$$R\text{-}CO\text{-}X\text{-}P \text{ (excess)} + NH_2\text{-}R' \longrightarrow R\text{-}CO\text{-}NH\text{-}R' + HX\text{-}P \qquad (3)$$

Reaction 3 has been successfully applied by Katchalski et al in the preparation of cyclic[2] and linear[3] peptides, by Blout et al,[4] and other groups.[5] The corresponding polymeric components of activated amino acids (P-XH, eq 3) which were previously used in peptide synthesis, include cross-linked poly-4-hydroxy-3-nitrostyrene,[2,3] branched copoly [DL-lysine-3-nitro-L-tyrosine],[2] linear and cross-linked copoly [ethylene-N-hydroxymaleimide],[4] and others.[5]

It appeared to us that in each of the above polymeric reagents (i) no substitutions in reactive functional groups are feasible, (ii) the distance between the reactive center and macromolecular backbone is fixed, (iii) there are no uniform methods of controlling the extent of reactive group substitution, and in some cases, (iv) no continuous controls of macromolecular cross-linking are available. In an attempt to provide access to these controls, and to demonstrate the feasibility of our approach, we wish to report (i) a general preparation of an insoluble polymeric reagent similar to the known[2,3] poly-4-hydroxy-3-nitrostyrene (eq 4), (ii) synthesis of corresponding polymeric amino acid active esters (eq 5), and (iii) stepwise synthesis of a tetrapeptide using the above polymeric reagents as intermediates (eqs 3,6).



Polymeric o-nitrophenol (II, eq 4) was prepared by treating chloromethylated copoly [styrene-2% divinyl benzene] with a solution of 3-hydroxy-4-nitrobenzoic acid and triethylamine in ethanol.[1] The suspension was stirred at 75° for 65 hrs, and the polymeric product, II, was filtered off, washed with ethanol, water, methanol, and dried in vacuo (II). The infrared spectrum of II (KBr pellet, Perkin Elmer 225 spectrophotometer) included characteristic bands of H-bonded phenolic OH 3250 cm^{-1} (w), phenylbenzoate ester 1718 cm^{-1} (s), and NO_2 group 1582 cm^{-1} (s).

Polymeric o-nitrophenyl esters of Boc-D-Phe-OH, Boc-Leu-OH, and Boc-Ala-OH (III, eq 5) were prepared by the dicyclohexylcarbodiimide (DCC) method.[6,2,3] Polymer II was suspended in a solution of Boc-D-Phe-OH and DCC in methylene chloride. The suspension was stirred for 18 hrs at room temperature, and the polymeric active ester, III, was filtered off, washed with water, ethanol, methanol, methylene chloride, and dried in vacuo (III). The infrared spectrum of polymer III (KBr pellet) included characteristic bands of amino acid phenyl ester, 1770 cm^{-1} (m), and Boc group 1360, 1385 cm^{-1} (w). Polymeric esters of Boc-Leu-OH and Boc-Ala-OH were similarly prepared.

Use of polymeric o-nitrophenyl esters as intermediates in synthesis of peptides listed in Table I (eq 6) was made in the following way. Polymeric active ester of Boc-amino acid (ca. 1.5 equiv) and amino peptide hydrochloride (1 equiv) were treated with triethylamine (1 equiv) and stirred in dry methylene chloride for ca. 9 hrs at room temperature. The polymeric by-product, II, was filtered and washed with methylene chloride. The combined filtrates were extracted with water, dried and flash-evaporated. The chromatographically pure products were once crystallized from ethyl acetate-hexane. An improved yield was obtained in one instance when the reaction was carried out in dimethylformamide. Removal of the Boc- protecting group from intermediate peptides was carried out with anhydrous HCl in dimethoxyethane.[4] Table I summarizes the results of stepwise syntheses of the tetrapeptide Boc-Ala-Leu-D-Phe-Gly-OBzl with polymeric o-nitrophenyl esters, N-Hydroxysuccinimide (HOSu) esters,[7,8] and mixed anhydride[9] intermediates.

Table I - Peptides Synthesized with Polymeric and Conventional Methods[a]

Peptide	Method of Activation	mp[b] °c	Yield,% Crude	Cryst.	$[\alpha]_D^{25}$[c]
Boc-D-Phe-Gly-OBzl	Polymeric Ester	131-133	72	48	+25
"	HOSu Ester	"	73	60	+25
"	Mixed Anhydride	"	84	78	---
Boc-Leu-D-Phe-Gly-OBzl	Polymeric Ester	160-162	50	23	+12
"	Polymeric Ester[d]	161-162	62	58	---
"	HOSu Ester	"	64	54	+12
"	Mixed Anhydride	"	89	80	---
Boc-Ala-Leu-D-Phe-Gly-OBzl	Polymeric Ester	110-111	61	41	-14
"	HOSu Ester	"	76	59	-14
"	Mixed Anhydride	"	83	76	---

a. All peptides were authenticated with C,H,N analyses and infrared spectra.
b. Melting points were determined on a Kofler block and are uncorrected.
c. Specific rotations were measured in ethanol (c 1).
d. Coupling reaction was carried out in dimethylformamide.

The results reported above demonstrate the feasibility of our general approach. The use of these polymeric o-nitrophenyl esters may serve as a satisfactory alternative to conventional methods of peptide synthesis, particularly in reactions which require difficult work-up procedures. The advantages of using polymer I in preference to previously reported macromolecular starting materials are: (1) It may be attached to other potential coupling reagents which correspond to N-hydroxysuccinimide[7] and N-hydroxyphthalimide[10] esters, alkylchloroformates,[9] and dialkylcarbodiimides.[11] (2) Chloromethylated polystyrene may be coupled to reagents of type $X-(CH_2)_n-Y$, thus providing a variable distance between the reactive center and the polymeric backbone, a potentially critical factor in coupling reactions.[12] (3) The extent of reactive group substitution on the macromolecule is controlled by chloromethylation and esterification-reactions which have been widely used in the Merrifield solid phase method[1] under a variety of conditions.

Acknowledgement. We appreciate the financial support of the Public Health Service, Grant GM-15538.

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DUAL FUNCTION SUPPORTS IN SOLID PHASE PEPTIDE SYNTHESIS

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Over the past several years, a number of natural peptide products have been isolated and characterized (1) which have either cyclic structures or amide function at their C-terminus.

In the synthesis of peptide amides, amomonolysis of simple alkyl or aryl esters is usually slow. Problems encountered in the synthesis of cyclic peptides include the synthesis of the open chain from its constituent units and secondly, the ring closure usually by activation at the carboxyl end. Application of the dilution principle results in intramolecular amide formation. Frequently, however, the desired products are contaminated with linear oligomers as well as with other cyclic products.

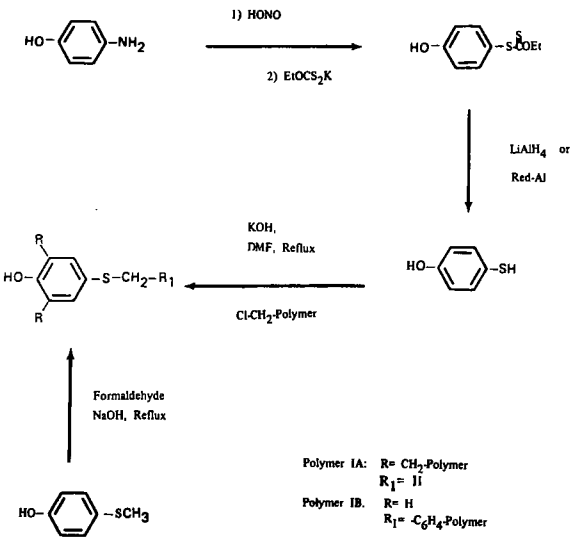

Fig. I. Synthesis of MTP Supports.

Polymeric supports have been demonstrated to be extremely useful in peptide synthesis for carboxyl protection as exemplified by the work of Merrifield (2) and for activation as shown by Fridkin (3) and Laufer (4). Recently supports have been introduced which allow the specific synthesis of peptide hydrazides (5) and amides (6) respectively. In a similar fashion, peptides have been removed easily as their hydrazides and amides from a polyphenol support (12). The modification of a support during synthesis to change its functional characteristics such as lability has been conceived; and such a polymer termed a Dual Function support.

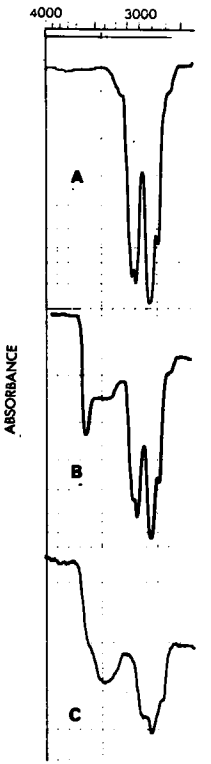

Fig. II. Infrared spectra of chloromethyl polymer (A), Phenol-Sulfide Polymer (B), Resitol Type-MTP polymer (C).

The 4-(methylthio)phenyl (MTP) group of Johnson and Jacobs (7) which is easily converted to the reactive 4-(methylsulfonyl)phenyl (MSP) group of Schwyzer (8) has been incorporated into two polymers, IA and IB. As shown in Fig. I. the diazonium salt of p-Aminophenol was treated with the potassium salt of ethyl xanthic acid. Subsequent reduction of the xanthate by $LiAlH_4$ or by Red-Al gave the p-mercaptophenol in 60-80% yields. Reaction of this product with the chloromethyl polymer containing an equivalent

amount of chlorine, in the presence of DMF and 2.0 equivalents of KOH gave the phenol-sulfide polymer, IB. The completeness of the reaction was monitored in the I.R. as shown in Fig. II. Bands at the 3500 cm^{-1} (s) are due to the hydroxy moiety.

Polymeric MTP esters (see Fig. III.) of protected amino acids were prepared by the dicyclohexylcarbodiimide (DCC), carbonyldiimidazole (CDI),

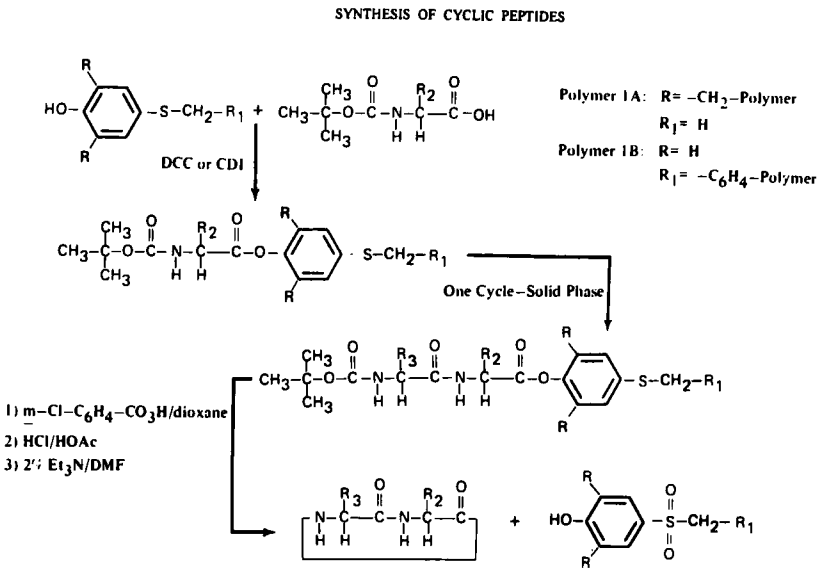

Fig. III. Synthesis on Dual Function Supports.

mixed anhydride and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) methods using CH_2Cl_2 or THF as solvents. When equal molar quantities of all reactants were used, the coupling efficiency was as follows: DCC>CDI>EEDQ>mixed anhydride. The extent of substitution on the polymers was estimated by amino acid analyses, the characteristic bands of Boc-α-amino acid phenyl esters (1752 (s), 1720cm^{-1} (s); KBr discs), and by chlorine analysis.

As shown in Fig. III., chain elongation to desired lengths were carried out according to Marshall and Merrifield (9). Cleavage of the ester link was observed occassionally when 1N HCl/HOAc was used for deprotection. In recent experiments, 20% TFA/CH_2Cl_2 has been used. The polymer could be activated by oxidation to the sulfone using either 30% H_2O_2/HOAc or 3 equivalents of m-chloroperbenzoic acid in dioxane (10). Complete conversion to the sulfone was followed using the infra-red bands at 1139 (s) and 1310 cm^{-1} (s) due to the unsymmetrical and symmetrical stretching of the SO_2 group respectively. By comparing the ratio of the absorbance of the standard aromatic stretching peak at 1602 cm^{-1} with that of the symmetrical sulfone stretch at 1139 cm^{-1} the extent of oxidation could be estimated. It can be seen from Fig. IV. that m-chloroperbenzoic acid is a much more efficient oxidizing agent. Losses of 2-12% of the peptide chain were

Table I Cyclic Peptide Products*

Product	mp. °C.	R_f (I)	R_f (II)	% Yield IA	% Yield IB
c-(Ala-Gly)	238-239	.59	.46	23	52
c-(Gly-Gly)	308-309	.51	.35	40	63
c-(Gly-Phe)	261-265	.71	.66	21	42
c-(Ala-Ala-Ala-Gly)	279-280**	.57	.49	-	40
c-(Gly-Val-Ala-Phe-Ala-Gly)	209-210	.64	.58	-	50

* All melting points are uncorrencted. Thin layer chromatography was carried out on Silica Gel G. Solvent Systems: (I) n-Butanol:HOAc:HOH (4:1:1); (II) sec-Butanol: EtOAc:HOH (65:60:25). Yields are based on amino acids initially esterified to the polymer. Amino acid analyses and molecular ions from mass spectrometry are consistent with the proposed structures.

** Decomposition.

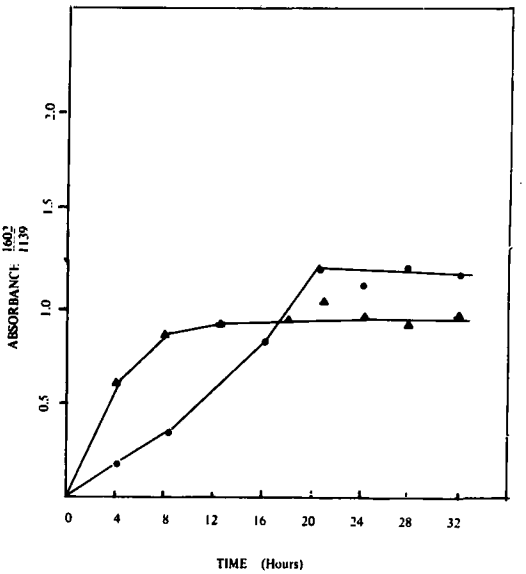

Fig. IV. Time study of oxidation of sulfide support (MTP) to activated sulfone (MSP) support. 30% H_2O_2 in HOAc (4:6); 4 equivs of m-chloroperbenzoic acid in dioxane.

detected when 30% H_2O_2 /HOAc was used. Fig. V. shows an I.R. comparison of unsubstituted Merrifield resin as well as the esterified modified supports.

Removal of the peptide chain from the support is accompanied by several means. Cyclic peptide products can be synthesized using the activated support by removal of the N-terminal protecting group. Intramolecular condensation is accomplished by neutralization of the protonated amine with Et_3N in DMF for 18-24 hours. Evaporation of the solvent and recrystallization of the solid from appropriate solvents yielded chromatographically pure cyclic peptides. The cyclic products gave negative ninhydrin tests in the tlc, but were identified with starch-hypochlorite-KI (11). Results of several preparations are shown in Table I. Low yields on IA may reflect the presence of the two orthomethylene groups or the degree of cross-linking in the polymer. Table II summarizes other procedures employed to remove peptides from the supports. As was shown by Inukai (12) the un-oxidized polymeric esters are readily reactive toward NH_3/MeOH, NH_2NH_2/DMF, to sponification or to transesterification.

The tetrapeptide of gastrin was synthesized in comparatively low yield due to the considerable amount of cleavage of the phenyl ester linkage when the β-Benzyl ester was removed from the aspatate residue by HBr/TFA at 0°C for 30 minutes. Subsequent removal by NH_3/MeOH gave only 25% overall of the expected yield. D. L. Marshall (13) has removed protected peptides from the activated form of polymer IB by acylation with an amino acid salt thus lengthening the chain by one amino acid at the C-terminal end. In a similar fashion peptides have been removed as thiophenyl esters.

TABLE II

REMOVAL FROM PHENOL-SULFIDE SUPPORTS

Nucleophile	Product	Support	Time	% Yield
NH_2NH_2/DMF	t-Boc-L-Tyr(OBz)-L-Val-L-Phe-$NHNH_2$	MTP,,IB	34	60
NH_3/MeOH-DMF	t-Boc-L-Val-NH_2	MTP, IA	36	40
NaOH/EtOH-DMF	t-Boc-L-Pro-L-Pro-L-Ala-L-Phe-OH	MTP, IB	5	59%
EtOH/Et_3N	t-Boc-L-LeuOEt	MTP, IA	8	51%
NH_3/MeOH-DMF	L-Try-L-Met-L-Asp-L-Phe-NH_2	MTP, IB	36	25

REMOVAL OF PEPTIDES FROM ACTIVATED SUPPORTS

NH_2NH_2/DMF	t-Boc-L-Tyr(OBz)-L-Val-L-Phe-$NHNH_2$	MSP, IB	6	Quan.
L-PheOMTP/DMF	t-Boc-L-Asp(OBz)-L-PheOMTP	IA	30	41
C_6H_5-SH DMF-H_2O	t-Boc-L-Leu-SC_6H_5	IB	18	70
NH_3/MeOH-DMF	Glu-L-His-(ImBz)-L-Pro-NH_2	IB	40	73

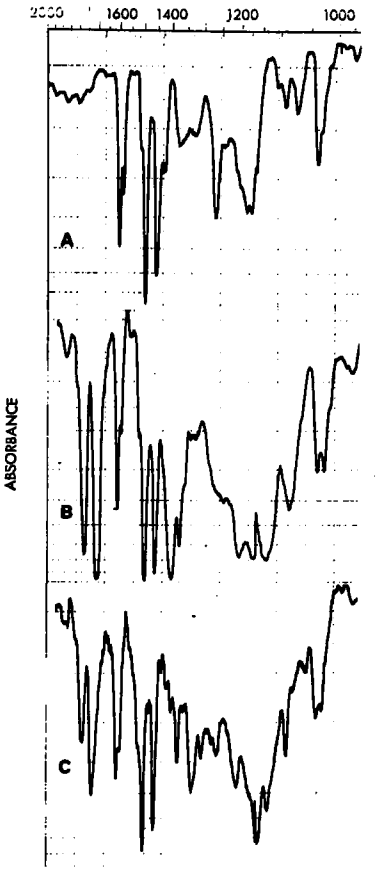

Fig. V. Infrared spectra of chloromethyl polymer (A), 5-Boc-Phe-phenyl-sulfide polymer, IB (B), t-Boc-Phe-phenyl-sulfonyl polymer, IB (C).

Attempts to remove a decapeptide from the resin as its cyclic product has resulted in cleavage from the polymer to give the linear product. Thus, the removal of peptides by thiophenyl esters, or amino acid MTP esters promise still another possible route to cyclic peptides, since the thiophenyl esters are also very reactive.

While the oxidative or basic conditions of this system limit its applications, a number of biologically active peptide can be synthesized on this support. Protected intermediates, such as hydrazides or active esters can also be prepared. The advantages of the Dual Function support include higher overall yields, a minimization of racimization during activation and subsequent cyclization as well as the normal conveniences of the solid phase method.

Support by NIH Grants AM13025 and GM714 is acknowledged.

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Angiotensin II: A Competitive Inhibitor and Analogs With Restricted Conformations

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Angiotensin II has been the subject of intensive investigation due to its probable role in the normal regulation of blood volume and possible pathological role in the etiology of various hypertensive states. This vasopressive peptide contracts a wide variety of smooth muscle preparations and releases catecholamines and aldosterone, in addition to having a probable intrarenal role and possible effects on the CNS. Recent studies in our laboratory have resulted in a specific competitive inhibitor of angiotensin II as well as active analogs in which the possible conformations of the peptide backbone have been severely limited by chemical modification.

The peptides described were prepared by solid phase peptide synthesis. Two modifications from the original synthesis of angiotensin II by this procedure (1) were incorporated. The im-dinitrophenyl group of Shatiel (2) was used for histidine protection and was removed by treatment with 25-fold excess of thiophenol in DMF for 1 hour (J. M. Stewart, personal communication) prior to cleavage from the support by HF, which was the other major modification. The peptides were purified by partition column chromatography on Sephadex-G-25 according to Smeby et al. (3).

Khairallah and co-workers (4) have reported an analog of angiotensin II, [5-Ile,8-Ala]-angiotensin II, which antagonized the action of angiotensin on guinea pig ileum strips at high doses [500 ng/ml], but had an activity equivalent to 0.1% that of angiotensin II when assayed at greater dilution. This compound showed only agonistic activity in pressor assays in vivo or when tested on isolated rat uterus. Jorgensen and co-workers (5) have found a cyclic hexapeptide which showed angiotensin-like activity on guinea pig ileum, but is inactive in other assays.

[4-Phe,8-Tyr]-angiotensin II, on the contrary, competitively inhibits the action of angiotensin both on isolated rat uterus and in the in vivo pressor assay (6). Fig. I shows the 100-fold shift in the angiotensin dose-response curve seen in the uterus assay in the presence of 10 µg/ml of the inhibitor. The inhibitor is easily washed out and the dose-response curve returns to normal. A similar shift in the angiotensin dose-response curve is also seen in the presence of 10ng/gm inhibitor in the in vivo pressor assay both in normotensive and in genetically hypertensive animals. The specificity of the inhibitor was then examined. In rat uterine strips, [4-Phe,8-Tyr]-angiotensin II did not alter any portions of the dose-response curve to bradykinin, oxytocin, vasopressin, or serotonin. In addition,

the in vivo vasopressor response to epinephrine, and the vasodilator response to nitroglycerin were not altered.

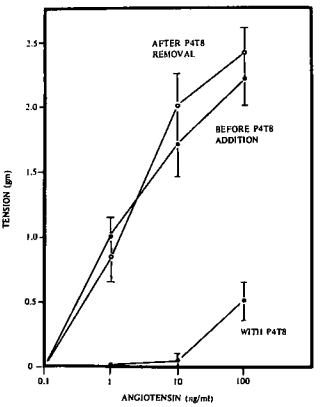

Figure 1
Effect of [4-Phe,8-Tyr]-angiotensin II
on the dose-response relationship for angiotensin II-induced contraction of isolated rat uterus strips.

Reciprocal plots of contraction of rat uterine strips against angiotensin II in the presence of various concentrations of inhibitor are shown in Fig. 2. The linear plots and common intersection with the vertical axis are consistent with the hypothesis that the antagonist competes with angiotensin for an active site with an affinity of 1/36th that of angiotensin II. At higher concentration, the inhibitor shows an intrinsic activity which is less than 0.005% that of angiotensin II in both uterine and pressor assays.

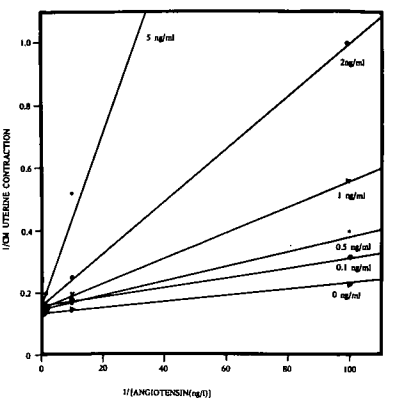

Figure 2
Reciprocal plot of uterine strip response
versus angiotensin concentration in the
presence of different levels of [4-Phe,8-Tyr]-angiotensin II

Consideration of other analogs, [4-Phe]-angiotensin II and [8-Tyr]-angiotensin II, which have 10% and 83% of the biological activity of angiotensin respectively, makes the inhibitory activity of [4-Phe, 8-Tyr]-angiotensin II somewhat surprising. In addition, the report that [1-Asn, 4-Phe, 8-Tyr]-angiotensin II has 0.2% activity, with no report of inhibition, makes it clear that the molecular requirements for competitive antagonism require further study.

In order to define the biologically active conformation of angiotensin II and other peptide hormones, we have investigated chemical modifications of the peptide backbone which would restrict the sterically-allowed conformations available to the peptide. Our attention was originally directed to the replacement of the proton with a methyl group, as N-methyl amino acids are quite prevalent in cyclic antibiotics; two good examples would be enniatin B and actinomycin D. The effect of the substitution on glycine, alanine, and valine with an N-methyl group on the sterically-allowed area as shown in Ramachandran plots has been calculated (7). In addition, the replacement of the alpha proton with a methyl group has also been examined. Only angles corresponding to a right- and left-handed helix are allowed for α-methyl amino acids in peptide linkage (8). The results are summarized in Table I.

Table I
STERICALLY-ALLOWED AREAS FOR THE PEPTIDE BACKBONE
FOR VARIOUS AMINO ACID DERIVATIVES

Residue	Total Possible Area	Allowed	%
Gly	1024	559	54
N-Me-Gly	1024	298	29
Ala	1024	184	18
N-Me-Ala	1024	93	9
Val	1024	170	16
N-Me-Val	1024	45	4.4
N-Me-Leu	1024	92	9
N-Me-Ile	1024	47	4.6
α-Me-Ala	1024	10	1
α-Me-Ala	4096	32	0.78
α-Me-Ala	16384	122	0.75

Substitution of α-methyl alanine [α-aminoisobutyric acid, Aib] for the two valines in angiotensin II does not abolish angiotensin activity. [3-Aib]-angiotensin II and [5-Aib]-angiotensin II both show 1% of the biological activity of angiotensin II in the rat uterine assay. The activity is not as high as one might have expected when compared with other analogs of angio-

tensin with substitution in the 3 and 5 positions (Table II). This could be explained by many hypotheses. Two which we favor are: (1) the biologically active conformation has angles near, but not identical to the helical angles which the Aib substitution requires; (2) the accommodation of the α-methyl group has required movement of other parts of the peptide chain slightly from the optimal arrangement. Preparation of other analogs such as the 3- and 5- N-methyl valine derivatives may help the interpretation of the above observations.

Table II
ANGIOTENSIN II ANALOGS
IN THE 3 AND 5 POSITIONS

	% Activity
Asp-Arg-Val-Tyr-Aib-His-Pro-Phe	1
Asp-Arg-Aib-Tyr-Val-His-Pro-Phe	1
Asn-Arg-Leu-Tyr-Ile-His-Pro-Phe	100
Asn-Arg-Val-Tyr-Leu-His-Pro-Phe	25
Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe	35
Asp-Arg-Val-Tyr-Ala-His-Pro-Phe	7.5
Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe	40

†Established Investigator, American Heart Association (AHA 70-111), supported by the National Institutes of Health (AM-13025).

*Predoctoral trainee in neurobiology (NS-05613).

‡Graduate Fellow, Medical Scientist Training Program (GMO-2016).

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PEPTIDE SYNTHESIS BY MEANS OF t-BUTYLOXYCARBONYL AMINO ACID DERIVATIVES OF COPOLY-(ETHYLENE-N-HYDROXYMALEIMIDE)

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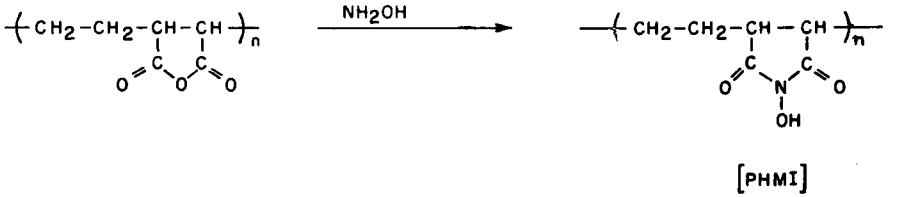
The successful use of polymeric reagents in the synthesis of peptides has been described previously. [1] The reagents used were high molecular weight, insoluble, active esters derived from cross-linked polymers, such as poly-4-hydroxy-3-nitrostyrene and N-blocked amino acids. High yields of N-and C-blocked peptides were obtained on coupling amino acid or peptide esters, possessing free α-amino groups, with excess of the insoluble poly-functional reagent. The newly formed peptides could be separated readily from the insoluble reagent, and elongated after removal of the N-blocking group and coupling with an insoluble polyfunctional active ester of a desired N-blocked amino acid.

One of the main difficultes encountered in peptide synthesis according to the above procedure is the relatively low reactivity of the polmeric active esters of N-t-butyloxy-carbonyl (t-Boc)-amino acids. [2] Moreover, long reaction times and a large excess of polymeric reagent were required to ascertain completion of the coupling reaction with amino acid or peptide derivatives containing bulky side chains. [2] In an attempt to overcome these difficulties N-t-Boc-amino acid esters of a cross-linked copoly-(ethylene-N-hydroxymaleimide) were prepared and their use in peptide synthesis and aminoacyl transfer reactions was investigated. [3] Because of the well known high activity of the N-hydroxysuccinimide esters of t-Boc-amino acids, [4] high activity of the corresponding polymeric esters might have been predicted.

Copoly-(ethylene-N-hydroxymaleimide), [PHMI], was prepared by reacting copoly-(ethylene-maleic anhydride) with hydroxylamine hydrochloride in a mixture of water and pyridine at room temperature (see Scheme I).

SCHEME I Synthesis of copoly-(ethylene-N-hydroxymaleimide)

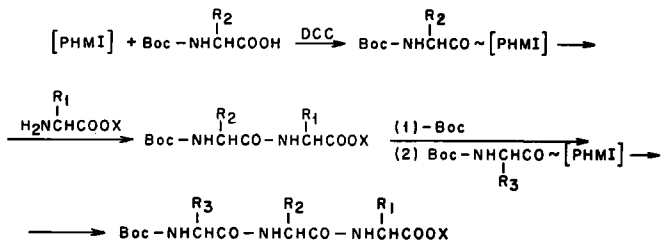


SCHEME I
Synthesis of copoly-(ethylene-N-hydroxymaleimide)

Cross-linking was effected by means of hexamethylene diamine, hydrazine, spermidine or spermine. The structure of PHMI was ascertained by infrared analysis. The absorption bands at 1780-1790 cm^{-1} and 1710 cm^{-1} (nujol) revealed the presence of the two carbonyls of the cyclic imide. These replaced the absorption bands at 1870 cm^{-1} and 1775-1785 cm^{-1} (nujol) of the starting ethylene-maleic anhydride copolymer. The cross-linked PHMI polymers synthesized are insoluble in water, methanol, eth anol, chloroform, acetonitrile, dimethylformamid (DMF), and acetic acid. All of them swell in DMF in accord with the degree of cross- linking and the nature of the cross- linking agent. The most suitable polymers for the synthetic procedures to be described below were those obtained by using spermidine (2-5%), spermine (2-4%), or hydrazine (5-10%) as the cross-linking agent. PHMI-esters of different t-Boc amino acids were prepared by coupling the appropriate components in DMF or mixtures of DMF and acetonitrile, using dicyclohexylcarbodiimide (DCC)[4] as the coupling agent. The amount of the amino acid derivatives bound to the polymer (1.0-1.7 mmole per g) was derived from the increase in weight due to esterification, or from amino acid analysis after acid hydrolysis.[1b]

The preparation of t-Boc-amino acids- PHMI esters and their use in peptide synthesis is summarized in Scheme II.

SCHEME II Peptide synthesis with the aid of t-butyloxycarbonyl amino acid esters of copoly-(ethylene-N-hydroxymaleimide)



SCHEME II
Peptide synthesis with the aid of t-butyloxycarbonyl amino acid esters of copoly-(ethylene-N-hydroxymaleimide)

The synthesis of the heptapeptide L-Ser-L-Pro-L-Cys-L-Ser-L-Glu-L-Thr-L-Tyr, corresponding to residues 159-165 of bovine

carboxypeptidase A, by means of the polymeric active esters listed in Table I.

Table I. N-Butyloxycarbonyl amino acid esters of cross-linked copoly-(ethylene-N-hydroxymaleimide)

Compound bound to polymer [a]	mmoles of amino acid bound per g of polyester [d]
N-Boc-L-Thr [b]	1.5
N-Boc-(γ-Bzl)-L-Glu [b]	1.45
N-Boc-(O-Bzl)-L-Ser [c]	1.5
N-Boc-(S-Bzl)-L-Cys [b]	1.6
N-Boc-L-Pro [b]	1.3
N-Boc-L-Ala [c]	1.4

[a] Binding was effected in DMF by the DCC method.

[b] Copoly-(ethylene-N-hydroxymaleimide) cross-linked with 10% hydrazine was used.

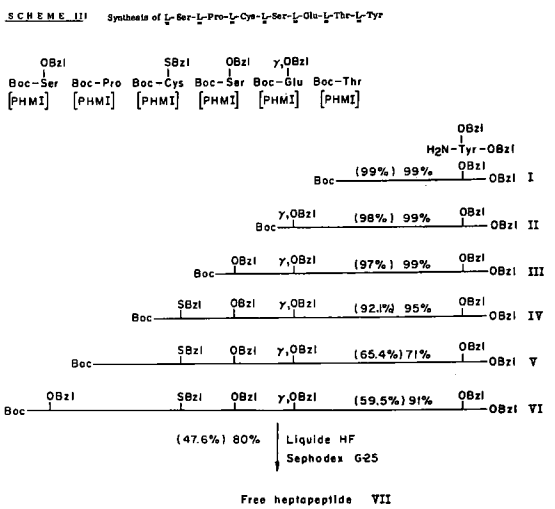
[c] Copoly-(ethylene-N-hydroxymaleimide) cross-linked with 5% spermidine was used.

[d] Assayed by increase in weight of starting polymer and by quantitative amino acid analysis after total acid hydrolysis.

The t-butyloxycarbonyl group was used to protect the N-terminals, whereas the benzyl group was used to protect the thiol of cysteine, the hydroxyl groups of serine and tyrosine, and the γ-carboxyl of glutamic acid. The synthesis was initiated by reacting O-benzyl-L-tyrosine benzyl ester with excess (3-equiv) of polymeric insoluble active ester of t-Boc-L-threonine in DMF, to yield N-t-Boc-L-threonyl-O-benzyl-L-tyrosine benzyl ester. Removal of the t-Boc protecting group with HCL in dioxane and neutralization with triethylamine yielded a dipepdide ester which was coupled with the corresponding polymeric active ester to yield an N-blocked tripeptide ester. Repetition of this set of reactions led, by stepwise elongation of the peptide chain, to the formation of the blocked heptapeptide in 59.5% overall yield. Removal of the blocking groups with liquid HF [6] yielded the desired heptapeptide in 47.6% yield. The results of these studies are summarised in Scheme III.

Concomitantly with the synthesis of the heptapeptide according to Scheme III, a classical synthesis of the peptide by the DCC method [7] was performed. A comparison of the properties of the corresponding intermediate peptides obtained by both methods show that identical intermediates were obtained and that in most cases higher yields of intermediates and of the final product were obtained when PHMI-active amino acid esters were employed.

In order to determine the possible acylation of high molecular weight synthetic and native compounds with PHMI-amino acid esters, the alanylation of poly-ε-benzyloxycarbonyl-L-lysine (mol wt~10,000), and of insuline, by the polymeric active ester of t-Boc-L-alanine was investigated. The poly-ε-benzyloxycarbonyl-L-lysine was reacted with the polymeric active ester at room temperature in DMF, and the N-terminal amino acid of the product was assayed by the DNP-method. The assay was performed after removal of the terminal t-Boc group with anhydrous trifluoroacetic acid (TFA).



SCHEME III
Synthesis of L-Ser-L-Pro-L-Cys-L-Ser-L-Glu-L-Thr-L-Tyr

The acid hydrolysate of the dinitrophenylated product was found to contain DNP-alanine but no α-DNP-lysine as determined by thin layer chromatography (tlc).[8] The interaction of the polymeric active ester with insulin was similarly carried out at room temperature in DMF. The product was treated with anhydrous trifluoroacetic acid to remove the t-Boc blocking groups. Amino acid analysis of the unmasked product revealed the insertion of three additional alanine residues per protein molecule. Dinitrophenylation proved the presence of DNP-Ala instead of DNP-Gly, DNP-Phe and ξ-DNP-Lys to be expected from intact insulin.

The acylation of amines by the polymeric active esters was markedly enhanced at elevated temperatures. The reaction of L-H_2N-Val-OBzl with Z-L-Val-[PHMI] in DMF at room temperature (1:2 molar ratio) gives the dipeptide Z-L-Val-L-Val-OBzl in quantitative yield within 12-14 hours. When the same reaction was carried out at 70°, however, the coupling was completed within 45-60 min. The blocked dipeptides obtained at the two different temperatures were identical in their melting points, optical rotations, and chromatographic properties, with an authentic dipeptide derivative. It could thus be concluded that no racemization had occurred during the coupling step at 70°. A similar shortening of the time of the coupling reaction was observed when HNProOBzl was reacted in DMF with Z-Phe-[PHMI], and H_2NLeuOMe reacted with Z-Phe-[PHMI] at 70°.

The experimental findings summarized above show that the polymeric amino acid active esters described, obtained on coupling t-butyloxycarbonyl amino acid with crosslinked copoly-(ethylene-N-hydroxymaleimide), might serve as useful polymeric reagents in the synthesis of low and high molecular weight peptides. The coupling time in DMF is markedly shortened at elevated temperatures. Finally, it is worth mentioning that PHMI-active esters might be useful in the acylation of proteins and other biopolymers.

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SOME FUNDAMENTAL EXPERIMENTS IN SOLID-PHASE PEPTIDE SYNTHESIS

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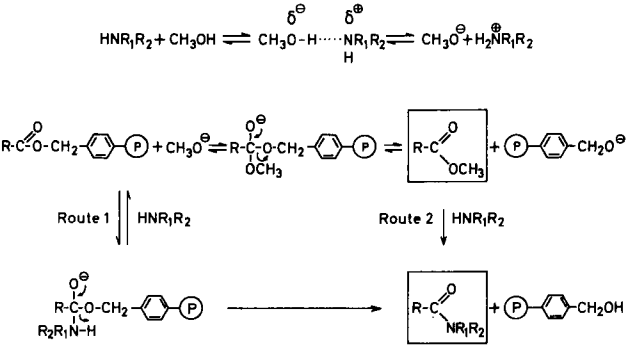
1. Introduction. Up to the present, acid cleavage of the completed peptide from the resin is the most widely used procedure in solid-phase peptide synthesis. Merrifield originally used hydrogen bromide in trifluoroacetic acid[1]. With this reagent many side-chain protecting groups are conveniently removed at the same time, but decomposition of acid-labile amino acids and occasionally low yields are definite disadvantages. Sakakibara[2] overcame these disadvantages by using liquid hydrogen fluoride, and this reagent, notwithstanding its dangerous properties, has now been found to be increasingly useful.

Recently, cleavage with a base such as ammonia[3], hydrazine[4], and sodium in ethanol[5] has been employed. Interaction between a peptide-on-resin and methanolic ammonia gave C-terminal peptide amides. This reaction was used successfully in several laboratories in the synthesis of oxytocin and vasopressin, inter alia in our Laboratory. The sequence of oxytocin could also be split off with methylamine, hydroxylamine, and hydrazine in methanol to give N-benzyloxycarbonyl-S,S'-dibenzyl-oxytocinoic acid N'-methylamide, N'-hydroxyamide, and hydrazide, respectively[7].

In a synthesis of oxytocin we found a considerable amount of nonapeptide ethyl ester when ethanolic ammonia was used in the cleavage of the peptide from the resin. Visser et al.[8], and Kessler and Iselin[4] isolated impure products after treatment of a peptide-resin with hydrazine hydrate in methanol and ethanol, respectively, possibly owing to the formation of esters. Bodanszky et al.[9] were even able to isolate esters, but considered this to be a special case, caused by steric hindrance of the C-terminal valine; these authors did not observe alcoholysis of the glycine-polymer bond.

2. Theory. We reasoned that in these cases a direct ammonolysis, hydrazinolysis, etc., (Scheme, route 1) competes with a base-catalysed transesterification followed by ammonolysis, hydrazinolysis, etc. (route 2). Furthermore we reasoned that in the second instance tertiary amines would analogously catalyse the first reaction, the transesterification, but would prevent the next step, the ammonolysis, thus yielding esters only. This proved indeed to be the case[10]. A similar finding was made

independently by R. Piasio[11].



Scheme
Ammonolysis, hydrazinolysis, etc., of the peptide-polymer bond (route 1).
Base-catalysed transesterification of the peptide-polymer bond eventually followed by ammonolysis, hydrazinolysis, etc. (route 2).

High yields of pure peptide esters could be obtained in this way. We will cite as an example our preparation of pure Z-Cys(BZL)-Tyr(BZL)--Ile-Gln-Asn-Cys(BZL)-Pro-Leu-Gly-OCH_3 in an overall yield of 70% (calc. on Gly/polymer and including the eight peptide couplings) when using 1 N methanolic N-methylpiperidine for 4 hours at room temperature[10,12].

We consider the alcoholysis to be useful in the following methods:

(i) In thin-layer chromatography (tlc) and gas-liquid chromatography (glc).

A solid-phase synthesis can be monitored by the conversion of peptide-on-resin samples with the aid of triethylamine/methanol into peptide methyl esters, followed by tlc/glc.

(ii) In quantitative amino acid analysis.

With the acid hydrolysis of peptides, esterified to a hydroxymethylstyrene-divinylbenzene resin, amino acids notably Glu, may escape determination by adsorption to the resin. An obvious answer is alcoholysis prior to the acid hydrolysis and subsequent quantitative amino acid determination.

(iii) With mass spectrometry.

It is a well-known fact that methyl esters of peptides are eminently suitable for mass spectrometric sequence analysis. We made use of this fact, e.g. in our automated synthesis[10] and in our syntheses mentioned later in this report.

(iv) In the synthesis of large peptides or proteins.

In our opinion the solid-phase method is not yet suited for the synthesis of proteins or large peptides in the pure form, mainly because of incomplete condensation reactions[13]. It might be preferable to prepare on the polymer first, e.g. with the use of a machine[14], peptides of the size of about 10-20 residues, followed by removal of the chain with methanol/tert base. The peptide methyl esters could then be coupled, e.g. after conversion into the hydrazide and azide, to yield a larger peptide or, possibly, a protein.

3.1. Transesterification experiments. The transesterification was, therefore, investigated in detail. We studied the influence of the alcohol and the tert base. The results are listed in Table 1.

TABLE 1

Transesterification of Z-Pro-Leu-Gly-resin [a]

Base (pK_a in water)	Alcohol					
	Methanol		Ethanol		i-Propanol	
	time h	yield %	time h	yield %	time h	yield %
--	4	∿6	4	∿3 [c]		
--	24	37	28	∿5 [b]	24	∿1 [c]
N-Methylmorpholine (7.4)	4	93	4	16		
			28	39		
N-Methylpiperidine	4	97	4	33		
			28	100		
Triethylamine (10.6)	2	89			60	∿6 [b]
	4	94	4	37		
			28	95		
Ethyldiisopropylamine (11.2)	4	96	4	53		
			28	100		
Amberlite IRA 400	2	82				
(treated according to Halpern et al., Reference[16])	4	76[d]				

a 1 N-solution of a tertamine in alcohol at room temperature; resin esterified according to Merrifield (Reference[1]), 0.6 mmole tripeptide/g of resin.

b In part methyl ester, see note c.

c Only methylester, because of a trace of methanol in the alcohol used, according to tlc (diisopropylether/chloroform/acetic acid 6:3:1) and mass spectroscopy.

d Lower yield possibly caused by deterioration of the anion-exchange resin (see Reference[16a]).

Not much is known of the pK_a-values of the bases and the alcohols in the systems used. It seems plausible that with a given base the yield of ester will increase with decreasing pK_a of the alcohol. With methanol or ethanol as the solvent, the yield of the peptide ester will increase with increasing pK_a of the amine.

It seems remarkable that the methanolysis and ethanolysis at room temperature gives such high yields in so short a time, in spite of the absence of swelling of the resin in these alcohols. A resin-surface adsorption phenomenon of the alcohols wight be the explanation.

3.2. We also observed a transesterification without the addition of a tert base. BOC-Gly-OCH_3 could be cleaved from the polymer on being kept for two days in absolute methanol[15]. Several years ago, van Zoest[3] showed that in the Merrifield-esterification[1] a side-reaction occurs

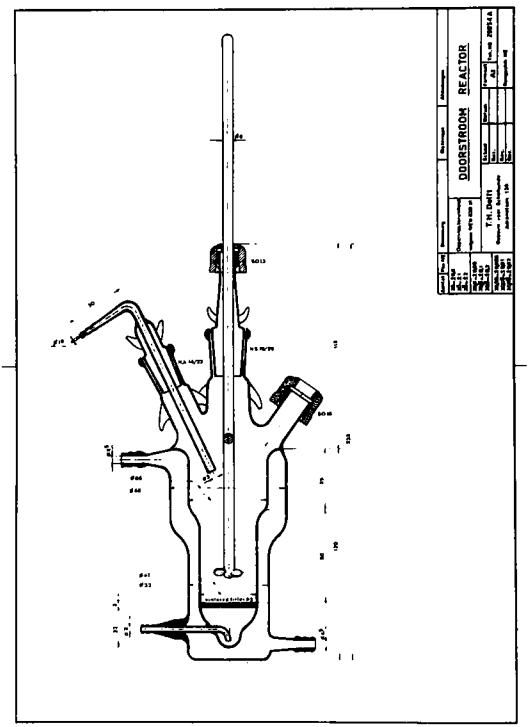
between triethylamine and the chloromethyl groups in which quaternary ammonium compounds not unlike certain ion-exchange resins are formed:

$$NEt_3 + ClCH_2 - C_6H_4 - P \rightarrow Cl^-.\overset{+}{N}(Et_3) - C_6H_4 - P$$

Apparently these groups are able to exchange chloride ions for methoxide ions. The course of the reaction resembles the transesterification according to Halpern et al.[16], in which a strong anion-exchange resin is used (see Table 1)[17].

A logical conclusion is to avoid methanol and ethanol as washing solvents in solid-phase peptide synthesis with a Merrifield-esterified resin, and use non-alcoholic solvents or use isopropanol (see Table 1).

4. Kinetic experiments. The previous results were reported in a lecture[18]. I now wish to report on kinetic experiments by J. Hirt. A full-flow reaction vessel was constructed (Fig. 1).



Full-flow reactor for kinetic measurements.

Figure 1

This reactor was connected to a peristaltic pump and a recording ultra-violet spectrometer. The flow-sheet (Fig. 2) shows the arrangement, together with the set-up of the reference vessel (blank).

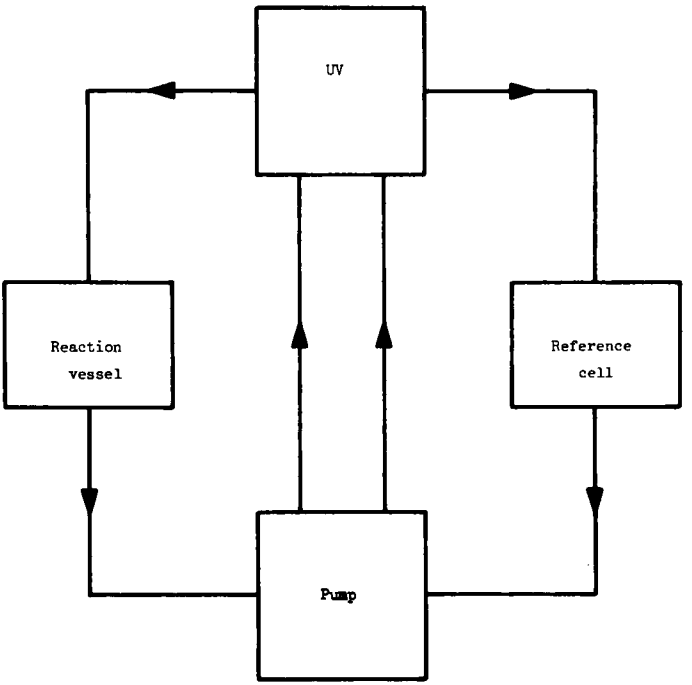
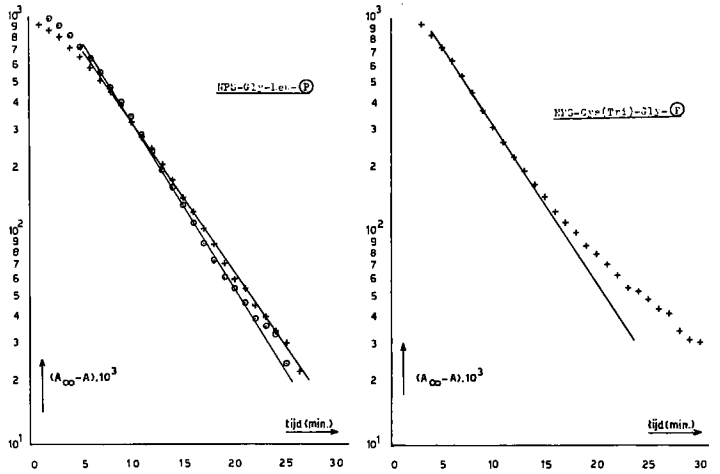


Figure 2

Flow-sheet showing arrangements of instruments for kinetic measurements.

A number of o-nitrophenylsulfenyl N-protected peptides-on-polymer were synthesized, e.g. NPS-Gly-Cys(BZL)-P
NPS-Gly-Gly-P
NPS-Gly-AsN-P
NPS-Gly-Leu-P
NPS-Cys(Tri)-Gly-P
NPS-Phe-Gly-P
NPS-Phe-Pro-P

The alcoholysis, by methanolic triethyl amine,was followed by measuring the extinction at λ= 368 nm (NPS-Gly-) or at λ= 378 nm (NPS-Phe-, NPS-Cys-) of the liberated NPS-peptide methyl ester in the solution. A_∞-A was plotted on a semi-logarithmic scale against time. In most cases this showed, after a starting effect, linearity, as is to be expected for a pseudo first-order reaction with one reaction velocity constant[19]. Typical examples are shown in Fig. 3, the methanolysis of NPS-Cys(Tri)--Gly-P. The half-reaction time, that is the time belonging to $\frac{1}{2}A_\infty$ of the last example is about 7 min. Log(A_∞-A) plotted against time represents a straight line until about 80% alcoholysis. It appears from measurements on the above-mentioned and of other peptides that the inhomogeneity of the resin is a negligible factor, at least with resins not loaded



Methanolysis of NPS-Gly-Leu-P (left) and of NPS-Cys(Tri)-Gly-P (right) in 1 N methanolic triethylamine.

Figure 3

excessively with peptide.

6. Syntheses. Some practical examples of our methods are given.

6.1. [5-Glutamine]-α-melanotropin. Blake, Crooks, and Li[20] in their solid-phase synthesis of [5-glutamine]-α-melanotropin obtained the tridecapeptide Acetyl-Ser(BZL)-Tyr(BZL)-Ser(BZL)-Met-GlN-His(BZL)-Phe-Arg-(Tos)-Try-Gly-Lys(Tos)-Pro-Val-OMe in a 81% yield, calculated on 0.8 mmole of Val-P as starting material and using triethylamine in methanol--dimethylformamide for 22 h at 42° for removal from the polymer. The ester was then converted into the C-terminal amide in nearly quantitative yield.

6.2. Human calcitonin. H. Hindriks used our mechanical synthesizer[14] and prepared the sequence 11-32 of human calcitonin on the Merrifield polymer starting with 0.6 mmole/g of Pro-P. Treatment of the peptide-on-polymer for 17 hours with 1 N methanolic triethylamine followed by column chromatography on Sephadex LH-20, performed by H.A. Billiet, yielded the peptide containing 22 amino acids BOC-Thr-Tyr-Thr-GlN-AsN--Phe-AsN-Lys-Phe-His-Thr-Phe-Pro-GlN-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro--OMe in the protected form. This was pure according to thin layer chromatography and quantitative amino acid analysis.
(Found: Lys 0.95, His 0.91, Asp 1.99, Thr 3.51, Glu 2.05, Pro 2.20, Gly 2.08, Ala 2.21, Val 1.10, Ile 1.12, Tyr 0.78, Phe 3.10).

In this synthesis all amino acids were N-BOC-protected with the exception of Thr, which was pMZ-protected. For side-chain protection we used 15-Asp(amide), 18-Lys(Z), 20-His(ZTF), 21-Thr(ZTF), and 25-Thr(ZTF). Most couplings were effected by DCCI in methylene chloride, with the exception of 15-AsN, 17-AsN, and 24-GlN, which were coupled by our triazole method[21] via-nitrophenyl esters and with the exception of the

sequence 11-13 in which we used the triazole method[21] in combination with pentachlorophenyl esters in dimethylformamide.

It should be noted that in the chromatographic purification a quite considerable amount of the sequence 24-32-OMe was obtained. The reason for the troublesome presence of this truncated sequence is at present not clear to us.

6.3. Human growth hormone (HGH). Some sequences of human growth hormone (HGH)[22] were synthesized by E.W.B. de Leer. From our computer-aided calculations on the statistical distribution of peptides in stepwise synthesis[13] it follows that a continuous solid-phase synthesis of this protein containing 188 amino acids will only result in an extremely complex mixture unless a yield of 99.9% or better is obtained in each coupling step (Table 2). We therefore chose for our approach first the stepwise-synthesis of peptides containing 10-20 residues only, followed by coupling of these fragments.

We synthesized the HGH-sequences 6-10 and 12-21 with appropriate side-chain protection, on the polymeric support, and split the pentapeptide and, respectively, the decapeptide as the methyl ester, from the resin with tert.base-catalysed methanolysis.

TABLE 2

Statistical distribution of peptides in the stepwise synthesis of human growth hormone (188 amino acids) as a function of constant coupling yield.

Number of amino acids \ Coupling yield %	Molfraction in % for 187 steps			
	98	99	99.5	99.9
188 *	2.3	15.3	39.2	82.9
187 **	8.7	28.8	36.8	15.5
186	16.6	27.1	17.2	1.4
185	20.8	16.9	5.3	.1
184	19.6	7.8	1.2	
183	14.6	2.9	.2	
182	9.0	.9		
181	4.8	.2		
180	2.2	.1		
179	.9			
178	.3			
177	.1			

* Pure HGH

** Mixture of all possible peptides with 187 amino acid residues.

6.4. Thyrotropin releasing hormone (TRH). Several workers recently reported the sequence of the thyrotropin releasing hormone (TRH) to be pyroglutamyl-histidyl-proline amide, PGA-His-Pro.NH_2.

I wish to report a convenient and relatively simple solid-phase synthesis, by J.L.M. Syrier and P. Kranenburg, which again exemplifies the utility of the alcoholysis.

BOC-His(BZL) was coupled to Pro-P with the aid of DCCI in methylene chloride in the usual way. Upon removal of the BOC-group, either with 4 N HCl/dioxan (30 min) or with trifluoracetic acid/methylene chloride (1:1), we coupled with pyroglutamic acid 2,4,5-trichlorophenyl ester in DMF with the addition of excess triazole. Splitting of the protected tripeptide from the polymer was effected with methanol/triethylamine (4:1). The yield of PGA-His(BZL)-Pro-OMe was nearly quantitative; m.p. 185-186° (130°). The benzyl-group was smoothly removed by Pd/C-catalysed hydrogenolysis in 80% acetic acid at room temperature in about 24 hours; this yielded the crystalline acetate with m.p. 105-107° (decomp.). The product was converted, upon standing for several days with 9 N ammoniacal methanol, into the acetate of TRH. The yields obtained and the ease of operation seem to be an improvement over the one synthesis (mainly performed to prove the correctness of the sequence) which has been reported in full[23].

Acknowledgements. The investigation was carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with support from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

We wish to thank Mr. H.A. Billiet and Dr. A. Dijkstra, Laboratory of Analytical Chemistry, Technische Hogeschool, Delft, for help with chromatographic separations and amino acid analyses.

We acknowledge with thanks the amino acid determinations by Ir. J.A. Goverde of the Analytical Department of Organon N.V., Oss.

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P. Kranenburg
E.W.B. de Leer
G.W.H.A. Mansveld
J.L.M. Syrier.

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THE SYNTHESIS OF AN IMMUNOLOGICALLY ACTIVE PENTAPEPTIDE USING A NEW INSTRUMENT ("THE PEPTIDER") AND STUDIES ON THE SYNTHESIS OF AN IMMUNOLOGICALLY ACTIVE EICOSAPEPTIDE OF TOBACCO MOSAIC VIRUS PROTEIN

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The peptide, Leu-Asp-Ala-Thr-Arg has recently been synthesized using a new instrument for manual solid phase peptide synthesis, "The Peptider", which was demonstrated at the symposium (Peninsula Laboratories, 1105 Laurel Street, San Carlos, California, 94070, Telephone: 415-592-5392). A schematic of the instrument is given in Figure 1.

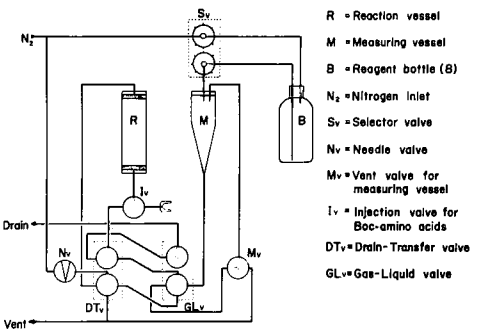

Figure 1. Schematic of "The Peptider"

Two separate syntheses of this pentapeptide were performed. The mole ratio of the amino acids in each peptide-resin synthesis was 1.0 ± 0.05. One of the peptide-resin preparations was cleaved by HF and fractionated on a Dowex 1X2 column. The isolated pentapeptide was obtained in 72% yield based on its recovery from the peptide-resin and was identical to that synthesized by the usual manual method of solid phase peptide synthesis in which the reagents are added by opening the reaction vessel (1).

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The criteria for comparison were: amino acid analysis, paper chromatography in two solvents and paper electrophoresis at pH 6.4 and pH 2.8.

The manual solid phase peptide synthesis of the eicosapeptide of tobacco mosaic virus protein representing residues 93-112 and having the sequence: Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg was attempted. The conditions for synthesis were those recommended by Stewart and Young (2). The chloromethylated resin was 2% crosslinked; a 2.5 molar excess of each Boc-amino acid was used (except for glutamine and asparagine) with methylene chloride as the coupling solvent; glutamine and asparagine were coupled in dimethylformamide using a four molar excess of their Boc-p-nitrophenyl esters; deprotection of the Boc group was performed using HCl-acetic acid.

The pentadecapeptide-resin was cleaved with HF and the eicosapeptide-resin was cleaved with HBr-trifluroacetic acid and reduced with hydrogen under atmospheric pressure. The Dowex 1X2 chromatographic elution pattern of each product is shown in Figures 2 and 3. The products were heterogeneous. Since only the acid gradient was used in the pentadecapeptide separation (Figure 2), the Dowex chromatography probably effected less separation. The native peptides eluted in positions identical to Peak II (Figure 2) for the pentadecapeptide and Peak III (Figure 3) for the eicosapeptide.

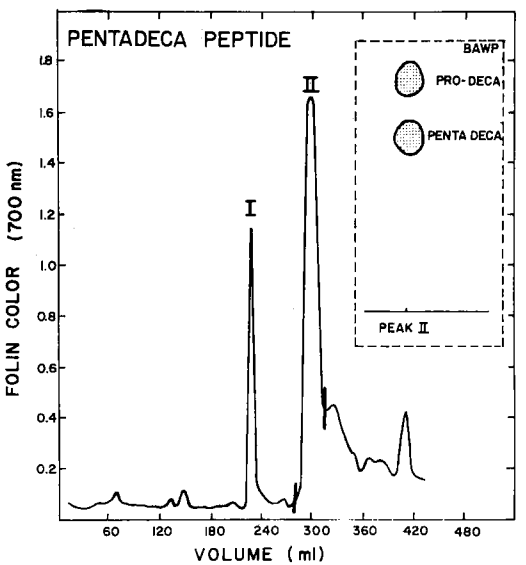

Figure 2. Separation of the pentadecapeptide on a Dowex 1X2 column (3). The insert represents the paper chromatographic pattern of the Peak II material using the solvent: 1-butanol-acetic acid-water-pyridine (15:3:12:10).

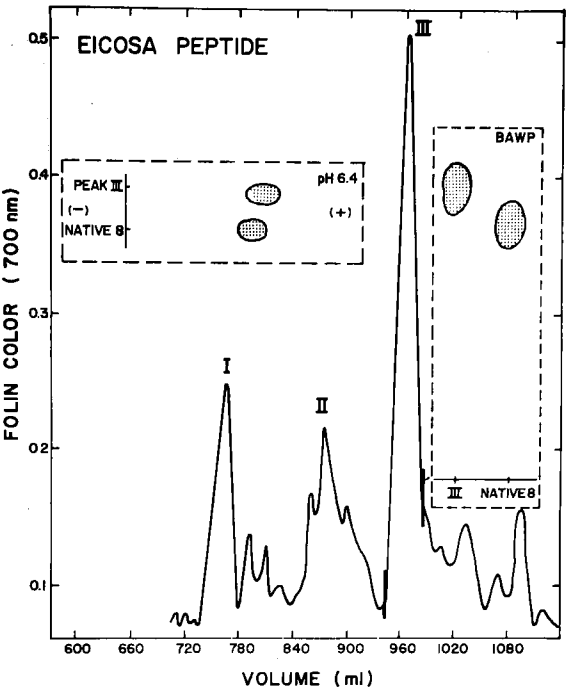

Figure 3. Separation of the eicosapeptide on a Dowex 1X2 column (4). The inserts give the paper chromatographic pattern using the same solvent as in Fig. 2 and the paper electrophoretic pattern at pH 6.4 of the Peak III material and the native eicosapeptide (native 8).

The Peak II material in Figure 2 was further separated by paper chromatography as indicated in the insert in the figure. Amino acid analyses of the pentadecapeptide fractions are given in Table I. The material migrating faster on paper gave an amino acid analysis and N-terminal analysis for the undecapeptide: Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg and was present in about the same concentration as the slower moving material. The slower moving material gave an amino acid analysis close to that expected for the pentadecapeptide. The yield was 10%, based on its recovery from the peptide-resin.

The amino acid analyses of the synthetic and native eicosapeptide are given in Table II. The analysis of the synthetic eicosapeptide is not as good as that obtained for the pentadecapeptide or of the native eicosapeptide. It is high in proline and low in aspartic acid compared to the analysis of native peptide. These values were not the result of the longer time of hydrolysis since a 15 hr. hydrolysis of the sample gave similar values. The inserts in Figure 3 show that the synthetic peptide migrated faster than the native eicosapeptide both on paper chromatography or paper electrophoresis.

Some equilibrium dialysis data are given in Table III for the properties of (^{14}C)-N-acetylated derivatives of the synthetic pentadecapeptide, the synthetic eicosapeptide and the native eisocapeptide toward various

TABLE I

MOLE RATIOS OF AMINO ACIDS IN PENTACEDAPEPTIDE PREPARATION

Amino Acid	Dowex-1 Peak II[1]	Expect	Paper Chrom. of Dowex-1 Peak II[2]			-1 PTH[3] Fast Spot
			Slow Spot	Fast Spot	Expect for Pro-DECA	
ARG	0.97	1	1.02	0.95	1	0.98
ASP	2.01	3	2.79	1.08	1	1.05
THR	3.45	4	3.67	3.16	4	3.45
GLU	1.59	2	2.19	1.06	1	1.05
PRO	0.81	1	1.18	0.64	1	0.11
ALA	2.53	3	3.28	2.08	2	2.06
VAL	0.0	0	0.0	0.0	0	0.0
ILE	0.0	0	0.0	0.0	0	0.0
LEU	1.03	1	1.05	0.91	1	0.92

[1]Peak II refers to Figure 2. The Peak II material was obtained in 31% yield (66 μmoles) based on the recovery from the peptide-resin (196 μmoles, 1.05 g).

[2]The paper chromatographic pattern is given in Figure 3.

[3]Amino acid analysis of Pro-decapeptide after removal of one phenylthiohydantoin.

TABLE II

MOLE RATIOS OF AMINO ACIDS IN EICOSAPEPTIDE

Amino Acid	Synthetic Peak III[1]	Expect	Native[2]
ARG	0.97	1	1.03
ASP	2.31	3	3.07
THR	3.21	4	3.95
GLU	4.24	4	4.02
PRO	1.44	1	1.01
ALA	2.92	3	2.95
VAL	0.90	1	0.93
ILE	1.64	2	0.56
LEU	1.22	1	1.02

[1]Sample was hydrolyzed for 72 hours; Peak III refers to Figure 3.

[2]Sample was hydrolyzed for 15 hours.

rabbit antibodies. The native eicosapeptide had a higher binding constant than the synthetic peptides and was found to bind to a higher concentration of antibodies in the range of high concentrations of peptide. All three synthetic peptides had similar binding constants and bound the same amount of either the anti-SBSA-pentapeptide or the anti-SBSA-decapeptide with the K values and antibody concentrations of each being higher toward the anti-SBSA-decapeptide. Supported in part by U. S. P. H. S. grant AI-06040.

TABLE III

EQUILIBRIUM DIALYSIS EXPERIMENTS

(^{14}C)-N-acetylated Peptide	Anti-TMVP		Anti-SBSA-DECA		Anti-SBSA-PENTA	
	$K(\frac{1}{M})$ x10^6	[Ab] (M) x10^{-6}	$K(\frac{1}{M})$ x10^6	[Ab] (M) x10^{-6}	$K(\frac{1}{M})$ x10^6	[Ab] (M) x10^{-6}
DECAPEPTIDE	2.2	0.23	9.4[2] 1.1	0.34 0.56	3.0	0.12
PENTA DECAPEPTIDE	4.2	0.46	10.3	0.29	2.7	0.15
EICOSAPEPTIDE	3.1	0.5	10.0	0.28	3.9	0.14
NATIVE EICOSAPEPTIDE	9.5	0.39	N.D[3]	N.D	N.D	N.D

[1]Antibodies were obtained in rabbits by injection of the antigens with Freund's complete adjuvant. The antigens, SBSA-DECA and SBSA-PENTA were prepared by conjugation of succinylated bovine serum albumin (SBSA) with either the decapeptide or the pentapeptide (3).

[2]The decapeptide bound differently at high and at low concentrations. The top figures were obtained for the lower concentrations of decapeptide.

[3]N.D., not determined.

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APPLICATION OF N-(2-HYDROXY-I-NAPHTHAL)-AMINO ACIDS AND THEIR PENTACHLOROPHENYL ESTERS IN PEPTIDE SYNTHESIS

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Solid phase peptide synthesis has evoked tremendous interest during the last few years and it has now been possible to synthesize by this method a number of biologically active peptides and at least one enzyme. However, difficulties have been encountered with some amino acid residues and certain sequences, and this has been mainly attributed to the inadequacy of presently employed amino protecting groups or their combinations in trifunctional amino acids. For example, in the preparation of nonalysine, Yaron and Schlossman (1) selected benzyloxycarbonyl group for protection of ε-amino function and tertiary butyloxycarbonyl group for protecting the α-amino function. BOC group was removed at each step with 1 N HCl in acetic acid. At the end of the synthesis it was noticed that the yield of the desired nonalysine was only 35% while the yield of peptides of short chain length was 22%, and the peptides containing longer chains, 10 to 17 residues of lysine, were obtained in 43% yield. Obviously, longer peptides were due to a side reaction in which partial cleavage of benzyloxycarbonyl group took place and branching occurred at ε-amino function. This side reaction has also been confirmed by Grahl-Nielsen and Tritsch (2) and by Wang and Merrifield (3). In order to avoid this difficulty, Yaron and Schlossman (1) repeated the synthesis with o-nitrophenylsulfenyl group as the α-amino protecting group and used 0.3 M HCl in acetic acid for its removal. As expected, the branching reaction did not occur but a higher proportion of shorter chains was obtained. Similarly, during the synthesis of γ-glutamyl decapeptide, Nitecki and Goodman (4) found that there was partial loss of α-benzyl esters during the removal of BOC group thus yielding side products. Difficulties have also been experienced with tryptophan, glutamine and serine residues indicating the need to investigate amino protecting groups which could be removed under very mild conditions. In this connection, Sieber and Iselin (5), and later on Wang and Merrifield (3), have advocated the use of 2-(p-biphenyl)-isopropyloxycarbonyl group for α-amino protection, and Ontjes and Anfinsen (6) investigated the use of trifluoroacetyl group for ε-amino protection of lysine. Working somewhat on similar lines, we have looked into the possibility of using arylidene derivatives.

Although benzaldehyde reacts with free monoamino acids to give Schiff

bases, these products are very unstable and have never been isolated. In 1947, McIntire (7) treated amino acids with o-hydroxy aromatic aldehydes and obtained yellow colored crystalline Schiff bases in reasonable yields. Of the various aldehydes used, 2-hydroxy-1-naphthaldehyde and 5-chloro-salicylaldehyde seemed to react most readily with free amino acids and to produce the most stable Schiff bases. The increased stability of these Schiff bases is perhaps due to intramolecular hydrogen bonding between the arylidene nitrogen and the phenolic proton (Fig. 1). Removal of N-arylidene residue can be accomplished easily with very dilute HCl in methanol at room temperature. These properties suggested their use as N-protective derivatives in peptide synthesis.

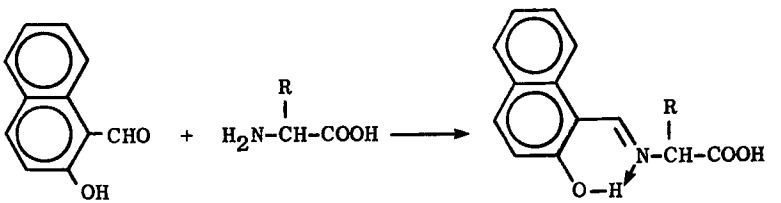

Fig. I

In 1962, Sheehan and Grenda (8) prepared 5-chloro-2-hydroxy-1-benzylidenevaline and 2-hydroxy-1-naphthalvaline. These derivatives were then coupled with glycine ethyl ester and phenylalanine methyl ester, respectively, to obtain corresponding dipeptides. Best results were obtained when the carboxyl component was first activated with DCC before adding the amino component. The products were very impure when the addition order was altered. However, during coupling with 5-chloro-2-hydroxy benzylidene derivatives there was partial removal of the amino protection and, therefore, impure products and lower yields of the desired dipeptides were obtained. On the other hand, peptides prepared with 2-hydroxy-1-naphthal derivatives were obtained in good yields without any complication. No racemization of the peptide derivatives was observed and in view of these results we decided to extend the use of 2-hydroxy-1-naphthal derivatives.

PREPARATION AND PURIFICATION OF N-(2-HYDROXY-1-NAPHTHAL)-AMINO ACIDS.

Sheehan and Grenda (8) prepared 2-hydroxy-1-naphthalvaline by a slight modification of McIntire's procedure (7) by stirring 2 g valine with 1 equivalent of 2-hydroxy-1-naphthaldehyde in a mixture of 480 ml ethanol and 35 ml methanol. The amino acid slowly dissolved as it condensed with the aldehyde. There are three difficulties encountered when using this as a general procedure. First, it is difficult to prepare large quantities of the Schiff bases because of the large volume of solvent required per gram of the amino acid. Second, these Schiff bases are not very soluble in cold alcohol and they begin to crystallize before the amino acid has completely dissolved. Third, the yields obtained are variable. For example, the yields obtained with leucine and glutamic

acid were less than 50% and with glycine 62%. We tried a number of solvents and found that all these difficulties are removed when dimethylformamide (DMF) is used as the solvent. The volume of this solvent required in most cases was approximately 50 ml for 5 g of amino acid. The Schiff bases formed were soluble in DMF and gave a homogeneous solution after the reaction was over. For recovery of the product, the DMF solution was concentrated to a small volume at which time the product crystallized immediately or upon treatment with ether. The yellow crystalline product was filtered and washed with ether. For a further recrystallization, 1 mole of the aldehyde was added to the DMF solution. The yields obtained in every case were quantitative and the compounds gave correct elemental analysis.

McIntire (7) as well as Sheehan and Grenda (8) crystallized these derivatives from hot ethanol. We have noticed that while using this procedure a part of the compound decomposes and becomes more impure than the original derivative. It was perhaps due to this reason that McIntire (7) was led to the belief that these compounds decompose without melting sharply and made no attempt to establish the constancy of their melting point, nor to recrystallize these compounds from other solvents. While in our hands, these compounds have been well characterized, except that in spite of a number of crystallizations from DMF or a mixture of DMF and ether, which were found to be the most suitable solvents for crystallization of these derivatives, it was difficult to judge the purity of these compounds through thin-layer chromatography using ethanol as the mobile phase. For example, with N-(2-hydroxy-1-naphthal)-L-leucine we always detected two spots, the yellow spot with R_f 0.47 was the desired Schiff base, and the ninhydrin positive spot with R_f 0.37 was due to free leucine. It appeared as if a part of the compound was being hydrolyzed on the chromatogram. To confirm this point, the yellow spot was eluted with DMF and rechromatographed on a fresh thin-layer plate. Once again the presence of leucine was detected on this new chromatogram. This clearly indicated that partial removal of the protecting group took place on the chromatogram when ethanol was used as the mobile phase. However, when the chromatogram was developed in an ethereal solvent, such as in a mixture of bis(2-ethoxyethyl)-ether and tetrahydrofuron, only one yellow homogeneous spot with R_f 0.7 was obtained. It is evident from this study that one should avoid solvents containing water and alcohols, both for crystallization as well as for chromatography of these compounds.

These derivatives could also be purified as their dicyclohexylammonium salts. Formation of these salts very much enhanced the solubility and stability of these compounds in organic solvents but their use was not explored because of danger of N-deprotection during neutralization with acid before the condensation step.

PEPTIDE SYNTHESIS BY THE CONVENTIONAL PROCEDURE.

For the synthesis of peptides, using DCC as the coupling agent, the procedure of Sheehan and Grenda (8) was followed. The carboxyl component was first activated with DCC before adding it to the amino component. The dipeptide, N-(2-hydroxy-1-naphthal)-L-valyl-phenylalanine methyl ester was obtained in 75% yield and was identical with that reported by the earlier authors (8).

Bodanszky suggested (private communication) the use of o-nitrophenyl esters for conventional and solid phase synthesis. We therefore prepared

o-nitrophenyl ester of N-(2-hydroxy-1-naphthal)-L-valine. It was difficult to crystallize this compound, but column chromatography on silica gel G (Stahl), using ethyl acetate as the solvent, gave the ester in about 40% yield. On condensation with L-phenylalanine methyl ester, the corresponding dipeptide was obtained in good yield. However, in view of the difficulties faced in the purification of these active esters, this approach was not further pursued.

Recently Johnson et al. described (9) N-t-butyloxycarbonyl-L-amino acid pentachlorophenyl esters, and showed these to be useful intermediates for extremely rapid peptide synthesis in the conventional (10) and solid phase procedures (11). For a further application of hydroxynaphthal derivatives we prepared N-(2-hydroxy-1-naphthal)-amino acid pentachlorophenyl esters by the procedure analogous to that of Kovacs et al. (12,13). These esters are high melting (> 140°) yellow crystalline compounds and are conveniently obtained in high yields. With the exception of the glycine derivative, which was comparatively less soluble, all these active esters are freely soluble in most organic solvents, e.g., ethyl acetate, methylene chloride, tetrahydrofuron, and DMF, and can be easily crystallized to a constant melting point from a mixture of methylene chloride or tetrahydrofuron and ether. Thin-layer chromatography gave homogeneous spots and the IR spectrum gave the characteristic band at 1780 cm^{-1}. Condensation of N-(2-hydroxy-1-naphthal)-L-alanine pentachlorophenyl ester and the corresponding L-valine ester with L-phenylalanine methyl ester gave the desired dipeptides in good yields.

PEPTIDE SYNTHESIS BY THE SOLID PHASE PROCEDURE.

In order to test the applicability of these derivatives on solid phase, we decided to synthesize a tetrapeptide, leucylalanylglycylvaline. A solution of 2-hydroxynaphthal glycine, in a mixture of DMF and methylene chloride was cooled to 0° and treated with exactly 1 equivalent of DCC. The activated carboxyl component so obtained was added to valine-polymer, which was obtained from the corresponding BOC derivative by the usual removal of BOC group and subsequent neutralization of the hydrochloride salt with triethylamine. The reaction mixture was shaken for 2 hours at 0° and 4 hours at room temperature. The solvents and reagents were filtered and the polymer washed as usual with suitable solvents such as DMF, methylene chloride and dioxane. At this stage the color of the polymer became deep yellow. For the removal of the hydroxynaphthal group, the polymer was shaken with 0.5 M aqueous HCl in dioxane, prepared by adding 5 ml 2 N aqueous HCl to 15 ml dioxane. After about 1 hour the typically yellow color was discharged. The mixture was further shaken for 30 minutes and then the polymer washed as usual with dioxane, DMF, EtOH and CH_2Cl_2. The hydrochloride salt was then neutralized with 10% triethylamine in DMF and the polymer again washed with DMF and CH_2Cl_2. At this stage the dipeptide polymer, which was white, gave ninhydrin positive reaction. Although in this synthesis we used 0.5 M aqueous HCl for the removal of protecting group, it appeared that 7% benzylamine in methylene chloride (v/v) (14) was a much superior deprotecting agent. This reagent was used in all subsequent syntheses. Amino acid analysis of the dipeptide polymer at this stage gave 1:1 ratio of valine and glycine. Likewise, the dipeptide polymer was further coupled with N-(2-hydroxy-1-naphthal)-L-alanine and N-(2-hydroxy-1-naphthal)-L-leucine, respectively, to give a tetrapeptide. At the end of the synthesis, N-terminal hydroxynaphthal group was removed and polymer, after proper drying, was treated with HBr in trifluoroacetic acid. TLC of the cleaved

product showed that it was a mixture of 2 major components and at least 3 minor components. Purification by thin-layer chromatography and column chromatography, by the procedure reported by Merrifield (15) on Dowex-50 using 0.1 M pyridine acetate buffer, gave 2 homogeneous components. Amino acid analyses of these components indicated that one of these was the desired tetrapeptide while the other was a tripeptide containing valine, glycine and alanine. The yield of the tetrapeptide was very low and analyses suggested that the terminal coupling with N-(2-hydroxy-1-naphthal)-L-leucine did not proceed to completion. These results are not surprising in view of a recent synthesis of this tetrapeptide by Dorman (16) who used BOC-derivatives, and DCC as the coupling agent. In this synthesis the extent of coupling with BOC-leucine was 69% and the yield of the tripeptide obtained was 38%. However, repetition of the synthesis with longer period of reaction did not yield satisfactory results.

For the synthesis of peptides from pentachlorophenyl esters, a tripeptide alanylglycylvaline was prepared. N-(2-hydroxy-1-naphthal)-L-valine was esterified on bromomethyl polymer by the method analogous to that reported by Tilak (17). Hydroxynaphthal protecting group was removed with 7% benzylamine (14) in a mixture of dimethylformamide and methylene chloride (60:40) (18). The polymer was washed with suitable solvents and then condensed with N-(2-hydroxy-1-naphthal)-glycine in dimethylformamide. The above process was then repeated with N-(2-hydroxy-1-naphthal)-L-alanine. The reaction was carried out in a specially designed vessel in which the mixture was stirred by bubbling N_2 and an aliquot of the active ester solution (0.1 ml) withdrawn by filtration after every 30 minute interval. The progress of the reaction, during the

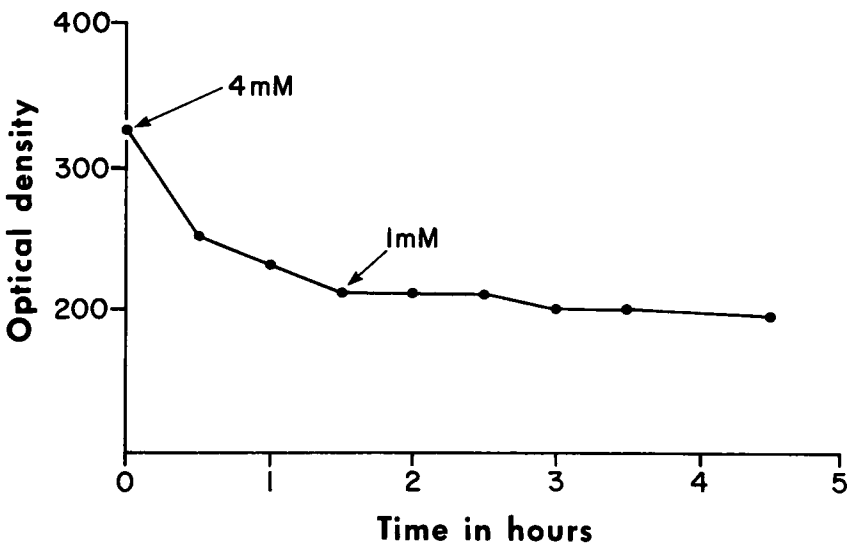

Fig. 2 - Condensation of N-(2-hydroxy-1-naphthal)-L-alanine pentachlorophenyl ester with glycyl-L-valine polymer in DMF

coupling step, was monitored by measurement of optical density on Klett-Summerson photoelectric colorimeter. Figure 2 summarizes the results graphically and indicates that almost 3 mmoles or one equivalent of the required amount of N-(2-hydroxy-1-naphthal)-L-alanine pentachlorophenyl ester condensed with glycylvaline polymer in about 1.5 hours and thereafter there was no further utilization of the active ester. At the end, N-terminal protecting group was removed and the tripeptide cleaved from the polymer and purified by the usual procedure to give a product with an amino acid ratio 1:1:1 of valine, glycine and alanine.

CONCLUSIONS.

The results obtained indicate that N-(2-hydroxy-1-naphthal)-monoamino acids can be obtained in high yields in crystalline characterizable form. This opens up the possibility of investigating other 2-hydroxy arylidene derivatives for solid phase peptide synthesis. Due to the color, one could follow deprotection at each stage. Since deprotection can be accomplished under very mild conditions, these derivatives may be useful in combination with tert.-butyloxycarbonyl or benzyloxycarbonyl group in dibasic amino acids.

Coupling with DCC in the conventional synthesis gave the desired products in good yields but did not give encouraging results on the solid phase. On the other hand, coupling through pentachlorophenyl esters was very fast and offered a procedure for the measurement of the extent of the coupling by the colorimetric method. Although transamination could not be ruled out during the coupling reactions with these N-protecting groups, this does not appear likely with pentachlorophenyl esters which condense, comparatively speaking, very fast.

These derivatives may also be useful for fragment condensation. This could be done either by protecting the N-terminal of peptide with 2-hydroxy-1-naphthal group or by removing the protected fragment from the polymer with HBr. The latter is feasible since this protecting group cannot be removed under strictly anhydrous conditions with HBr. The protected peptides, because of their color, may then be purified more conveniently.

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SOLID-PHASE PEPTIDE SYNTHESIS OF 1-AMINOCYCLOPENTANECARBOXYLIC ACID (ACPC) ANALOGS OF ANGIOTENSIN II, USING A NEW APPARATUS (*)

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(*) This investigation was supported by the Medical Research Council of Canada.

Introduction: Following the first synthesis of angiotensin II by the Basel and the Cleveland groups in 1957, many angiotensins or angiotensin analogs have been synthesized in several different laboratories by the classical method of peptide synthesis in solution or by the solid-phase method. These various peptides have been investigated in order to derive some correlation between the biological activity and the chemical structure. Must of the synthesized peptides thus studied are made of natural amino acids and only a very few contain amino acid analogs or derivatives. Due to the interesting properties of 1-aminocyclopentanecarboxylic acid (ACPC) (Berlinguet et al.) we have synthesized five new peptides containing this amino acid analog which has been substituted in position 1, 3, 6, 7 and 8 of 5-isoleucine-angiotensin II. For the synthesis of these ACPC-angiotensin II analogs, we have used a newly developed reaction vessel designed by us which greatly improve the solid-phase method of synthesis. The biological activities of these ACPC-angiotensin II analogs have been assayed and compared to the pressor activity of 5-isoleucine-angiotensin II.

NEWLY DEVELOPED APPARATUS.

The non-automatic, manual valving system of the new apparatus used in the solid-phase peptide synthesis method is basically composed of 4 parts which are (Figure 1); (i) the reagents and solvents reservoir, (ii) the reagents and solvents cutlet system, (iii) the shaker, and (iv) the reaction vessel.

(Shaker) (Reaction Vessel) (Solvents and Reagents Outlet System) (Solvents and Reagents Reservoir)

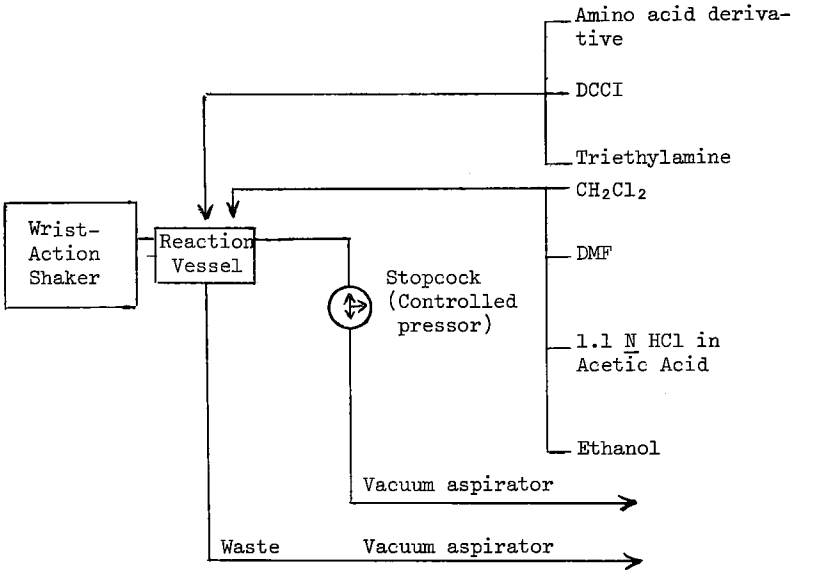

FIGURE 1: System of Non-Automatic Manual Valving Apparatus for Solid-Phase Peptide Synthesis.

The reaction vessel is the most important part of this system. The first reaction vessel was originally described by Merrifield (1963,1965). Since then, quite a large number of systems have been designed and used. The review of J.M. Stewart and J.D. Young (1969) cites the following; (a) the vessel of Samuel and Holybee which provides a complete inversion (180^{o}) of the vessel, (b) the vessel of Khosla et al (1967) which is always kept upright and consists of a side-arm with a special funnel, (c) the upright stationnary vessel designed by Grahl-Nielson and Tritsh (1969), and (d) the vessel of Sipos and Denning who have combined a titled rotary evaporator with the synthesis vessel. These are the most important non-automatic, manual valving systems used now in various laboratories. Although these vessels have some advantages over the original one, they are not entirely satisfactory. Stewart and Young have listed the qualities of an ideal vessel which should have the following characteristics; 1st, a volume which allows a reasonable variation in batch size; 2nd, allow rapid and thorough suspension of the polymer, so that all polymer is in good contact with the solvent; 3rd, allow the rapid and convenient addition of solvents in such a way that the vessel walls are washed down; 4th, allow convenient removal of polymer samples during the process of synthesis and 5th, provide for rapid and complete solvent removal.

Our newly developed reaction vessel almost satisfies the above requirements for an ideal vessel. The reaction vessel consists of a 200 ml (150-1000 ml) round bottom flask of pyrex glass with a side-arm and 3 other connections serving 3 stopcocks. The side arm of the vessel is closed with a ground jointed glass stopper used to insert the polymer (C-terminal amino acid esterified to the polymer), and to permit removal of samples of the polymer during the cycles of the synthesis. This vessel if fixed upright to a Wrist-Action Shaker and during shaking (complete time: 2 min.) the position of the vessel is continuously and vigorously changed to an angle of 90° . The vessel is connected with a flexible teflon or rubber tubbing to the reagent and solvent outlet system or reservoir. The reagents and solvents are introduced into the vessel or removed from the vessel by the controlling pressure of water suction, e.g. to introduce or to remove 50 ml of solvent from the vessel takes only 20 sec.

RESULTS AND DISCUSSION.

The following ACPC-angiotensin II analogs were prepared by the solid-phase method previously described by Marshall and Merrifield (1965) using our newly developed reaction vessel;

(1-ACPC, 5-Ile)-Angiotensin II ..... (I)
(1-Asp, 3-ACPC, 5-Ile)-Angiotensin II ..... (II)
(1-Asp, 5-Ile, 6-ACPC)-Angiotensin II ..... (III)
(1-Asp, 5-Ile, 7-ACPC)-Angiotensin II ..... (IV)
(1-Asp, 5-Ile, 8-ACPC)-Angiotensin II ..... (V)

To obtain these octapeptides, either C-terminal Boc-phenylalanine (I, II, III, and IV) or o-nitrobenzenesulfenyl-ACPC (V) were esterified to the chloromethylated polymer to yield Boc-phenylalanine polymer or NBS-ACPC polymer. The amino acids as the Boc derivatives (except ACPC which was protected by NBS or carbobenzoxy groups) were then added one at time in the desired sequence. Then followed the usual operations (Park et al, 1967): removal of the protecting Boc or NBS groups with approximately 1.1 N HCl in glacial acetic acid at room temperature; neutralization of the resulting hydrochloride with triethylamine in dimethylformamide coupling the free base with the new Boc-amino acid or NBS-ACPC, using N, N'-dicyclohexylcarbodiimide as the condensing agent. Methylene chloride was used as the solvent for all the condensations except for the introduction of Boc-nitro-arginine and Boc-im-benzyl-histidine for which DMF was the solvent. In all cases, excess reagents and by-products were removed by washing with glacial acetic acid, absolute ethanol, DMF and methylene chloride before proceeding to the next coupling step. At the end, the whole protected peptide polymer was cleaved from the polymer by bubbling HBr through a suspension of the peptide polymer in anhydrous trifluoroacetic acid for fifty minutes at room temperature under anhydrous conditions. This treatment also removed the benzyl group from both the aspartic acid and tyrosine residues. The partially protected octapeptides were reduced with hydrogen using palladium black as a catalyst for 48 hr at atmospheric pressure. The crude free peptides were purified by chromatography on Sephadex G-25, columns in BAW or BAPW systems. The final products were homogeneous on paper and thin-layer chromatography and electrophoresis. They all give a single spot with ninhydrin, diazotized sulfanilic acid, and Sakaguchi reagent after acid hydrolysis, amino acid determinations gave the correct amino acid ratios.

After each step of deblocking and of coupling during the peptide synthesis, a small sample of the polymer was removed from the reaction vessel and was reacted with ninhydrin. For the coupling reactions into the peptide chain attached on the polymer, usually a two-fold excess of the Boc-amino acids where used, except for histidine, arginine and ACPC for which a three-fold excess was used.

The biological activities of the new five ACPC angiotensin II analogs were determined by the standard pressor assays using ganglion-blocked and nephrectomized rats and guinea-pig ileum.

The results are summarized in table I.

Phenylalanine is known to be essential in position 8. It is thus normal that when ACPC is substituted in that position, the peptide is inactive. ACPC is unable to replace 7-proline, 6-histidine or 3-valine. However when replacing 1-aspartic acid, the peptide has 40% of the pressor activity of 1-aspartic, 5-isoleucine angiotensin II, or Hypertensin CIBA.

	Pressor Activity (%)
1 2 3 4 5 6 7 8 Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (1-Asp, 5-Ile)-Angiotensin II	100
ACPC-Arg-Val-Tyr-Ile-His-pro-Phe (1-ACPC, 5-Ile)-Angiotensin II	40
Asp-Arg-ACPC-Tyr-Ile-His-Pro-Phe (1-Asp, 3-ACPC, 5-Ile)-Angiotensin II	1.0 - 2.0
Asp-Arg-Val-Tyr-Ile-ACPC-Pro-Phe (1-Asp, 5-Ile, 6-ACPC)-Angiotensin II	0.1 - 0.5
Asp-Arg-Val-Tyr-Ile-His-ACPC-Phe (1-Asp, 5-Ile, 7-ACPC)-Angiotensin II	1.0 - 1.5
Asp-Arg-Val-Tyr-Ile-His-Pro-ACPC (1-Asp, 5-Ile, 8-ACPC)-Angiotensin II	0.1 - 0.5

* The pressor activity given is the mean value obtained from various method that include: ganglion-blocked and vagotomized rats, Pickens et al, (1965), Ing et al (1968). nephrectomized rats, Boucher et al (1964) and guinea-pig ileum, Gascon (1968).

Table 1: Biological activity of ACPC-angiotensin II analogs.

EXPERIMENTAL SECTION.*

1-Aminocyclopentanecarboxylic acid (ACPC) was synthesized according to the original synthesis by Zelinski and Stadnikov (1906, 1911) modified by Berlinguet et al (1961, 1962).

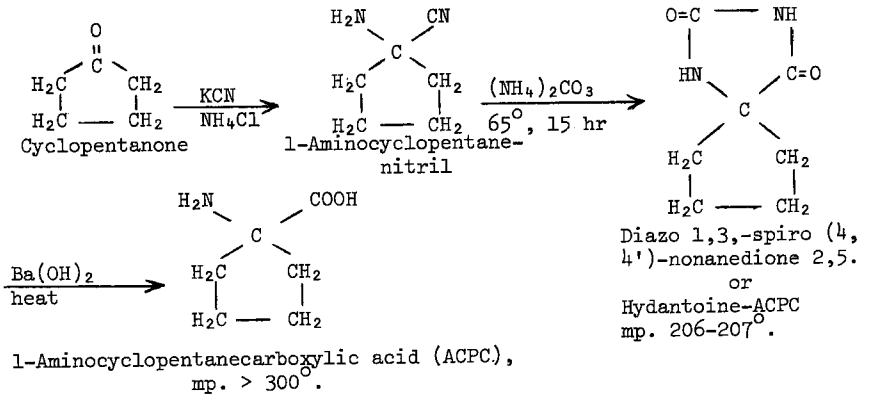

FIGURE 2: Synthesis of 1-Aminocyclopentanecarboxylic acid.

Boc-β-benzyl-aspartic acid, Boc-O-benzyl-tyrosine, and Boc-im-benzyl-histidine were obtained from Mam Research Lab. (New York). The other Boc-amino acid derivatives; nitro-arginine, valine-isoleucine, proline, phenylalanine, and ACPC were synthesized by us according to the procedure of Schwyzer et al (1959). N-carbobenzoxy-ACPC and o- nitrobenzenesulfenyl-ACPC were prepared by us according to the procedure of Zerval et al (1963) (Figure 3).

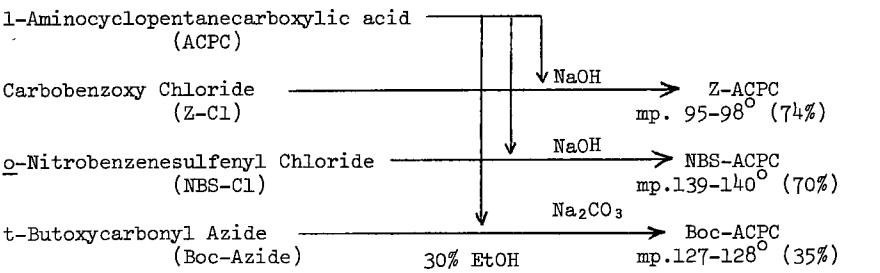

FIGURE 3: Synthesis of N-ACPC Protected Derivatives.

The purity of all the amino acid derivatives and peptides was checked by paper or thin-layer chromatography using three different solvents in an ascending system; n-BuOH: OHAc: H_2O (4.1:5) (BAW), (ii) n-BuOH: OHAc:

* L-isomers were used for all amino acids except for ACPC.
Abbreviations used: ACPC, 1-aminocyclopentanecarboxylic acid; NBS, o-nitrobenzenesulfenyl group; Boc, t-butyloxycarbonyl; DMF, dimethylformamide; DCCI, N,N'-dicyclohexylcarbodiimide; PC, paper chromatography; TLC, thin-layer chromatography.

Pyridine: H_2O (30:6:20:24) (BAPW), (iii) Methylethylketone: Pyridine: H_2O (40:10:16) (MPW). PC and TLC were carried out on Whatman No. 1 filter paper and Eastman Kodak 6060 silica gel chromatogram sheets. The condition used for paper electrophoresis were; formic acid - acetic acid buffer (pH 2.1) for three hours at 450 v. Electrophoretic mobilities are reported as the ratio of the distance the peptide and glutamic acid migrated, and abbreviated as E_G. Melting points were taken on a Fisher-Johns Melting Point Apparatus and are uncorrected. Optical rotation were determined. Microanalyses were done by Organic Microanalyses, Montreal, Canada. For amino acid analysis, the samples were hydrolyzed in 6 N HCl in a sealed tube (under nitrogen) at 110° for 38 hr and the analyses were performed on Technicon Amino Acid Auto-Analyzer. The biological activities were determined by pressor assay in ganglion-blocked, vagotomized rat (Pickens et al, 1965), ganglion-blocked rat (Ing et al, 1968), nephrectomized rat (Boucher et al, 1964), and guinea-pig ileum (Gascon, 1968).

o-Nitrobenzenesulfenyl-1-Aminocyclopentanecarboxylic acid (NBS-ACPC).

ACPC (2.58 g., 0.02 moles) was dissolved in a mixture of 10 ml of 2 N NaOH and 25 ml of dioxane. During a period of 15 min. o-nitrobenzenesulfenyl chloride (4.17 g., 0.022 moles) was added in 10 equal portions while 2 N NaOH (12 ml) was added dropwise, with vigorous stirring by magnetic stirrer. The reaction solution was diluted with 200 ml of water and filtered free of any insoluble matter: the filtrate then was acidified with 1 N sulfuric acid in an ice-water bath. The appearing precipitate was collected by filtration and washed with water. The crystalline product was recrystallized from ethyl acetate and pet-ether; yield 3.81 g. (60%); mp. 139-140°. Anal. Calcd for $C_{12}H_{14}N_2SO_4$(282.31): N, 9.92. Found: N, 9.86.

t-Butyloxycarbonyl-ACPC (Boc-ACPC).

ACPC (5.16 g., 0.04 moles) and Na_2CO_3 (8.58 g., 0.08 moles) were added in 60 ml of 30% ethanol stirred for 3 hr at 40-50°. An almost clear solution resulted; 12.16 g. (0.085 moles) of t-butyloxycarbonyl-azide were added dropwise and the mixture was stirred two days at room temperature. 40 ml of water were then added. The reaction mixture was EtOAc (3 x 60 ml). The aqueous phase was adjusted at pH to 3-4 with cold concd. HCl in an ice-water bath and extracted with EtOAc (2 x 80, 2 x 65 ml); the EtOAc phase was then washed with water (3 x 50 ml) and with saturated NaCl solution (50 ml). After drying over anhydrous Na_2SO_4, the solvent was removed in vacuo and the residue was crystallized from EtOAc with pet-ether; yield, 3.21 g. (35%), mp. 127-128°. Anal. Calcd for $C_{11}H_{19}NO_4$ (229.268): N, 6.11. Found: N, 6.01.

NBS-ACPC Polymer.

A solution of 1.28 g. (4.7 mmoles) NBS-ACPC and 0.59 ml (4.7 mmoles) of triethylamine in 25 ml of abs. EtOH and EtOAc (1:1) mixture was added to 5.0 g. of chloromethylated copolystyrene-2%-divinylbenzene. The mixture was stirred with magnetic stirrer under reflux at 90° for 24 hr, an additional 0.3 ml of triethylamine was added to the reacting mixture which was then shaken vigorously at room temperature for 20 hr. The esterified polymer was washed with abs. EtOH-EtOAc mixture, abs. EtOH, water and abs. MeOH and was then dried over P_2O_5 and paraffin in vacuo. The treated polymer was found to contain 0.63 mmoles of NBS-ACPC/g. of esterified polymer.

Boc-phenylalanine Polymer.

Boc-phenylalanine polymer was prepared according to the above method. Analysis of this substituted polymer gave 0.65 mmoles of Boc-phenylalanine of esterified polymer.

Carbobenzoxy-ACPC-nitro-Arginyl-Valyl-O-benzyl-Tyrosyl-isoleucyl-im-benzyl-Histidyl-Prolyl-Phenylalanine Polymer.

Boc-phenylalanine polymer (2.5 g.) was placed in the newly developed reaction vessel and the following cycle of deprotection, neutralization and coupling was used to introduce each new residue; (1) washed (3 x 50 ml) with glacial acetic acid; (2) Boc group was cleaved with approximately 1.1 N HCl in glacial acetic acid (40 ml) for 30 min. at room temperature; (3) washed (3 x 50 ml) with glacial acetic acid;(4) washed (3 x 50 ml) with abs. ethanol; (5) washed (3 x 50 ml) with DMF; (6) neutralized with 6 ml of triethylamine in 50 ml of DMF for 10 min.; (7) washed (3 x 50 ml) with DMF; (8) washed (3 x 50 ml) with methylene chloride; (9) addition of 3.0 mmoles of appropriated Boc-amino acid in 40 ml methylene chloride and allowed to mix for 10 min. (10) addition of 3.0 mmoles of DCCI in 6 ml of methylene chloride. The mixture was shaken with shaker for three hours at room temperature. (11) washed (3 x 50 ml) with methylene chloride; (12) washed (3 x 50 ml) with abs. ethanol. For Boc-im-benzyl-histidine and Boc-nitro-arginine cycles, step 8 was deleted and DMF was substituted for methylene chloride in steps 9-11. For these two Boc-amino acids and for Z-ACPC 4.0 mmoles were used. At the end of the synthesis, the protected peptide polymer in the reaction vessel was dried in desiccator over P_2O_5 and paraffin in vacuo. The whole protected peptide obtained gave an app. 95% yield by weight.

ACPC-Arginyl-Valyl-Tyrosyl-Isoleucyl-Histidyl-Prolyl-Phenylalanine (1-ACPC, 5-Isoleucine)-Angiotensin II. (Peptide I).

The protected polypeptide polymer (app. 3.35 g.) was suspended in 50 ml of anhydrous trifluoroacetic acid in the reaction vessel and a gas dispersion tube was fixed to the side arm of the vessel replacing the stopper. HBr gas was bubbled slowly through it into the suspension with occasional shaking for 50 min. at room temperature under unhydrous conditions. The reaction mixture was filtered and the polymer was washed (3 x 10 ml) with anhydrous trifluoroacetic acid. The combined filtrate was evaporated to an oil at room temperature in vacuo. The peptide was precipitated by the addition of anhydrous ether, collected by filtration and washed with anhydrous ether several times. After this, the partially protected peptide was dried over P_2O_5, paraffin and NaOH in vacuo (Yield was app. 85-90% from whole protected peptide polymer). It was then dissolved in a mixture of 80 ml of methanol, 10 ml of acetic acid and 10 ml of water. Hydrogen was bubbled through the solution for 48 hr at atmospheric pressure in presence of palladium black as catalyst; 0.8 g. of catalyst was added at the beginning of the reduction and an additional 0.5 g. added after 24 hr. The catalyst was removed by filtration and washed (3 x 15 ml) with the same solvent mixture. The combined filtrate was evaporated to dryness in vacuo at 20° and the residue was dissolved in minimum volume of 50% acetic acid and precipitated with ether - acetone (1:1) to yield a crude product (80% from partially protected peptide) which on paper chromatography gave three spots; Rf (BAW): solvent front, 0.85, and 0.65. The first two spots reacted with ninhydrin and the Pauly's reagent but not with Sakaguchi's reagent. The peptide was purified by chromatography on a 4.5 x 75 cm column of coarse Sephadex G-25, using BAW as the developing solvent. Individual fractions of 7 ml

were collected. From paper chromatography and ultraviolet absorption data, fractions 67-98, 112-156, and 175-245 were pooled and evaporated to dryness at room temperature in vacuo. The pooled fractions 175-245 (major component) in solution in 50% acetic acid were precipitated with ether-acetone (1:1) mixture to give the peptide with a yield of 55% based on 0.65 mmoles of Boc-phenylalanine esterified on the polymer. A sample was reprecipitated twice from same solvent mixture and dried over P_2O_5, NaOH and paraffin in vacuo. The physical constants of the peptide are given in Table 2. Amino acid ratios found: ACPC, 0.97; Arg, 0.95; Val, 1.05; Tyr, 0.95; Ile, 1.00; His, 1.03; Pro, 1.00; Phe, 1.06. ($C_{52}H_{75}N_{13}O_{10}.2CH_3COOH.H_2O$). The biological activity is given in Table 1.

Aspartyl-Arginyl-ACPC-Tyrosyl-Isoleucyl-Histidyl-Prolyl-Phenylalanine (3-ACPC, 5-Isoleucine)-Angiotensin II. (Peptide II).

This free octapeptide was prepared by the above procedure in a 53% yield. Physical constants are given in Table 2. Amino acid ratios found: Asp, 0.93; Arg, 0.92; ACPC, 0.92; Tyr, 0.90; Ile, 0.97; His, 1.00; Pro, 1.00; Phe, 1.07. Anal. Calcd for $C_{51}H_{71}N_{13}O_{12}.2CH_3COOH.2H_2O$ (1226.32): C,54.84; H,6.82; N,14.85. Found: C,54.15; H,6.59; N,14.92. Biological activity is given in Table 1.

Aspartyl-Arginyl-Valyl-Tyrosyl-Isoleucyl-ACPC-Prolyl-Phenylalanine (6-ACPC, 5-Isoleucine)-Angiotensin II. (Peptide III).

The peptide was prepared from Boc-β-benzyl-aspartyl-nitro-arginyl-valyl-O-benzyl-tyrosyl-isoleucyl-ACPC-prolyl-phenylalanine polymer and was purified by the use of a column of Sephadex G-25, fine (3.5 x 70 cm) with BAPW solvent (Table 2). A yield of 51% of the free peptide was obtained. Amino acid ratios found: Asp, 1.02; Arg, 0.98; Val, 1.00; Tyr, 0.95; Ile, 1.04; ACPC, 0.98; Pro, 1.00; Phe, 1.03. ($C_{50}H_{73}N_{11}O_{12}.CH_3COOH.2H_2O$). The biological activity is given in Table 1.

Aspartyl-Arginyl-Valyl-Tyrosyl-Isoleucyl-Histidyl-ACPC-Phenylalanine (5-Isoleucine, 7-ACPC)-Angiotensin II.(Peptide IV).

The peptide was prepared as describe aboved to give a 52% yield of the desired peptide. Physical constants are given in Table 2. Amino acid ratios found: Asp, 0.98; Arg, 0.96; Val, 1.00; Tyr, 0.93; Ile, 1.02; His, 0.94; ACPC, 0.92; Phe, 1.00. Anal. Calcd for $C_{51}H_{73}N_{13}O_{12}.\frac{1}{2}CH_3COOH.5H_2O$ (1180.30): C, 52.92; H, 7.26; N, 15.43. Found C, 42.34; H, 7.54; N, 15.85. Biological activity is given in Table 1.

Aspartyl-Arginyl-Valyl-Tyrosyl-Isoleucyl-Histidyl-Prolyl-ACPC (5-Isoleucine, 8-ACPC)-Angiotensin II. (Peptide V).

The peptide was prepared from Boc-β-benzyl-aspartyl-nitro-arginyl-valyl-O-benzyl-tyrosyl-isoleucyl-im-benzyl-histidyl-prolyl-ACPC polymer and purified by chromatography on a 3.5 x 70 cm column of Sephadex G-25 coarse, using BAW as the developing solvent. A yield of 55% of 8-ACPC-angiotensin II was obtained. Table 2: Amino acid ratios found: Asp, 1.03; Arg, 1.03; Val, 1.01; Tyr, 1.00, Ile, 1.00; His, 1.06; Pro, 1.05; ACPC, 0.98. Anal. Calcd for $C_{47}H_{71}N_{13}O_{12}.2CH_3COOH.H_2O$ (1148.26): C, 53.34; H, 7.11; M, 15.86. Found: C, 53.69; H, 7.52; N, 15.53. The biological activity is given in Table 1.

	Chromatography					E_G	°mp (dec)	% Y	Sephadex G-25 (Solvent)
	PC		TLC						
	BAW	BAPW	BAW	BAPW	MPW				
(1-ACPC, 5-Ile)-Angiotensin II	0.63	0.76	0.41	0.56	0.84	1.23	218-220	55	Coarse (BAW)
3-ACPC, 5-Ile)-Angiotensin II	0.48	0.53	0.34	0.65	0.80	1.18	234-237	53	Coarse (BAW)
(5-Ile, 6-ACPC)-Angiotensin II	0.68	0.60	0.44	0.75	0.85	1.04	225-228	51	Fine (BAPW)
(5-Ile, 7-ACPC)-Angiotensin II	0.50	0.58	0.33	0.60	0.68	1.21	221-225	52	Coarse (BAW)
5-Ile, 8-ACPC)-Angiotensin II	0.46	0.47	0.32	0.58	0.55	1.22	225-230	55	Coarse (BAW)

Table 2: Physical Constants of ACPC-Angiotensin II Analogs.

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HISTIDINE IN SOLID PHASE PEPTIDE SYNTHESIS: THYROTROPIN RELEASING HORMONE AND THE ANGIOTENSINS.

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Although the presence of histidine in many important natural peptides has provided a strong impetus for development of suitable methods for synthesis of histidine-containing peptides, no fully satisfactory methods have so far been described. While in some cases it has been possible to incorporate histidine into peptides without protection of the imidazole, this approach does not appear to be generally satisfactory. In solid phase peptide synthesis, (SPPS) the use of unblocked histidine cannot be recommended generally, in spite of literature reports of its use in certain syntheses (1,2). Carbobenzoxy imidazoles are too unstable to be generally useful.

In most peptide synthesis, both classical and solid phase, im-benzyl histidine has been used. This blocking group imparts low solubility to its derivatives, and powerful solvents (e.g., dimethyl formamide) must be used in synthetic reactions. More seriously, the only reliable method for removal of the im-benzyl group from the finished peptides has been sodium in liquid ammonia, a reagent which causes rapid cleavage of many peptides at proline bonds. Catalytic hydrogenolysis is frequently very slow, and sometimes fails completely.

The carbobenzoxamidotrifluoroethyl (ZTF) group introduced by Weygand (3) would appear to avoid these difficulties, but the great bulk of the ZTF group has caused chemists to fear adverse steric effects in coupling reactions, especially in SPPS, and the very high cost and general unavailability of the derivative have inhibited its utilization.

Two other recently introduced imidazole blocking groups, the 2,4-dinitrophenyl (Dnp) (4) and p-toluenesulfonyl (Tos) (5) appear to offer much more promise for satisfactory solid phase synthesis of histidine peptides. At least one report of the use of Dnp histidine in SPPS has appeared (6).

A strong nucleophile is required for removal of the Dnp group from imidazoles. Chillemi and Merrifield (6), following the recommendation of Shaltiel, treated the finished peptides with 2-mercaptoethanol for this purpose. When this technique was applied to the synthesis of angiotensin I, it was found that di-Dnp angiotensin I is insoluble in aqueous buffers at pH 8, and satisfactory thiolysis could be done only when a high proportion of DMF was added to the solution. This low solubility also made it necessary to use a powerful solvent (glacial acetic acid) for extraction

of the peptide from the resin after hydrogen fluoride cleavage. To overcome these difficulties, we have developed a method for removal of Dnp blocking groups while the peptide is still attached to the solid phase resin. Of several reagents tried for this purpose, thiophenol was the most satisfactory. Satisfactory removal of Dnp groups from the imidazole ring was achieved by swelling the peptide-resin in a solid phase synthesis vessel in the minimum amount of DMF needed to slurry the resin, adding 20 moles of thiophenol for each mole of Dnp groups, and rocking the vessel at room temperature for one hour. Thiolysis under these conditions was rapid, and was probably complete in most cases within fifteen minutes. The resin was washed thoroughly with DMF, water, ethanol and dichloromethane, and dried. Following cleavage of the peptide from the resin with anhydrous hydrogen fluoride in the usual way, the peptide was extracted with an appropriate solvent. A small amount of intensely yellow Dnp-thiophenol is usually adsorbed to the resin and is removed in the peptide extraction, but it can be readily separated from the peptide by standard purification procedures.

The use of im-tosyl histidine in solid phase synthesis has the advantage that the imidazole blocking group is removed from the peptide simultaneously with other blocking groups during cleavage of the peptide from the resin with HF. In addition, as is the case with Dnp histidine (7), the α-Boc derivative can be esterified to the chloromethyl resin in the standard way when histidine is the C-terminal residue of the peptide being synthesized. Both Boc-Dnp-histidine and Boc-Tos-histidine are quite

Table I. Esterification of Histidine to SPPS Resins

Derivative	Esterification to Chloromethyl Resin	Comments
Benzyl	No	Use hydroxymethyl resin
Dnp	Yes	Normal
Tosyl	Yes	Normal

soluble, and solid phase coupling reactions may be carried out in dichloromethane or chloroform, without the addition of DMF, which is necessary for Boc-Bzl-histidine. The only restriction imposed upon the technique of SPPS using tosyl histidine is that trifluoroacetic acid (TFA) must be used for deprotection of Boc groups, since the tosyl group is readily removed from the imidazole by anhydrous HCl in organic solvents and appears to be converted to tosyl chloride. In our work we have used a 30-minute treatment with TFA:$CHCl_3$ (1:3) satisfactorily; the resin is prewashed once with the same mixture. Table II summarizes imidazole deblocking reagents.

Table II. Removal of Imidazole Blocking Groups

Group	6N HCl 110°	HCl-Dioxane 25°	TFA-$CHCl_3$ 25°	NH_3-DMF 25°	H_2-Pd
Benzyl	-	-	-	-	+
Dnp	-	-	-	+	-
Tosyl	+	+	-	+	+

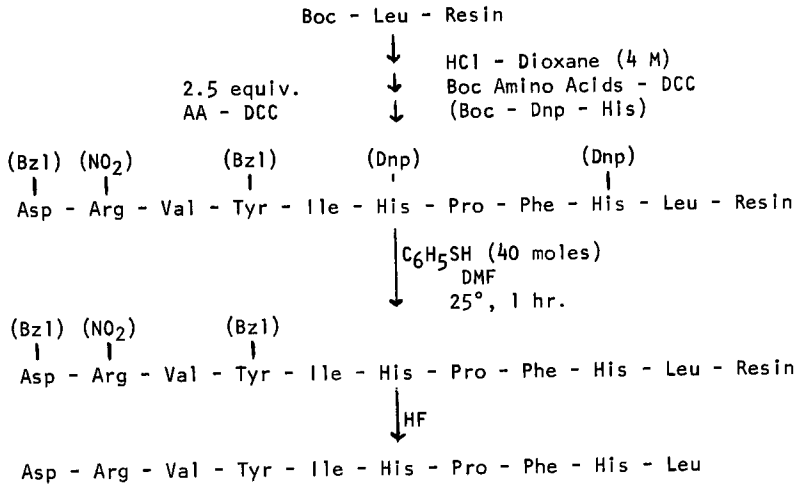

Figure 1. Angiotensin Synthesis with Dnp Histidine

Asp^1-Val^5-Angiotensins I and II were synthesized readily using Boc derivatives of both Dnp- and Tos-histidine, using the methodology outlined above. An automatic instrument (8,9) was used for the synthesis, and coupling reactions were done in chloroform with 2.5 moles of DCC and Boc amino acids per mole of peptide-resin. Although HCl-dioxane removes the tosyl group from histidine, it could be used without difficulty as a deprotection reagent in the synthesis of angiotensin II with tosyl histidine. When the synthesis of angiotensin I was attempted with Tos-histidine using HCl deprotection, the chief product was des-Arg^2-angiotensin I, and angiotensin I was a minor product. A repetition of the synthesis using four moles of amino acids and diimide per mole of leucine-resin gave a crude product which contained approximately equal amounts of these two products. Apparently the free imidazole group on the peptide-resin (liberated by the HCl) prevented the normal coupling reactions from taking place. For this reason the use of unblocked imidazoles cannot be

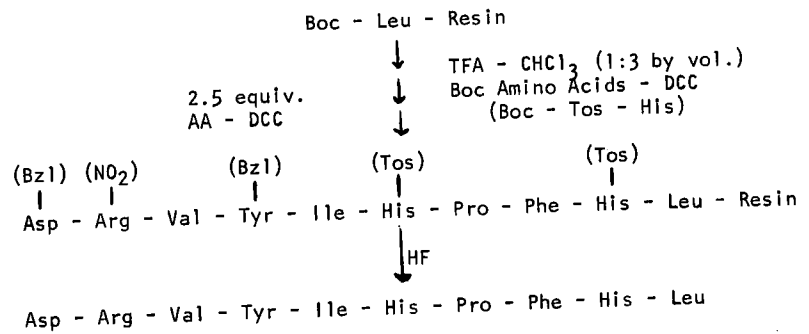

Figure 2. Angiotensin Synthesis with Tosyl Histidine

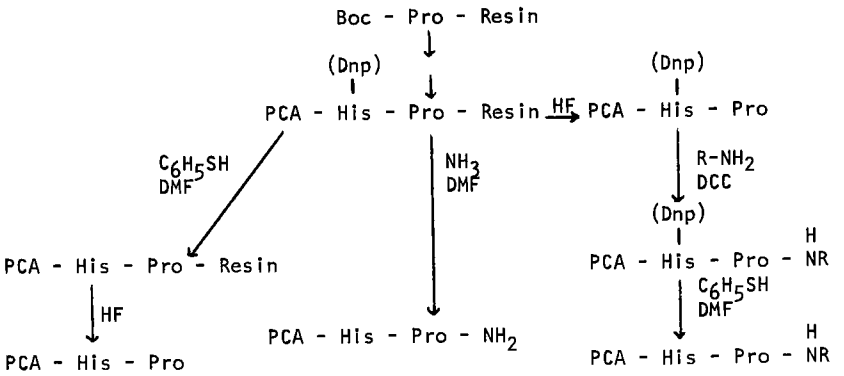

Figure 3. Synthesis of Thyrotropin Releasing Hormone Using Dnp Histidine

recommended in SPPS, and TFA must be generally used as a deprotecting reagent with tosyl histidine.

Thyrotropin releasing hormone, pyroglutamyl-histidyl-proline amide, was synthesized readily by SPPS using either Dnp or Tos histidine. Pyroglutamic acid (PCA) was coupled in the standard way, using DCC for coupling and a solvent composed of DMF-chloroform (1:1). For ammonolysis, the peptide-resins were stirred for three days at room temperature in a pressure bottle with either anhydrous methanol or DMF saturated with ammonia at -20°. The ammonolysis also removed Dnp or Tos groups from histidine. TRH acid, PCA-His-Pro, was prepared by HF treatment of the Tos-His tripeptide-resin, or from the Dnp-His tripeptide-resin by treatment with thiophenol for removal of the Dnp group, followed by HF cleavage. Direct HF cleavage of the Dnp-His tripeptide-resin gave PCA-His(Dnp)-Pro, which was used for coupling in solution with other amines to form a group of substituted amides of TRH.

A benzhydrylamine resin for direct solid phase synthesis of peptide

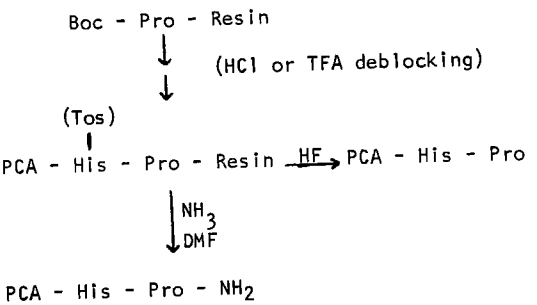

Figure 4. Synthesis of Thyrotropin Releasing Hormone Using Tosyl Histidine

amides was described recently by Marshall (10). A sample of a similar resin, prepared by a different method, was obtained from Schwarz BioResearch, through the courtesy of R. Piasio and M. Guiducci, and was used in a simple synthesis of TRH. Boc proline (0.5 mmole/g) was first

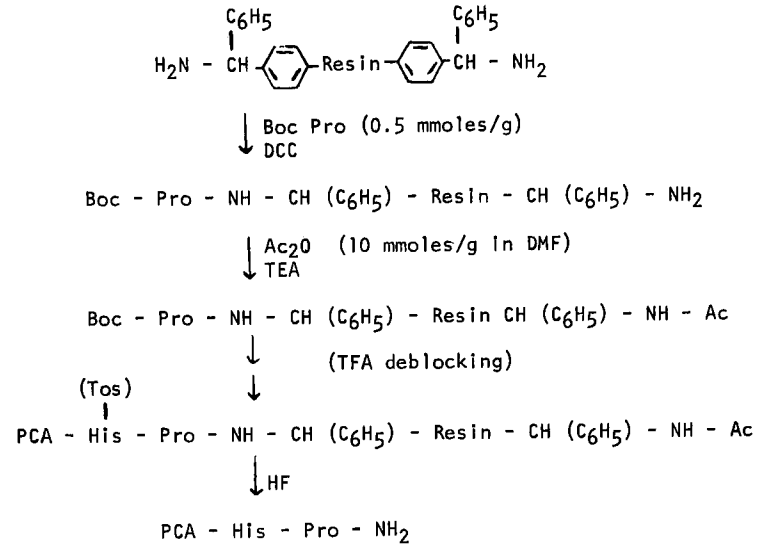

Figure 5. Synthesis of Thyrotropin Releasing Hormone Using Benzhydrylamine Resin

coupled to the amine resin, and all remaining amino groups were then blocked by acetylation of the resin with acetic anhydride and triethyl amine. Tosyl histidine and pyroglutamic acid were then coupled in the usual way, and the tripeptide amide was cleaved from the resin with anhydrous HF.

Both dinitrophenyl and toluenesulfonyl groups appear to be quite satisfactory for blocking of histidine in SPPS, and offer the peptide chemist versatility for the synthesis of a range of types of peptides and derivatives.

This work was aided by Grant HE-12325 from the USPHS, and Grants M68.172 and M70.64C from the Population Council. The authors thank Misses Katherine Hill and Alice Gordon for excellent technical assistance.

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SOLID PHASE SYNTHESIS OF GLUTAMINE - CONTAINING PEPTIDES

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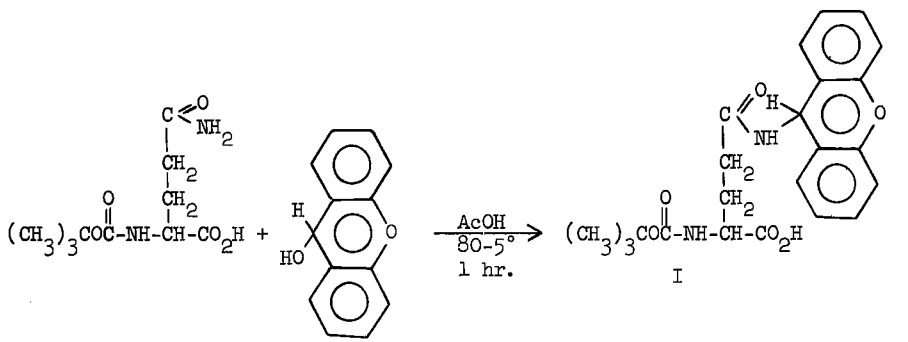
Side reactions and other complications are well known in the synthesis of glutamine-containing peptides (1). For example, there is a strong tendency of glutamine and derivatives to undergo pyroglutamyl formation (1,2) as well as a tendency of the carboxamido group of glutamine to undergo dehydration durring carbodiimide (3) coupling.

One of the more serious problems associated with the solid phase method is the synthesis of C-terminal glutamine peptides. BOC-L-glutamine resin esters cannot be obtained with hydroxy methylated resin (4) by carbodiimide coupling. We had very little success trying to prepare BOC-L-glutamine resin esters directly from BOC-L-glutamine, triethylamine, chloromethylated resin and ethanol (5). After 48 hours of reflux the material obtained was virtually void of carbonyl absorption in the infrared. It had a low chloride equivalent value of 0.12 mmole Cl/g. after deblocking with 4 N HCl in dioxane for 0.5 hour, washing, basifying with triethylamine in DMF and titrating the acidified triethylamine-DMF filtrate with standard silver nitrate. On the other hand, we found that BOC-L-glutamine could be esterified conveniently in yields of 87-95% with dimethyl(arylmethylene)-sulfonium bicarbonate resin (6), the resin capacities being 0.52-0.94 mmole Gln/g., determined as described previously.

Deblocking of H-Gln-O-Resin succeeded quite well with 3-4 N HCl in dioxane without any apparent tendency toward pyroglutamyl formation. This was assessed by comparison of the resin amine capacity (mmoles H-Gln-O-Resin/g.) determined after acid deblocking with the capacity (mmoles BOC-Gln-O-Resin) indicated by nitrogen analysis of the starting ester, e.g., 0.52-0.53 mmoles H-Gln-/g. vs 0.53 mmoles BOC-Gln-/g., respectively. Of interest to us at this point was the stability of H-Gln-O-Resin under various solid phase synthesis conditions. Suspending H-Gln-O-Resin in DMF for nearly 3 days caused only a 4% drop in available amine. Suspension in the resin in 3% trimethylamine in DMF for 18 hours caused a 3% lowering of available amine while N HCl in acetic acid for 0.5 hour caused a 5% drop in amine content. These results show that BOC-L-glutamine resin ester can be cleanly deblocked in 0.5 hour with 3-4 N HCl in dioxane and that subsequent steps, as commonly used in solid phase peptide synthesis, can be followed without substantial chain termination resulting from pyroglutamyl or imide formation. Following in this manner, we prepared H-Ala-Gln-OH (7) and H-Arg-Thr-Gly-Gln-OH ($HGH^{131-134}$) (8) in yields of 37-46%.

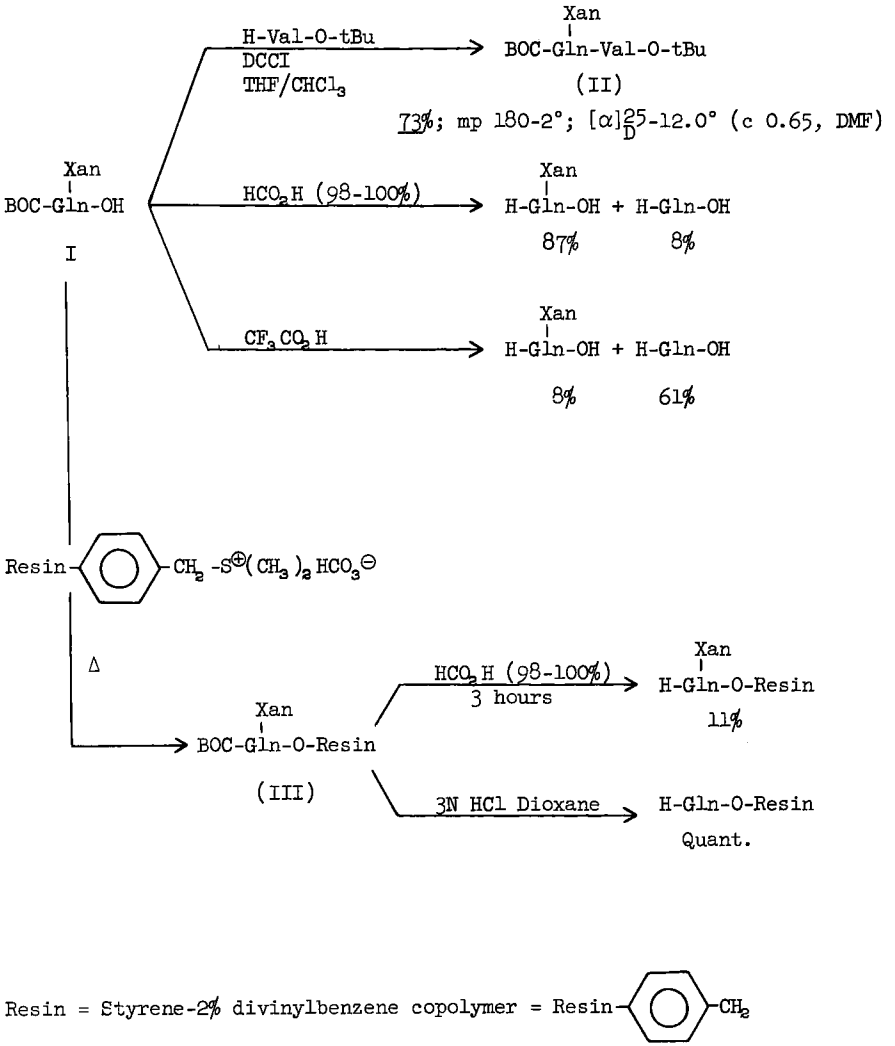
Next we directed our attention to the solid phase synthesis of peptides containing glutamine elsewhere in the chain. Akabori and co-workers (7) described the preparation and use of N^{α}-Cbz-N^{δ}-xanthyl-L-glutamine for the synthesis of C-terminal glutamine peptides. We desired to determine whether the amide protecting xanthyl group could be adapted for solid phase work, viz., for use in conjunction with the α-amino protecting BOC group. N^{α}-BOC-N^{δ}-xanthyl-L-glutamine (I) was prepared in 67% yield by heating BOC-L-glutamine and xanthydrol in glacial acetic acid at 80-85° for 1 hour. The crude product was precipitated with water, dried and recrystallized from chloroform/hexane, mp. 151-152°.

Reactions of I are summarized in Figure 1. As indicated, attempt to effect selective deblocking of the N^{α}-BOC group of I with 98-100% formic acid (9) was only partially successful, resulting in a 87% yield of N^{δ}-xanthyl-L-glutamine and a 8% yield of free glutamine. Treatment of I with



trifluoroacetic acid for 1 hour also did not proceed very cleanly. As expected, I could be coupled by dicyclohexylcarbodiimide as illustrated by the formation of BOC-Gln(Xan)-Val-O-tBu (II) from H-Val-O-tBu and I in 73% yield. Attempts to effect BOC deblocking of the resin ester (III) of I with formic acid was even less successful leading only to ca. 11% deblocking of the BOC group. However, treatment of III with 3 N HCl in dioxane effected a clean and quantitative removal of both protective groups.

These reactions show that the N^{δ}-xanthyl group cannot be used as a permanent amide protective group for glutamine when used in conjunction with the N^{α}-BOC group in solid phase applications. However, we feel that I may still be useful in solid phase synthesis since it can be readily prepared and coupled directly with carbodiimides. This may be particularly useful with automated synthesizers. Once I has been coupled to a peptide chain, both N-protective groups should be cleanly removable by 3-4 HCl in dioxane and the synthesis continued in the usual manner.



Resin = Styrene-2% divinylbenzene copolymer = Resin--CH_2

Figure 1

Reactions of I

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SYNTHESES OF POLYPEPTIDES BY SOLID PHASE FRAGMENT COUPLING

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Introduction. Solid phase (SP) stepwise synthesis by Merrifield has provided a rapid method for preparation of several peptides. Since this method is composed of successive chemical reactions on resin without purification of intermediates, quantitative completion of each reaction is essential in order to obtain an objective peptide in high purity. If some steps of successive reactions do not proceed quantitatively, a final product may be contaminated with numbers of very similar deficient peptides.

Therefore the introduction of fragment condensation technique to SP synthesis may offer advantages of fewer steps in a long sequence and of convenience in purification of polypeptide product. Several studies in this field have been reported recently. Some six papers[1] have described the use of BOC-dipetide acid or BOC-dipeptide acid active ester (n=2) for the peptide bond formation with amino group in a resin. Similarly, there have been found some four papers (n=3),[2] three papers (n=4),[3] four papers (n=5),[4] and one or two papers on BOC-hexa,[5] BOC-hepta[4] and BOC-octapeptide acid.[6]

Weygand reported that the presence of a bulky amino acid such as Ile or Val in C-terminal position in BOC-peptide acid caused the delay on complete formation of a peptide bond with peptidyl-resin.[7]

The present paper reports on our several experiments of basic studies related to SP fragment coupling, and on synthesis of basic pancreatic trypsin inhibitor by SP stepwise method which was conceived by sequences of experiments related to SP fragment coupling.

Reaction of BOC-Tripeptide Acid and Chloromethylated Resin. If a BOC-peptidyl-resin will be prepared by the reaction of corresponding BOC-peptide acid with chloromethylated resin in a good yield without racemization, the BOC-peptidyl-resin thus obtained will be a useful starting material for further SP stepwise or fragment coupling. We studied on a model case of BOC-Gly-L-Ala-L-Leu-OH and chloromethylated resin and obtained a satisfactory results.

An example of our results will be described as follows. A mixture of BOC-Gly-L-Ala-L-Leu-OH (1.5 equiv), chloromethylated resin (1 equiv) (2%; Cl 1.6 mM per g), NEt_3 (1.5 equiv) in ethanol and chloroform (2:1) was heated for 48 hr. The protected peptidyl-resin was treated with HF, and the filtrate was analyzed directly by an amino acid analyzer; content of BOC-tripeptide acid in the resin obtained was 0.89 mM per g and no racemization was observed at Leu component as judged by our technique detecting possible racemization.[8]

Possible Racemization during Reaction of BOC-Gly-L-Ala-OH and H-L-Leu-resin. It is recognized at present that the use of DCC and HOSu does not cause any racemization during coupling of BOC-peptide acid and amino group in a resin. However, Weygand reported the occurrence of some 9% racemization on pMZ-L-Leu-L-Phe-OH with H-L-Val-resin at a certain condition (DCC + HOSu, DMF, 24 hr shaking at room temp).[9] We observed, however, that almost no racemization occurred when BOC-Gly-L-Ala-OH was coupled with H-L-Leu-resin with the same condition. Furthermore, we recognized that the use of HOBT (hydroxybenzotriazole)[10] instead of HOSu accerelated the reaction rate without racemization.

A Hexapeptide Fragment of Cobrotoxin.[2] Since there was no attempt to compare a definite peptide sequence obtained by two SP methods (stepwise and fragment), we attempted to prepare a hexapeptide sequence (47 to 52 position) of cobrotoxin. Amino acid sequence of the venom was determined by Yang.[11] Figure 1 indicates scheme of synthesis of a protected hexapeptidyl-resin (I) by two methods.

Each of IA~IC was treated with HF and each filtrate was evaporated to yield a powder (IA-HF~IC-HF). Amino acid analysis on acid hydrolysate of three samples gave reasonable values corresponding to component amino acids. However, column chromatography by an amino acid analyzer on I-HF samples indicated the difference between IA-HF and IB- or IC-HF. Several peaks of by-products were recognized on the chromatogram of IA-HF, moreover, the shape of the main peak was unsymmetrical with a shoulder. Whereas IB- or IC-HF showed a symmetrical main peak (93 ml by 0.9 x 50 cm column with pH 5.28 buffer). Analytically pure hexapeptide (II) was isolated in a good yield from a reaction mixture of IB or IC and HF.

The results obtained indicate that SP fragment coupling will give purer material than usual SP stepwise procedure for some oligopeptides.

Influence of Chain Length in BOC-Peptide Acid for Reaction Time. There are many fundamental investigations in usual SP stepwise synthesis. For example, Esko et al reported the results on the reaction time to be needed for the completion of peptide bond formation with several BOC-amino acid and an amino group in a resin.[12] However, there is a very few concerning the fundamental and quantitative experiments on SP fragment coupling. We are trying to know the influence of chain length in BOC-peptide acid for reaction time through the experiment shown in Fig. 2. We prepared BOC-(εZ-Lys)$_n$-OH (n = 1, 2, 4, 6) in quantity by usual solution method. The detail results will be reported later.

Synthesis of BPTI (Basic Pancreatic Trypsin Inhibitor) by SP Stepwise Method. a. Strategy of SP Fragment Coupling; Reason to undertook the Study of BPTI Synthesis. As it was described in the section of the Introduction, in SP fragment coupling method, a polypeptide will be built up by either rather shorter components such as BOC-dipeptide acid or longer components such as BOC-octapeptide acid.

Suppose that we attempt to prepare a natural polypeptide in considerably large size with specific biological activity by SP stepwise method. If we obtain a polypeptide with certain degree of activity, but cannot purify to attain the same level of the natural material, SP fragment coupling with shorter components may be applied favorably to afford purer material than stepwise method. In this case, we can insert a notorious amino acid such as Ile, Val or Thr(Bzl) like BOC-X-Ile-Y-OH (X, Y; a common amino acid). If we obtain a polypeptide without any activity by SP stepwise method, SP fragment coupling with longer components may give a material with certain activity.

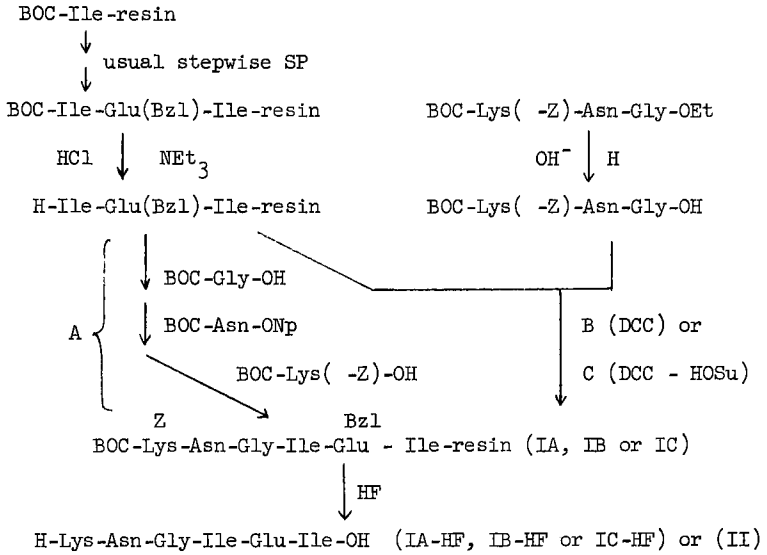

Fig. 1. Synthesis of hexapeptide by various reaction sequences.

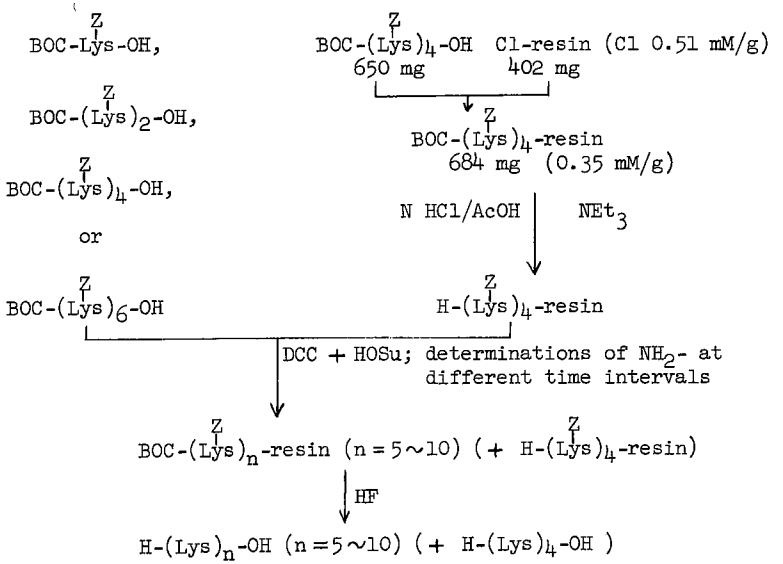

Fig. 2. Condensation of BOC-peptide acid in different sizes and a peptidyl-resin.

We had been heard that several laboratories attempted to prepare BPTI by SP stepwise method and obtained a material without activity. Therefore, we had expected that BPTI might be a good target to be synthesized by SP fragment coupling with rather longer components. Before we start this SP fragment coupling, we carried out the usual SP stepwise synthesis to confirm that a material obtained will be biologically inactive.

On the other hand, a group in our laboratory is continuing the studies concerning the mode of action of trypsin on synthetic peptide substrates and inhibitors; for example, we synthesized a N-terminal nonapeptide of trypsinogen, H-Val-Asp-Asp-Asp-Asp-Lys-Ile-Val-Gly-OH (III), by a solution method and observed that tetra Asp sequence of III possessed inhibition ability toward trypsin action for Lys-Ile bond in III.[13] Therefore, we were interested for the study of BPTI by synthetic approach.

b. Structure of BPTI. The sequence of 58 amino acid residues of BPTI was determined in several laboratories, the structure being shown in Fig. 3.[14] The amino acid sequence of kallikrein inactivator isolated from bovine lung or bovine parotid was reported to be identical with that of BPTI.[15]

c. Procedure of SP Stepwise Synthesis. Commercial 2% cross-linked polystyrene was chloromethylated by the usual manner,[16] and the resin with 0.88 mM Cl per g was obtained. This was changed to BOC-Ala-resin with 0.20 mM BOC-amino acid per g. The general procedure of SP method by mannual way was applied for synthesis of BOC-polypeptidyl-resin of 58 amino acid residues with the sequence of BPTI. The schedule of a step for the coupling of each BOC-amino acid was shown in Table 1. BOC-Ala-resin (1 g; 0.2 mM) was used as starting material.

The BOC-amino acids with protected side chains were: Lys(ϵ-Z), Arg(NO_2), Ser(Bzl), Thr(Bzl), Asp(Bzl), Glu(Bzl), Met(O) and Cys(MBzl). The coupling time for Val, Ile and Thr(Bzl) was 12 hr each, and that for amino acid next to them was 6 hr. For Ile-18 next to Ile-19 the coupling time was set for 24 hr. BOC-Arg(NO_2)-OH was reacted in DMF for 8 hr. Coupling of Gln and Asn was carried out in DMF for 12 hr using corresponding p-nitrophenyl esters.

Although S-Bzl group is used widely for SP synthesis, S-MBzl (p-methoxybenzyl) group was used for the protection of Cys because MBzl is sufficiently stable in HCl/AcOH but moderately labile in HF in contrast with S-Bzl group.[17] BOC groups were removed with N HCl/AcOH. As an exception, trifluoroacetic acid was used for BOC-Gln residue.

d. Purification of Polypeptide isolated from Protected Peptidyl-resin. Synthesis and purification of a polypeptide were summarized in Fig. 4. Cleavage of a polypeptide from its solid support together with the removal of all protecting groups was achieved by treatment with HF in the presence of anisole.[18] After evaporation of the solvent and drying in vacuo, the residue was washed with ethyl acetate and extracted with 10% AcOH. The crude peptide (79 mg) obtained by lyophylization of the extract was converted to S-sulfonate with sodium sulfite and cystein in 8 M urea,[19] and the S-sulfonate was purified on Sephadex G-25 column (0.9 x 50 cm) with 0.1 M AcOH. The fraction eluted at the same volume as natural BPTI(SSO_3^-)$_6$ were collected (yield, 62.9 mg).

The chromatographic patterns with Sephadex were shown in Fig. 5. Twenty and five mg from 62.9 mg BPTI(SSO_3^-)$_6$ was reduced by treatment with -mercaptoethanol. The reduced peptide was obtained from the reaction

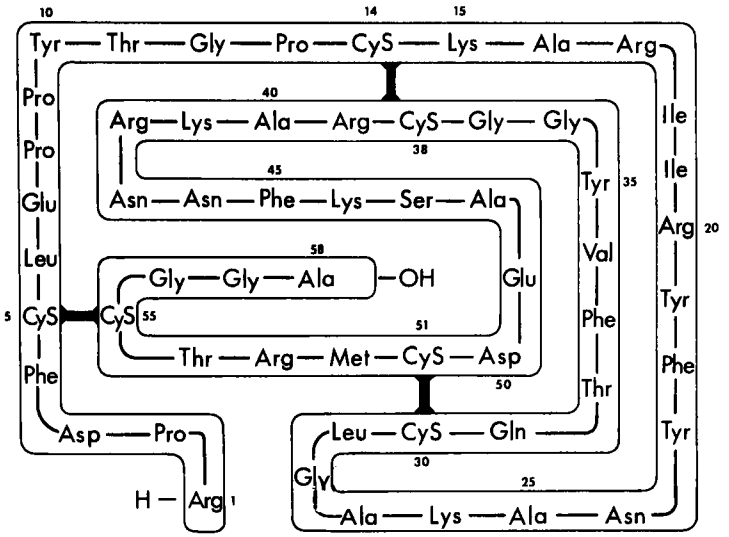

Fig. 3. Amino acid sequence of BPTI.

Table 1. Schedule for SP stepwise synthesis of BPTI.

Step	Reagent	Vol (ml)	Time (min)	Times
1	AcOH	7	5	3
2	N HCl/AcOH	7	30	1
3	AcOH	7	5	3
4	EtOH	7	5	3
5	DMF	5	5	3
6	10% NEt_3	5	10	1
7	DMF	5	5	3
8	CH_2Cl_2	5	5	3
9	BOC-AA/CH_2Cl_2	(0.8mM/5ml)	10	1
10	DCC/CH_2Cl_2	(0.8mM/5ml)	240	1
11	CH_2Cl_2	5	5	3
12	EtOH	7	5	3

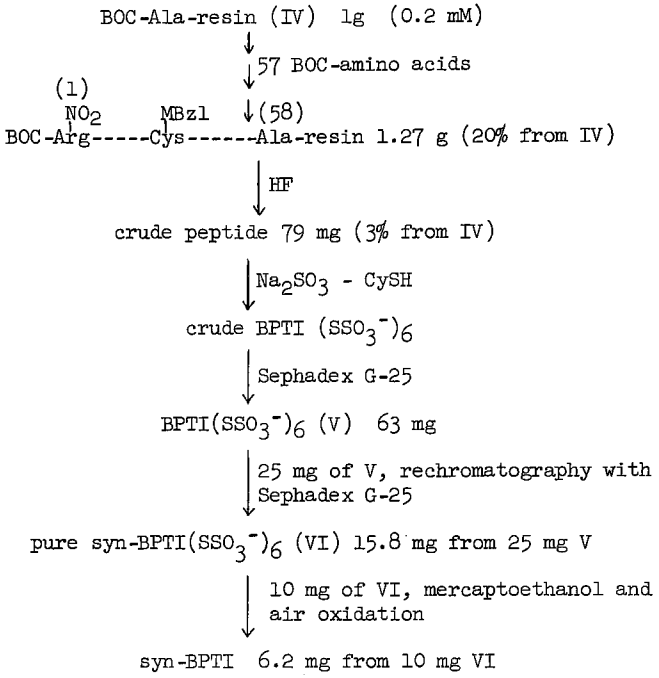

Fig. 4. Summary of BPTI synthesis.

mixture by Sephadex G-25 (0.9 x 50 cm) and oxidized by air to form the three disulfide bonds. The solution was chromatographed by Sephadex and peptide portions were lyophylized to yield a synthetic BPTI (6.2 mg).

To obtain a control material, native crystalline BPTI (10 mg) purchased from Worthington Biochem. Corp. was converted to the S-sulfonate (8.1 mg), reduced, and reoxidized as described above. The final peptide obtained was designated as regenerated BPTI.

e. Properties of Synthetic and Native BPTI. The synthetic BPTI ($\underline{R}$ 0.81 x His) was indistinguishable from the native inhibitor by paper electrophoresis at pH 3.6 (Fig. 6).

The both peptides afforded similar patterns on polyacrylamide gel disc electrophoresis (Fig. 7). When the synthetic BPTI was mixed with trypsin and the mixture was developed on the disc gel, the furnished enzyme-inhibitor complex moved as a single band identically to the native EI complex.

Amino acid analyses of acid hydrolysate of the purified synthetic BPTI$(SSO_3^-)_6$ gave reasonable values compared with those of the native BPTI (Table 2).

The fingerprints of the both materials were similar (Fig. 8). Each (2 mg) of the synthetic BPTI$(SSO_3^-)_6$ and the native BPTI was treated with mercaptoethanol and subsequently monochloroacetic acid in alkaline solution. The modified peptide was digested with trypsin (0.1 mg) for 24 hr

and the digest was applied for paper chromatography and electrophoresis.

f. Biological Activities of Synthetic and Native BPTI. We assayed an esterase inhibition activity with native and synthetic peptide for a system of trypsin and Tos-L-Arg-OMe as described in literature.[20] It was found that the regenerated native BPTI showed 35-39% activity of native crystalline BPTI and the synthetic BPTI showed 30-34% activity. These results were reported briefly.[21]

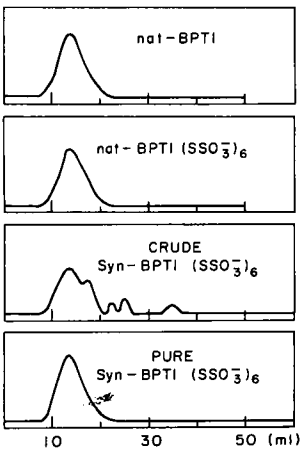

Fig. 5. Patterns by Sephadex G-25 column (0.9 x 50 cm) with 0.1 M AcOH on several polypeptides. Vertical, 280 mμ.

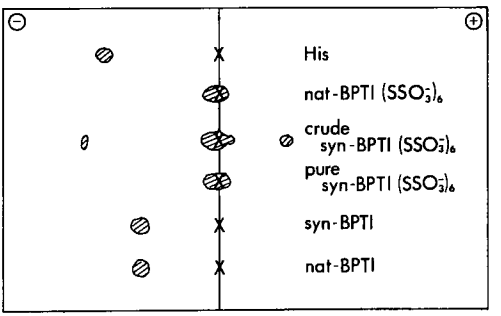

Fig. 6. Patterns in paper electrophoresis on several polypeptides. Solvent, pyridine-AcOH-H_2O pH 3.6; 600 V/25 cm, 2 mA/cm; 2 hr.

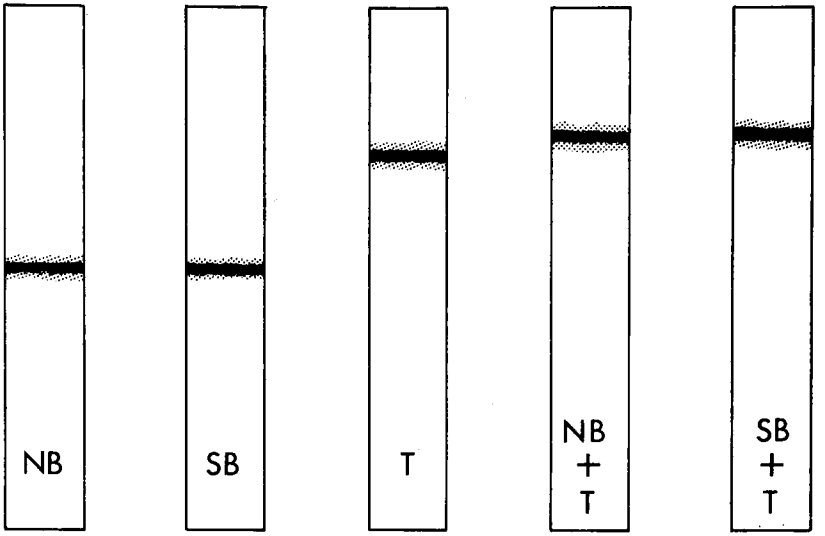

Fig. 7. Polyacrylamide gel electrophoresis of several materials. NB, native BPTI; SB, synthetic BPTI; T, trypsin. Buffer, β-Ala/AcOH pH 4.5; 3 mA per tube; 2 hr; amide black stain.

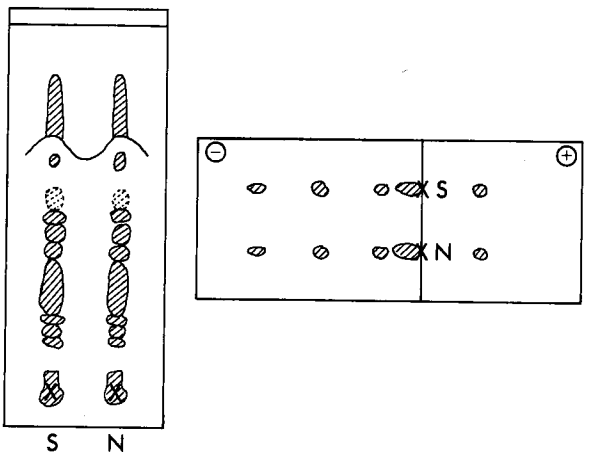

Fig. 8. Paper chromatography (left) and electrophoresis (right). S, tryptic digest of CM-synthetic BPTI; N, tryptic digest of CM-native BPTI.

Table 2. Amino acid analyses of native and synthetic materials.

Samples were hydrolyzed in 6 N HCl for 24 hr at 110 , and analyzed by Hitachi KLA3B amino acid analyzer. Cys and Met were not analyzed for synthetic BPTI(SSO_3^-)$_6$, and Cys for native BPTI.

Amino acid	Native BPTI		Synthetic BPTI(SSO_3^-)$_6$
	Lit.	Found	
Lys	4	4.27	3.66
Arg	6	4.72	4.80
Asp	5	5.11	6.38
Thr	3	2.79	3.46
Ser	1	0.81	0.99
Glu	3	3.50	3.23
Pro	4	3.41	3.11
Gly	6	6.05	6.00
Ala	6	5.23	6.47
Val	1	0.91	0.81
Met	1	0.31	
Ile	2	1.62	1.12
Leu	2	1.89	1.49
Tyr	4	2.49	2.27
Phe	4	3.94	3.45

Table 3. Proteinase inhibitory activities of polypeptides. System, 0.3% casein - trypsin; assay, Folin method.

Amount added (g)	Inhibition (%) native BPTI	syn BPTI
40	100	100
20	100	100
10	93	50
5	42	17
2.5	15	

Dr. Sawai of Teikoku Hormone Mfg. Co. in Japan carried out an assay of a proteinase inhibition activity with our samples for a system of trypsin and casein, the results being indicated in Table 3. Dr. Sawai also observed that the synthetic material showed strong kallekrein inactivation activity.

g. Remark. In view of the results obtained above, it seems that we prepared considerably pure polypeptide correspond to the sequence of BPTI though the presence of very closely related molecules may be possible in slight extent. The results yielding active material was somewhat unexpected for us. We are at present carrying out further purification of synthetic BPTI and native regenerated BPTI by procedures such as a complex formation with pure trypsin. When it cannot be obtained a fully pure BPTI by SP stepwise method, SP method with short fragments may be applied efficiently to yield purer product.

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SOLID PHASE SYNTHESIS AND SOME PHARMACOLOGICAL PROPERTIES OF 4-THREONINE ANALOGS OF THE NEUROHYPOPHYSIAL HORMONES

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Introduction. Molecular biologists like to talk about B.C. and A.C.; 1953 being their year one. We peptide chemists on the other hand like to talk of B.D. and A.D.; 1953 also being our year one. We are now in the year 17 A.D. (after du Vigneaud) and since the original synthesis of oxytocin (1), over 200 analogs, at least, of oxytocin and vasopressin have been synthesized (2). You may well wonder why I should be here today talking about yet another analog of oxytocin. Well, I can assure you that this time last year I had no idea that I would be here talking about the analog I am now going to tell you about.

The story I shall unfold concerns the discovery of a new analog of oxytocin, 4-threonine-oxytocin (3,4), in which the glutamine residue in the four position is replaced by threonine. This analog has been found to have most intriguing and surprising properties; properties which would not have been predicted on the basis of structure-activity relationships. I would like to begin by telling you how we came about the discovery of this new and intriguing peptide analog.

What Prompted the Synthesis of [4-Threonine]-Oxytocin. The structures of the seven known naturally occuring neurohypophysial hormones are shown in Table 1.

AMINO ACID SEQUENCES OF KNOWN NATURAL NEUROHYPOPHYSIAL PRINCIPLES

Common structure with variable amino acids in position 3, 4, and 8 denoted by X:

1 2 3 4 5 6 7 8 9

Cys-Tyr-(X)-(X)-Asn-Cys-Pro-(X)-Gly-NH_2

Known Neurohypophysial Principles	Amino Acids in Position 3	4	8
1. Vasotocin	Ile	Gln	Arg
2. Mesotocin	Ile	Gln	Ile
3. Isotocin	Ile	Ser	Ile
4. Glumitocin	Ile	Ser	Gln
5. Oxytocin	Ile	Gln	Leu
6. Arginine vasopressin	Phe	Gln	Arg
7. Lysine vasopressin	Phe	Gln	Lys

Table 1.

These include the basic-pressor principles and the neutral oxytocin-like principles. I would like you to focus your attention on the fact that all of these possess the same basic cyclic octapeptide structure with differences occuring in the 3, 4, and 8 positions. You might also note the presence of either a glutamine or a serine residue at position 4. A few years ago, after adapting the Merrifield method toward the successful synthesis of oxytocin (5), I began a collaboration with Bill Sawyer on a study centered around the attempted characterization of a new oxytocic principle isolated from the pituitary gland of the spiny dogfish (Squalus acanthias) termed EOP 1 (Elasmobranch Oxytocic Principle 1) (6). We had been trying to determine its structure using educated guesswork based on a) the pharmacological properties of the unknown principle; b) the structures as shown in Table 1; c) the genetic code; and d) the synthetic approach. The idea was to compare the pharmacological properties of the synthetic analog with those of the natural principle and thus hopefully arrive at the identity of the latter.

We had not had much luck in this. As a diversion about two years ago we tried to follow up a speculation which had been put forward by Vliegenthart and Versteeg (4) and by Geschwind (8) that there might, in fact, be a "missing link" between the 4-serine and 4-glutamine containing neurohypophysial hormones. This "missing link" was postulated on the basis that a mutation from serine to a glutamine residue can only occur by a double mutation in their nucleic acid codons; with codons for proline or nonsense codons serving as an intermediate. We made the 4-proline analogs of oxytocin, mesotocin and glumitocin (9) and at the same time Joe Rudinger made the 4-proline derivative of mesotocin (10). We found that these analogs were very weakly active. We then decided to have a look at other possible amino acids in the four position. This led to the synthesis of [4-lysine mesotocin]. This was also weakly active (11). Since a serine to threonine mutation required only a single base change, we thought it might be worthwhile having a look at the properties of [4-threonine-oxytocin] and set about synthesizing it.

[4-Threonine]-Oxytocin: Unexpected Findings. After the synthesis was completed, I sent a sample off to Bill Sawyer and thought very little more about it until a couple of months later when I met him at the Winter Symposium Meeting in Miami. He greeted me rather excitedly, "Maurice, this analog you sent me is extremely hot, incredibly potent, much more potent, in fact, than oxytocin!" I was most surprised and delighted, but also somewhat skeptical. In fact, on talking it over we both became a little worried by this totally unexpected finding. Bill worried about whether he might be weighing the sample inaccurately, and I worried about the possibility of a "goof-up" somewhere along the line in the synthesis. It all appeared too good to be true. I said, "Let's go back and repeat everything. I'll repeat the synthesis; you repeat the pharmacology." We did this and found that the analog did indeed have striking properties. In Table 2, you will see why we were so excited by this new analog.

PHARMACOLOGICAL ACTIVITIES (IN USP UNITS PER MILLIGRAM) OF 4-THREONINE-OXYTOCIN AND OXYTOCIN

COMPOUND	RAT UTERUS	FOWL VASO-DEPRESSOR	RABBIT MILK EJECTION	RAT VASO-DEPRESSOR	RAT ANTI-DIURETIC
4-Thr-Oxytocin	900	1480	540	0.43	3
Oxytocin	450	450	450	5	5

Table 2.

Properties of [4-Threonine]-Oxytocin. Table 2 shows [4-threonine]-oxytocin to be twice as potent as oxytocin in the rat uterus assay system; to be three times as active as oxytocin in the fowl vasodepressor assay system and 20% more active than oxytocin in the rabbit milk ejection assay. So you can see it is indeed an incredibly potent oxytocic substance. Of further interest is the observation that the vasopressin-like qualities of this hormone are greatly diminished. The pressor activity is only 1/10 that of oxytocin and the antidiuretic activity is only about 1/2; in fact, Bill Sawyer tells me that in direct assays, one against the other, [4-threonine]-oxytocin possesses only about 1/3 the antidiuretic activity of oxytocin.

The Implications of the Finding. So, here we had created by sheer serendipity in the laboratory a peptide with very specific oxytocin-like activities, more specific than oxytocin itself and with diminished vasopressin-like activities. This finding gave a new impetus to our thinking in three broad areas related to the neurohypophysial hormones as indicated as follows:

A) PHYLOGENY
B) STRUCTURE-ACTIVITY RELATIONSHIPS
C) POSSIBLE CLINICAL APPLICATION

I do not have time today to go into detail concerning the phylogenetic implications. This is a very intriguing story. We at first thought it might lead to all possible kinds of ramifications in terms of whether or not this analog might occur in nature. At least, I did; I tended to get somewhat carried away in speculation only to be brought back to earth by my more experienced colleague, Bill Sawyer. Now, the consensus seems to be that it probably does not occur in nature. I do not have time either to discuss the possible clinical applications other than to mention the possibility that use of [4-threonine]-

oxytocin might offer distinct advantages over oxytocin by virtue of the enhanced oxytocic activity and diminished antidiuretic activity should studies indicate that it possesses similar activities in humans. The third area--that of structure-activity relationships--offered the possibility of immediate investigation. The remainder of my talk will thus be devoted to this area.

[Deamino-4-Threonine]-Oxytocin. Would This Be Even More Potent? Those of you in the oxytocin field will recall the great excitement caused by the report from du Vigneaud's laboratory a number of years ago on deamino-oxytocin (12). Deamino-oxytocin is simply oxytocin without the amino group. It was found to be much more potent than oxytocin. By analogy we wondered what might happen if we were to synthesize the deamino derivative of [4-thr]-oxytocin. We had created what we liked to call "supertocin"; by taking off the amino group we wondered whether we might succeed in making a "superdupertocin." Well, unfortunately, as you shall see from the data in Table 3, this was not to be.

SOME PHARMACOLOGICAL ACTIVITIES (IN USP UNITS PER MILLIGRAM) OF [1-DEAMINO-4-THREONINE]-OXYTOCIN COMPARED WITH THOSE OF [4-THREONINE]-OXYTOCIN, [1-DEAMINO]-OXYTOCIN, AND OXYTOCIN

	RAT UTERUS	FOWL VASO-DEPRESSOR	RABBIT MILK EJECTION	RAT ANTI-DIURESIS	RAT VASO-PRESSOR
[1-Deamino-4-Thr]-Oxytocin	150	780	---	0.9	<0.1
[4-Thr]-Oxytocin	900	1480	542	1 to 3	0.43
[1-Deamino]-Oxytocin	803	975	541	19	1.44
Oxytocin	450	450	450	5.0	5.0

Table 3.

Our [deamino-4-threonine]-oxytocin is even less potent than oxytocin in the rat uterus assay system. We don't have an accurate figure for the milk ejection activity but Bill Sawyer tells me that it appears to be low. We were very surprised at this finding and still do not have an adequate explanation for this. The antidiuretic and the pressor activities, as was expected, are very low. You might also note the dramatic differences between the antidiuretic activities of deamino-oxytocin and [4-threonine]-oxytocin.

[3-Leucine-4-Threonine]-Oxytocin: An Unwanted Analog. With the results so far, we were suspended between elation on one hand and great disappointment on the other. Not knowing whether in fact what we had found with [4-threonine]-oxytocin was truly for real. Why had the threonine residue in the 4-position of oxytocin given enhanced oxytocin-like activities, but in the deamino-oxytocin given diminished oxytocin-like activities? So, we said, "let's make another analog of oxytocin." The analog we chose was mesotocin. Mesotocin differs from oxytocin only in having isoleucine in the position 8 instead of leucine. We set about making this analog and had synthesized and purified it in about three weeks. We were just about ready to send off a sample to Bill Sawyer when, lo and behold, the amino acid analysis on the protected nonapeptide came back and we found that instead of having two isoleucines in the molecule we had two leucines, which meant that we had made [3-leucine-4-threonine]-oxytocin and not the desired [4-threonine]-mesotocin. We

traced the error back to the bottle labelled "Boc-L-Isoleucine" obtained from the supplier and found that it contained, in fact, Boc-L-Leucine. After the initial burst of irritation had subsided it was possible to be a little more sanguine about this unexpected set-back; 3-leucine-oxytocin had been synthesized and its pharmacological properties published (2) so we thought it might be a good idea to compare the properties of our unplanned analog with those of [3-leucine]-oxytocin. The results are shown in Table 4.

SOME PHARMACOLOGICAL ACTIVITIES (IN USP UNITS PER MILLIGRAM) OF [3-LEUCINE, 4-THREONINE]-OXYTOCIN AND [3-LEUCINE]-OXYTOCIN

ANALOGUE	ISOLATED RAT UTERUS	FOWL VASO-DEPRESSOR	RAT VASO-PRESSOR
[3-Leu, 4-Thr]-Oxytocin	26	54	<0.1
[3-Leu]-Oxytocin	4.4	10	0.3

Table 4.

Interestingly enough, we found that the new analog followed the trend established by [4-threonine]-oxytocin; it exhibited enhanced oxytocin-like characteristics and diminished vasopressin-like activities. So at least we felt that we were on the right track.

[4-Threonine]-Mesotocin Is Also Very Active! In the meantime, we synthesized [4-threonine]-mesotocin and again very interestingly we found, as shown in Table 5, that the oxytocin-like activities were enhanced; we had increased rat uterus activity and increased fowl vasodepressor activity (11). We do not have an accurate value yet for the milk ejection activity.

SOME PHARMACOLOGICAL ACTIVITIES (IN USP UNITS PER MILLIGRAM) OF [4-THREONINE]-MESOTOCIN AND ISOTOCIN

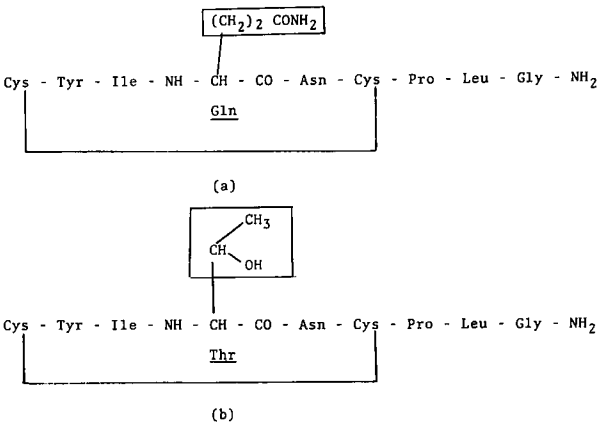
	RAT UTERUS IN VITRO	FOWL VASO-DEPRESSOR	RAT ANTI-DIURESIS	RAT VASO-PRESSOR
[4-Thr]-Mesotocin	520	1545	2.6	1.08
Mesotocin	290	500	4.3	6.3
Isotocin	132	320	0.7	0.04

Table 5.

You will notice that in Table 5 we have [4-threonine]-mesotocin, mesotocin and isotocin with threonine, glutamine, and serine occupying the 4 position in this order. You might note the gradual diminishment of oxytocin-like activities in these analogs. This will have a bearing on our discussion of why the threonine residue brings about such a marked enhancement of activities. In Table 5 you can see that the vasopressin-like activities are again diminished. When assayed directly against mesotocin, [4-threonine]-mesotocin exhibits only 1/3 of the antidiuretic activity of the former compound.

How Is the Enhancement of Activities Being Brought About? A Possible Explanation (11). We were very excited by these findings. This

now confirmed the fact that the threonine residue was exerting an unprecedented influence in the oxytocin molecule. The big question, of course, was: How was it doing it? If you look at the structures of oxytocin and 4-threonine-oxytocin (as shown below), you can see that there are very minor differences between these two structures.



Structures of (a) Oxytocin and (b) 4-[Thr]-Oxytocin.

In fact, the only differences occur at the β-carbon atom in the 4 position. I ask you to bear this in mind and to keep your thinking focused on this position because in Table 6 you will see that we have here a number of side-chains of 4-substituted oxytocin analogs showing the substituents attached to the common β-carbon atoms. Also given is the value for the rat uterus activity of each analog.

CORRELLATION OF SIDE-CHAIN STRUCTURES WITH RAT UTERUS ACTIVITIES OF 4-SUBSTITUTED OXYTOCIN ANALOGS (11)

AMINO ACID IN 4-POSITION	STRUCTURE OF SIDE-CHAIN	RAT UTERUS ACTIVITY
Threonine	$-\overset{CH_3}{\underset{}{CH}}-OH$	900
Glutamine	$-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-NH_2$	450
Serine	$-CH_2-OH$	195
Valine	$-\overset{CH_3}{\underset{}{CH}}-CH_3$	140
Asparagine	$-CH_2-\overset{O}{\overset{\|}{C}}-NH_2$	108
α-aminobutyric acid	$-CH_2-CH_3$	72
Norvaline	$-CH_2-CH_2-CH_3$	61
Isoleucine	$-\overset{CH_3}{\underset{}{CH}}-CH_2-CH_3$	37
Alanine	$-CH_3$	36
Leucine	$-CH_2-\overset{CH_3}{\underset{}{CH}}-CH_3$	13

Table 6.

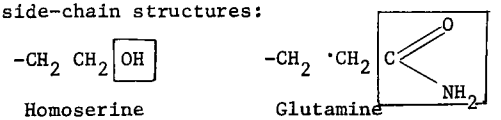
I do not have all the reported synthetic analogs listed here; otherwise, it would not be possible to read the slide. Analysis of the data presented here will reveal a number of interesting features. 1. For optimal activities there appears to be a requirement for the presence

of both hydrophilic and lypophilic substituents on the β-carbon in the 4 position. This is reflected by the threonine and glutamine residues in 4-threonine-oxytocin and oxytocin, respectively. 2. Further distinctions can be made with regard to (a) the relative contributions of the hydrophilic and lypophilic substituents in position 4, (b) the size and inductive effects of the lypophilic substituents, and (c) the nature of the hydrophilic substituents. This leads to the following observations.

(a) It appears as if hydrophilic characteristics predominate over lypophilic in contributing to the oxytocic activity of the hormone. This is evident by a comparison of the activities of [4-serine]-oxytocin with [4-α-amino-butyric acid]-oxytocin. (b) There also appear to be critical size and inductive effect factors involved in the contribution of the lypophilic substituents. (c) However, from the point of view of this discussion, the most intriguing observation concerns the comparative effects of the hydroxyl and carboxamide functions. Since [4-serine]-oxytocin is almost twice as potent as [4-asparagine]-oxytocin, it is evident that the hydroxyl group exerts a greater effect than the carboxamide group in the manifestation of oxytocic potency.

A Theory: From all of this, one might put together a theory explaining why the replacement of glutamine by threonine in the 4 position should lead to enhanced activity. The answer appears to be very simple: the methyl group of threonine can be equated with the methylene group of glutamine, thus the lypophilic components are about equal. The hydroxyl group of threonine in exerting a greater hydrophilic effect than the carboxamide group of glutamine might thus be viewed as the key factor involved in leading to the observed enhancement of activity.

How to Test the Theory? After formulating this theory, I outlined it to one of my colleagues at the Medical School, Doctor Wuu. He then came up with what I think is a very neat way to test it. He suggested that we synthesize the [4-homoserine] analog of oxytocin. Homoserine and glutamine differ only by a hydroxyl/carboxamide interchange, as shown by their side-chain structures:



Homoserine Glutamine

Therefore, if the theory is correct, 4-homoserine-oxytocin should also be more active than oxytocin. If it isn't, then, of course, we have to find a new theory.

A [4-Threonine]-Vasopressin: After finding that the [4-threonine]-oxytocin analogs exhibited enhanced oxytocin-like activities and diminished vasopressin-like qualities, we were very curious to know what would be the effect of replacing a threonine for a glutamine in one of the vasopressins. There has been some very interesting and elegant work done in this area. Very recently du Vigneaud and Gillessen reported that putting α-amino butyric acid in place of glutamine in lysine-vasopressin greatly enhanced the antidiuretic activity of lysine-vasopressin (13). On the other hand, the replacement of glutamine by

serine decreased the antidiuretic activity (2), so we somehow felt that threonine would lie somewhere in between. This, in fact, is what we found (Table 7).

PHARMACOLOGICAL ACTIVITIES (IN USP UNITS PER MILLIGRAM) OF [8-LYSINE]-VASOPRESSIN OF [4-THREONINE]-LYSINE-VASOPRESSIN, [4-SERINE]-LYSINE VASOPRESSIN AND [4-α-AMINOBUTYRIC-ACID]-LYSINE VASOPRESSIN

	RAT UTERUS	ANTIDIURETIC	VASOPRESSOR
[8-Lysine]-Vasopressin (LVP)	5	260	285
4-Thr-LVP	11	155	47.4
4-Ser-LVP	0.9	70	3.3
4-Abu-LVP	1.54	707	10.2

Table 7.

We found enhancement of rat uterus activity slightly decreased antidiuretic activity and remarkably decreased vasopressor activity. It would appear, then, that at least as far as the 4 position is concerned, the hydrophilic quality of the side-chain predominates in oxytocin and its analogs. Whereas in the vasopressins the lypophilic quality of the side chains predominates.

What Has Emerged from This Study? The analogs we have looked at are shown below:

ANALOGS DISCUSSED

[4-THR]-OXYTOCIN

[4-THR]-MESOTOCIN

[3-LEU, 4-THR]-OXYTOCIN

[DEAMINO-4-THR]-OXYTOCIN

[4-THR-LYSINE]-VASOPRESSIN

[4-threonine]-oxytocin, [4-threonine]-mesotocin, and [3-leucine-4-threonine]-oxytocin all show enhanced oxytocin-like activities and diminished vasopressin-like qualities. The enigma here is the [deamino-4-threonine]-oxytocin. We were very surprised at the results on this analog and cannot still offer an adequate explanation for why this does not have enhanced activities. The last one is [4-threonine-lysine]-vasopressin, which I have just discussed.

Conclusion: How the Merrifield Method and Luck Helped Out. By way of final conclusion, I would like to mention that this work would not have been possible without the use of the Merrifield Solid Phase Method (14). As far as I'm concerned, this is the method for making peptides of this type. I work together with only one assistant, and we have managed to synthesize all of these analogs since the beginning of the year. I am very happy with the method. We also have a purification method, developed in our laboratory, which has greatly facilitated the

synthetic aspect of the work (15). However, one cannot eliminate a third aspect, an element which has been of major importance in getting this whole study started; and that has been the element of luck.

I think those of you who watched the final-round action of the PGA yesterday will know what I'm referring to. I think luck played a great part in that struggle yesterday. Dave Stockton got the breaks; Arnie just didn't have it going for him. Luck is very much acknowledged in sports. I think it also plays a great part in scientific discoveries. This talk could have been given by any one of a number of other people; but Bill Sawyer and I just happened to get a lucky break, so I'm the one who has given it here.

Thank you very much . . .

Acknowledgements. This investigation was supported in part by Research Grants GB 4932 from the National Science Foundation, AM 01940 from the National Institute for Arthritis and Metabolic Diseases, by the Medical College of Ohio at Toledo, by a Contract (69-2193) from the Center for Population Research of the National Institute of Child Health and Human Development, and a General Research Support Grant to Columbia University from the National Institutes of Health. We thank Mrs. Esther Coy for her assistance in synthesizing the peptides, Mrs. Sarah Crumm for performing the amino acid analyses, Mrs. Margot Acosta for performing the biological assays, and Drs. T. Wuu and M. Saffran for stimulating discussion and criticism.

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REFLECTIONS ON SOLID PHASE PEPTIDE SYNTHESIS

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I have been asked to comment on the proceedings of this session on solid phase peptide synthesis and, I suppose, to give some prognosis for this approach to the synthesis of peptides.

It has been pleasing to me to see the solid phase method receive so much attention at this symposium, but I realize, of course, that much of the new work is being done because the original method was not as good as it should have been. If it had worked perfectly there would have been no need for the improvements that have been reported today.

The basic problems have been evident almost from the outset and, inspite of all the work that has followed, most of them are still with us. They involve the solid supports, coupling methods, selective protecting groups, quantitation of reactions, monitoring methods, purification procedures and automation. We have heard papers today covering most of these subjects.

Solid Supports. It may be of interest to point out that the original idea for solid phase peptide synthesis was to perform the reactions in a column. However, the polystyrene resin beads were not well adapted to such a process and the commonly used batch process was developed instead. The new, rigid, non-swelling coated supports described by Dr. Parr do lend themselves to the column application and this approach now appears to hold much promise. As pointed out, the mass transfer is accelerated in the thin films of the coated glass beads and if diffusion is the rate limiting step in the synthesis these new supports could be a very important improvement in the technology. Although it was not mentioned here it is possible that the newly developed continuous belt systems will replace all of these procedures. In regard to the standard copolystyrenedivinylbenzene beads I want to add that we find considerable differences in the various preparations that have been available to us, and I believe that a good part of the discrepancies in results from various laboratories can be attributed to differences in the resins. Several new ways to evaluate resins are being developed to enable us to specify much more precisely which parameters must be controlled.

Coupling Methods. The coupling reactions referred to today have been the standard ones. I think most would now agree that the dicyclohexylcarbodiimide method is the fastest and gives the best yields and is generally

the most useful. The nitrophenyl esters are the best of the active esters and have certain advantages, especially for coupling glutamine and asparagine. There is no doubt, however, that they couple much more slowly than carbodiimide-activated amino acid derivatives. Unfortunately, one of the newest and potentially most promising coupling methods will not be reported on at this meeting. It is Mukaiyama's new oxidation-reduction method involving triphenylphosphine and dipyridyldisulfide, with the formation of triphenylphosphine oxide providing the driving force in the reaction (1). In reactions in homogeneous solution the couplings are rapid, yields are high, and the mechanism of the reaction minimizes racemization. He has recently applied it to the synthesis of a dipeptide on solid phase with good results. I have also found a nearly quantitative (± 3%) yield at the first step. Based on the results of only a few experiments, further couplings have not been quite as quantitative, but the application of this new method to solid phase synthesis deserves a thorough examination.

In several instances couplings in solid phase syntheses have been observed to be incomplete in spite of efforts to drive them to completion. An interesting new paper by Westall and Robinson (2) has provided a possible explanation and a solution to the problem. They found poor incorporation of Boc-glutamine nitrophenyl ester at the hexapeptide stage of a synthetic peptide when the coupling was carried out in DMF. When 1.5 M urea was included in the reaction mixture the coupling rate was accelerated and the reaction was quantitative after 24 hr. In addition it was found that a mixture of methylene chloride and DMF was more effective solvent for DCC couplings than either of them alone. The authors attributed the incomplete coupling steps to a tertiary structure of the peptide that prevented access of reagents. The solvent mixtures were thought to disrupt the structure and allow better reaction. Whether the explanation is correct or the cure is a general one remains to be determined, but the results are very promising.

Protecting Groups. The protecting groups for histidine which Stewart has discussed have also greatly improved the situation with this troublesome amino acid. Results from our laboratory and others all seem to agree with his conclusions that both N^{im}-DNP-His and N^{im}-Tosyl-His are very satisfactory for solid phase synthesis and are significant improvements over the benzyl derivative. In addition to the other advantages of his method Mukaiyama observed no nitrile formation after coupling asparagine and glutamine. This finding together with the new amide protecting groups such as the xanthyl protection that Dorman has just described may eliminate the nitrile problem and avoid the need to use active ester couplings.

Optical Purity. One of the very important considerations, not only in solid phase synthesis but in conventional synthesis as well, is the quality of the starting materials, especially their optical purity. I believe the method of Manning and Moore (3) is a most valuable development in that area. Amino acids and their derivatives can now be rapidly and conveniently tested for the presence of traces of racemic impurities. We are able to exclude the presence of as little as 0.01% of D isomers in our starting materials. The application of the method to the evaluation of optical purity of synthetic peptides, which Dr. Manning will describe later in this symposium, is also a very valuable addition to the enzymatic methods that are usually employed.

Monitoring. Monitoring of reactions is still one of the most pressing problems in solid phase synthesis (4). The first efforts to follow the reactions involved the spectrophotometric measurement of nitrophenol

liberated during the coupling reaction. However, I never was able to get really reliable results, and I think others would now agree with these findings. Gut and Rudinger have reported similar measurements with 5-chloro-8-hydroxyquinoline esters. This procedure is good for following initial rates of reaction but even if it works perfectly it is not sensitive enough to detect the last 1% or less of reaction which is so important in solid phase synthesis. Our reaction rate data are in agreement with the trans-esterification data just described by Beyerman. We find good first order kinetics for most of the coupling range and only begin to see evidence for heterogeneity of reactive sites after about 99.5% of amino groups have reacted. Again, the differences between these results and those mentioned by Dr. Rudinger may reflect differences in resins or in the particular chemical and analytical techniques employed.

The newer $HClO_4$ titration procedure of Brunfeldt and the application of the Edman method to the monitoring problem by Weygand, and Beyerman, and recently by Niall are difficult for me to evaluate at the moment. Probably the most useful techniques have been the chloride method of Dorman and the Schiff base method of Esko. We find both of these to be very good, but still not fast enough. When the right method is finally found it should be possible to feed the results of the monitoring data back into the synthesizer program and to achieve true automation of the synthesis.

Applications of Solid Phase Peptide Synthesis. Ultimately, the value of a synthetic method depends on the use to which it is put. We have heard of several interesting applications of the solid phase method today. Most have dealt with relatively small peptides and I think they continue to demonstrate that peptides up to 10 or 20 residues can be made quickly and in good yield and can be purified easily enough to make this a useful method for such problems. Some of the most interesting results are still coming from the area of peptide hormones. For example, Marshall's competitive inhibitor, [Phe^4, Tyr^8]-angiotensin II, Park's 1-aminocyclopentanecarboxylic acid derivatives of angiotensin, the [Thr^4]-oxytocin of Manning, the bradykinin potentiating peptides of Ondetti and the TRF synthesis of Stewart.

I think the future will see further extention of the method to the synthesis of larger and more complex molecules. The successful synthesis of the bovine pancreatic trypsin inhibitor just announced today by Izumiya is a very interesting new development. As I understand his results, the synthetic 58-residue peptide was homogeneous by disc gel electrophoresis and was 90% as active as the natural protein that had been subjected to the same work-up conditions. The synthetic ribonuclease A which we reported last year has been further purified by tryptic digestion and column chromatography. Dr. Gutte has now obtained synthetic RNase A with 60% as much enzymic activity as pure natural RNase A.

I would like to report now on a new finding that has been made at Rockefeller University in a collaborative effort with Bernd Gutte, Michael Lin and Stanford Moore (5). We wanted to know if an interaction existed at the carboxyl end of ribonuclease which was analogous to the S-peptide-S-protein system at the amino end of this enzyme. That is, is it possible to combine a C-terminal peptide with an inactive protein component of RNase and regenerate activity. The new experiments have shown that it is.

Since an enzymic cleavage analogous to that with subtilisin, but with specificity for a bond near the carboxyl end of ribonuclease, was not known we chose the synthetic approach. Dr. Gutte made the C-terminal tetradeca-peptide of RNase, H-Glu-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp-Ala-Ser-Val-OH,

because it was the longest peptide sequence without a CysH residue. Our standard methods of synthesis were used, and the peptide was purified by Sephadex chromatography and free flow electrophoresis. The reconstitution idea was first tested by inactivating RNase by carboxymethylation at His^{119} and then trying to displace the modified tail of the protein with the synthetic peptide. After several attempts in which the protein was unfolded in urea, acid, base, or by heat and was then annealed in the presence of the peptide (at ratios up to 100:1) we conclude that the intramolecular interactions were too strong to allow the peptide to enter into the proper position to regenerate activity.

It was then discovered that by removing the last four residues of RNase A the peptide could regenerate appreciable activity. RNase (1-120) retains much of the original conformation and ability to bind substrate, but has only 0.5% activity. The tetradecapeptide restored about 30% of the activity. Dr. Lin then found that he could further remove first Phe^{120} and then His^{119} enzymatically and that the products were completely inactive. When either of these was mixed with the synthetic C-terminal tetradecapeptide nearly full RNase activity was regenerated. At a 1:1 ratio they gave about 50% activity and at 3:1 the activity was over 80%. The binding was remarkably efficient even though there was an overlap of 8, or 9, residues between the peptide and the proteins. In the combination of RNase (1-120) or RNase (1-119) with peptide (111-124) a His^{119} was present in both the peptide and the protein and it is not known which was acting catalytically, but with RNase (1-118) it is clear that if His^{119} is involved in the catalytic mechanism then it was being supplied by the peptide. We conclude that there is a peptide-protein interaction at the C-terminus of RNase which is quite analogous to the S-peptide-S-protein system at the amino end.

Even more interesting to me is the finding that peptides representing both ends of the molecule and containing both of the catalytically important histidines can be combined with a central core protein to give an active ribonuclease. Thus, S-protein could be degraded to RNase (21-118) and then mixed with S-peptide plus the C-terminal tetradecapeptide and about 30% RNase activity could be regenerated. It seems to me that this is a beginning toward the physical separation of the catalytic sites of enzymes from their binding and specificity sites which may someday lead to the synthesis of simplified enzymes with modified structures and functions.

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THE CHEMISTRY AND IMMUNOCHEMISTRY OF CALCITONINS

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Since the initial discovery of the hypocalcemic hormone, calcitonin, (1) our knowledge of its chemical, immunochemical and biological properties has advanced rapidly through the efforts of a number of laboratories. Its origin from the C cells of the thyroid in mammalian species and from the ultimobranchial body in lower vertebrates is well established. (2,3) The hormone is released during hypercalcemic challenge and acts physiologically to prevent bone resorption. The true mode of action and phylogenetic significance of calcitonin have not yet been established. Despite this, there is steadily accumulating evidence for an important biological role for calcitonin. Calcitonin has already proven to be of great therapeutic benefit in reversing the rapid bone turnover in Paget's Disease and the possible use of the hormone in other therapeutic situations such as hypercalcemia is being evaluated. (4)

Progress so far in this field has been greatly aided by the isolation, chemical characterization and synthesis of calcitonins from several species, and through the development of sensitive radioimmunoassay methods for detecting the trace amounts of hormone present in biological fluids. These chemical and immunological approaches, to be reviewed here, should continue to provide information concerning the control of secretion of calcitonin, structural features requisite for biological activity and the pattern of metabolic degradation of the hormone.

CHEMISTRY OF CALCITONINS

1. Isolation

The methods developed in our laboratory for the isolation of porcine and salmon calcitonins have been fully reported elsewhere. (5,6) Bovine and ovine calcitonin were purified by essentially the same procedure as for porcine. Since all calcitonins so far studied contain a 1,7 disulfide bridge, care must be taken to avoid conditions during isolation which might lead to oxidation or interchange reactions of the disulfide bonds. The experience of the Ciba group suggests that dimer formation may be particularly

favored in the human calcitonin molecule (7); the dimers are not necessarily preexisting in the glands but are generated to some extent during purification. Dimer formation is less well established for the other calcitonins but there have been indications of immunologically reactive fractions of both bovine and salmon calcitonins which eluted earlier from Sephadex G 25 columns than the 32-residue peptides. These could be dimers or simply aggregates of the native hormone molecules.

Oxidative reactions may alter the calcitonin molecule at sites other than the amino terminal disulfide ring. Oxidation of the methionine at position 8 in human calcitonin, which occurs readily, leads to complete loss of biological activity. However, oxidation of the methionine at position 25 in porcine, bovine, and ovine calcitonin is not accompanied by biological inactivation.

Another important aspect of the purification work has been the discovery of intraspecies microheterogeneity of calcitonin in salmon. Careful monitoring of column effluents for biological activity during the isolation of the salmon hormone led to the identification of two additional calcitonin components of differing composition but of similarly high potency, eluting earlier from CMC than the major form of the hormone.(6) These minor components have been designated S2 and S3, the original major component being S1. Because only minute quantities of the S2 and S3 components were available, and because contaminating peptides were present which were very similar in size and charge, their actual isolation in pure form proved extremely difficult and could be achieved only after performic acid oxidation. This was carried out in order to convert the two half-cystine residues into cysteic acids, thus providing two additional negative charges and shifting the elution position of the calcitonins on ion exchange. This allowed the isolation and provisional sequence analysis of the two salmon minor components. Since the purified performic acid oxidized material is biologically inert, estimates of specific biological activity must be based on the partially purified product. It is apparent, however, that the specific hypocalcemic activity of S2 and/or S3 is at least equal to and could perhaps exceed that of S1. Further attempts to isolate the minor components in the native state are in progress. Synthesis is being undertaken on the basis of presumed structure so that accurate estimates of potency can be made. This cumulative experience obtained during isolation of different calcitonin variants has important implications for future attempts at isolation of hormones from other species.

2. Sequence Determination

The primary structure of porcine calcitonin was established by two independent approaches in our laboratory (5) in 1967. (Fig. 1) The proposed structure was confirmed

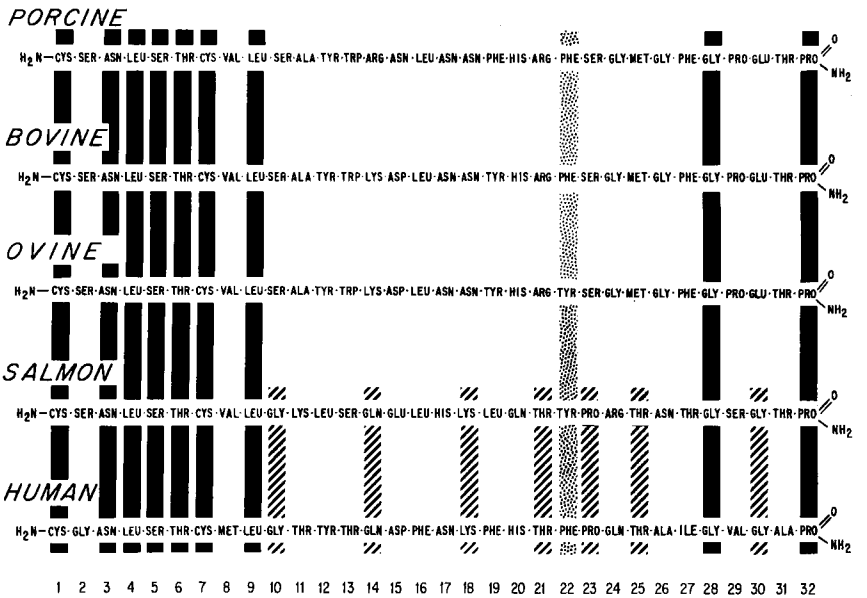

Fig. 1 Amino Acid Sequences of Various Calcitonins

by independent structural analysis carried out by two other laboratories (8,9), and was also validated by synthesis of biologically fully active material (10,11).

Much smaller quantities of the bovine, ovine and salmon calcitonins were available than of the original porcine hormone and it was necessary to devise extremely sensitive methods for sequence analysis. This was accomplished through the use of several new approaches employed in our sequence analyses of bovine, ovine and salmon calcitonins.

The accelerated form (12) of the manual Edman degradation was adapted for use at high sensitivity through modification of the actual degradation conditions and through the use of a gas chromatographic system for detection and quantitation of the amino acid phenylthiohydantoin derivatives. Using this procedure the entire sequence of the major salmon calcitonin was established in a single degradation on about 5 mg of the hormone. (13) (Fig. 2)

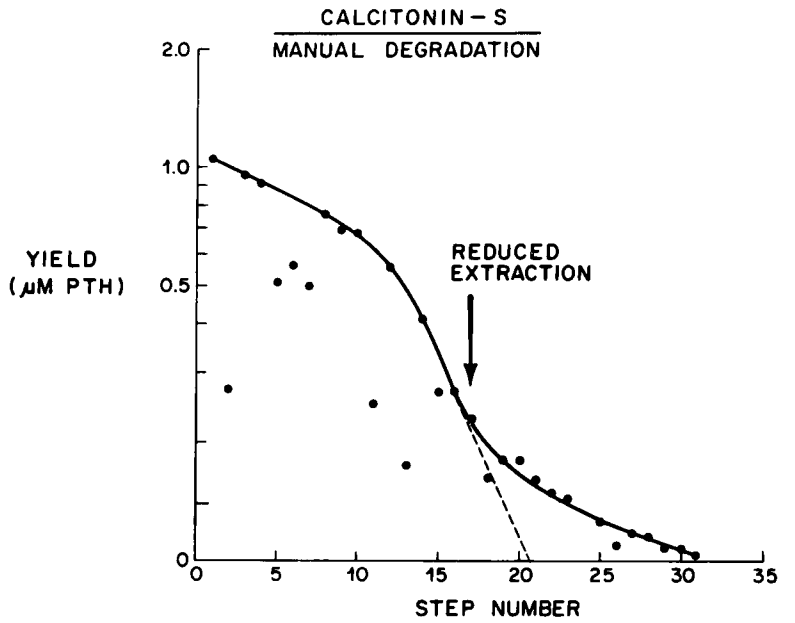

Fig. 2 Repetitive yields of PTH amino acids obtained during total sequence analysis of salmon calcitonin 1 by manual Edman degradation.

We have also developed automated procedures (14,15) for sequence determination of peptides in the protein-peptide sequenator (Beckman Spinco Sequencer, Model 890). Special programs have been devised which allow degradations of up to 25 cycles on as little as 400-800 micrograms of calcitonin. With the automated system a run of this length takes only 36 hours so that it is entirely feasible to obtain the complete sequence of a molecule the size of calcitonin within a week.

A third approach was used which is based upon Edman degradation of unfractionated peptide mixtures.(12) The information obtained from this can be correlated with the results of degradation on other mixtures or on single peptides or with amino acid compositional data. In this way information regarding absolute sequence may be obtained, as previously reported for the major component of salmon calcitonin.(13) Recently we have used this approach to determine a provisional amino acid sequence for the two minor components of salmon calcitonin (II and III). The basic, trypsin sensitive residues were found to be positioned as in the major salmon molecule. On tryptic digestion four peptides were generated. These were separated by preparative thin layer chromatography and subjected to amino acid analysis. This showed the substitution of a methionine for a valine in the amino terminal tryptic peptide in S3. Salmon 2 had the same composition as Salmon 1 in this peptide. Both minor components differed from the major components in having a substitution of aspartic acid for glutamic acid in the second tryptic peptide and substitution

of one residue of valine and one of alanine for serine and threonine in the carboxyl terminal tryptic peptide. Recently the exact positions of these substitutions have been shown by Dr. R. Lequin of our laboratory to be at residue 8(met) residue 15(asp) residue 29(ala) and residue 31(val), using a new modification of the mixture analysis technique as described elsewhere.(16)

In summary, a combination of approaches based upon manual and automated Edman degradations has been developed. This has allowed complete sequence analysis of calcitonins on only a few milligrams of material. This is important for future work when one considers the cost and difficulty of purifying calcitonin from other species of interest - for example, avian calcitonin. Since, as described below, we have available a large variety of specific antisera to different calcitonins and to different sequence regions of these calcitonins, it is likely that these antisera could be used in preliminary screening of crude tissue extracts from further species. The pattern of cross reactivity with these multiple antisera may indicate which species contain calcitonins with novel structural features that make them of interest for further characterization.

3. Comparative Chemistry

The determination in our laboratory of the primary structure of six distinct calcitonins (porcine, bovine, ovine and three variants of salmon) together with the structure of human calcitonin(7), has already provided a distinctive structural pattern quite unlike any other peptide hormone so far studied. Common to all seven molecules are the 1,7 disulfide bridge and the carboxyl terminal prolinamide residues. Only 9 sequence positions are completely constant in all structures (Fig. 1). Most of these positions are concentrated at the amino terminal end of the molecule in the disulfide loop region. At the carboxyl end only positions 28 and 32 are constant. The amino acid sequence between residue 10 and residue 27 is particularly remarkable. Though there is considerable homology in this region between porcine, bovine and ovine calcitonins, there is not a single sequence position between 10 and 27 common to all 7 calcitonins. At first sight this might seem to indicate that this middle region merely represents a biologically inert "string" holding together the functionally important conserved regions at either end of the molecule. In support of this, synthetic analogues of calcitonin where the chain length is shortened by the omission of one or more residues from the middle sequence (preserving the constant terminal regions) are almost totally inert. This would merely suggest that the actual length of the string is critical.

Though the great sequence variations in the middle region suggest that the "string" hypothesis may have some validity, the weight of evidence suggests that there is really a great deal of unobtrusive conservation throughout this region of calcitonin. This conservation is based more on the chemical properties of the amino acid sidechains than on their actual identity. For example, hydrophobic amino acids (leucine, phenylalanine or, tyrosine) occur at almost regular intervals along the chain, at positions 4, 9, 16, 19, and 22. Other evidence suggests that at one additional position it is

necessary to have a hydrophobic or at any rate a non-polar derivative for preservation of activity. Valine occupies position 8 except in human calcitonin and the salmon 3 hormone, which contain methionine at that residue. Mild oxidation of this methionine to the polar sulfoxide causes total loss of activity in human calcitonin. Oxidation of the methionine at position 25 in porcine, bovine or ovine calcitonin, however, causes no change in biological activity. This fits in with the observation that a polar residue (threonine) is found at position 25 in salmon and human calcitonins. Polarity at this position is thus consistent with unchanged or even increased biological potency. Further conservation in the molecule is seen in the positioning of acidic residues (found at positions 15 and 30 only) and in the localization of basic sidechains again to relatively few positions.

So far unexplained is perhaps the most important feature of all the calcitonin structural chemistry.(6) This is the extremely high potency of the salmon calcitonins relative to all others so far studied. The elucidation of two further salmon structures provides further information. Though exact biological potency figures are not yet available it is clear that the hypocalcemic activities of Salmon 2 and Salmon 3 are at least of the same order as that of Salmon 1. Hence the three structures must contain in common the features requisite for very high biological potency. Some features present in the major component can now be eliminated as possibilities on the basis of new structural information. For example, the tyrosine at position 22 in Salmon 1 is not crucial since phenylalanine is present at that position in Salmons 2 and 3, and tyrosine appears there in the low specific activity ovine hormone. Similarly, the hydroxy amino acids present in Salmon 1 at positions 29 and 31 are replaced by alanine and valine respectively in Salmons 2 and 3. This new evidence suggests that an explanation proposed(18) for the high potency of salmon calcitonin (that it is indirectly due to its possessing a greater number of hydroxy amino acids than other calcitonins) is less likely to prove correct. At present no firm conclusions can be drawn about the cause for the high activity of salmon calcitonin. Structural analysis of further calcitonins and studies based on peptide synthesis however should help to resolve the question.

IMMUNOCHEMISTRY OF CALCITONINS

Specific antibodies have been developed in our laboratory for porcine, bovine, ovine, salmon, and human calcitonin by the immunization of guinea pigs and rabbits with purified preparations of each of these peptides, respectively. Each of the pure peptides has been radioiodinated according to the method of Hunter and Greenwood (17) and studied together with the various specific anti-sera. The details of the immunoassay procedure have been previously described (18).

1. Properties of Calcitonin Immunoassays

There are striking structural differences among the calcitonins which have been isolated. Human calcitonin and salmon calcitonin are markedly different from each other and from the porcine, bovine, and ovine molecules. The latter three calcitonin molecules, however, have many structural similarities. It could be anticipated, therefore, that varying degrees of immunochemical cross-reactivity would be found among the calcitonins. This has, in fact, been observed in our various immunoassay systems.

Fig. 3 shows typical standard curves for the radioimmuno-

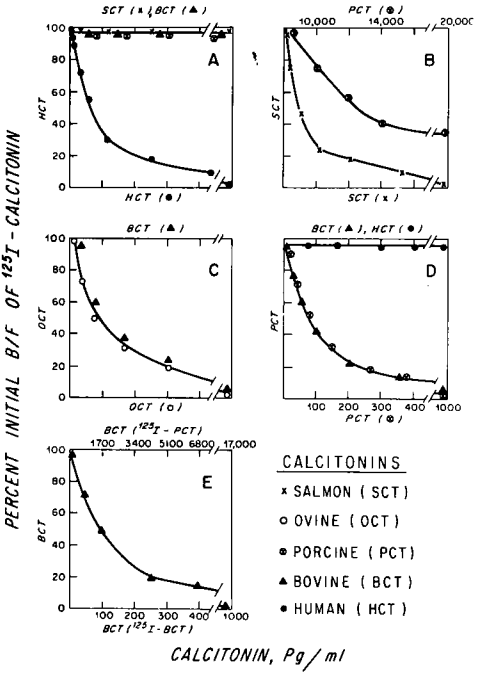

Fig. 3 Immunoassays for human (A), salmon (B), ovine (C), porcine (D) and bovine (E) calcitonin. In addition to each homologous immunoassay system, also illustrated are certain immunochemical relationships among the various calcitonins (see text for further discussion)

assay of each of the calcitonins, namely, human, salmon, porcine, ovine and bovine. Each of the assay systems can readily detect picogram quantities of the appropriate peptide. This degree of sensitivity makes the assays useful for a variety of applications. Not only can calcitonins be readily detected in appropriate tissue extracts, but they can also be detected in the tissue culture preparations of the appropriate

organs. Furthermone, the assays can be used to directly measure peripheral concentrations of the peptide circulating in blood of various species. (19,20)

(a) Human Calcitonin

Fig. 3A illustrates that there is no cross-reactivity between human calcitonin and salmon, bovine and porcine calcitonin (nor with ovine calcitonin which is not illustrated in this figure). Fig. 3D further illustrates that human calcitonin does not crossreact with porcine calcitonin. Therefore, in these immunoassay systems, there is no cross-reactivity between human calcitonin and any of the other calcitonins which have been isolated. This is not surprising in view of the marked structural differences between human calcitonin and the other calcitonins. We have, however, seen weak, but definite, cross-reactivity between human calcitonin and porcine calcitonin with several anti-porcine calcitonin antibodies other than those illustrated. It seems possible that these particular antibodies were directed against the amino terminal end of the porcine calcitonin molecule; in this region there is structural similarity between porcine and human calcitonin and, in fact, among all of the isolated calcitonins.

(b) Salmon Calcitonin

Fig. 3B illustrates that there is a slight but definite cross-reactivity between salmon calcitonin and porcine calcitonin. In the salmon immunoassay system, an excess of porcine calcitonin can displace I^{125} labelled salmon calcitonin from antibody raised against salmon calcitonin. This is somewhat surprising in view of the marked structural differences between these peptides. Again, it seems possible that this particular antibody to salmon calcitonin is directed against the amino terminal end of that molecule. In this region, salmon and porcine calcitonin are structurally identical (whereas human differs in 2 of 9 positions).

(c) Bovine and Ovine Calcitonin

Fig. 3C illustrates that there is virtually complete cross-reactivity between bovine calcitonin and ovine calcitonin. This was not unexpected since the two molecules are identical except for substitution of tyrosine for phenylalanine at position 19. Human calcitonin does not react in this immunoassay system.

(d) Porcine calcitonin

Fig. 3D illustrates that porcine and bovine calcitonin are immunologically indistinguishable in an immunoassay system consisting of radioiodinated porcine calcitonin and anti-porcine calcitonin antibody. This close degree of cross-reactivity is not surprising since porcine and bovine calcitonin differ only in positions 14, 15 and 19. Fig. 3E, however, reveals that bovine and porcine calcitonin are not immunologically similar in all immunoassay systems. In this instance bovine calcitonin was measured by two immunoassay

systems: radioiodinated bovine calcitonin with antibody to bovine calcitonin and radioiodinated porcine calcitonin (identical to the tracer used in Fig. 3D) with the same anti-bovine calcitonin antibody. It can be seen that bovine calcitonin was at least seven times more reactive in the bovine tracer-bovine antibody immunoassay (bottom and left scales) than in the assay where porcine tracer and the anti-bovine calcitonin antibody were used (top and right scales). It is, therefore, apparent that, for at least this anti-bovine calcitonin antibody, there was an immunological (structural) difference between bovine and porcine calcitonin. Presumably, this antibody was directed against those portions of the bovine and porcine molecule, respectively, where the two peptides are dissimilar. This observation points out the need for using multiple antibodies of differing specificity to investigate structural relations. Even if only one of scores of antibodies establishes immunological differences between two molecules, structural differences, must, in fact, exist.

2. Applications of Calcitonin Immunoassays

(a) Porcine Calcitonin Immunoassay

Our assay for porcine calcitonin was the first immunoassay used to study the control of secretion of this peptide. (18) We demonstrated by measurements in peripheral blood that calcitonin is continuously secreted at physiological concentrations of blood calcium, that hormone secretion is under the directly proportional control of blood calcium, and that hormone, once secreted, disappears rapidly from the blood with a T 1/2 of 2 to 20 minutes. In addition to studies in peripheral blood, the immunoassay for porcine calcitonin was used to study the secretion of calcitonin by direct measurements of the peptide in the thyroid effluent blood of the pig during alterations in blood calcium. These studies demonstrated that the secretion of calcitonin is directly related to changes in blood calcium. (19) Furthermone, the administration of oral loads of calcium insufficient to cause a perceptiable rise in serum calcium have also been demonstrated to result in an increase in the secretion of calcitonin. (21A)

(b) Bovine Calcitonin Immunoassay

The immunoassay for bovine calcitonin has been applied to the study of the control of secretion of this peptide in the bovine species. As with other mammals the secretion has been shown to be directly proportional to the serum calcium.

Fig. 4 illustrates that calcium infusion in a cow causes a

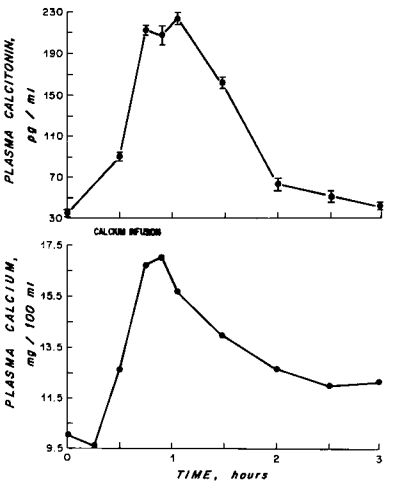

Fig. 4 Effect of a calcium infusion on peripheral calcitonin concentration in the cow

rapid increase in the peripheral concentration of calcitonin. The assay has also been used to study a syndrome commonly seen in older bulls which consists of medullary carcinoma of the thyroid, pheochromocytomas, and parathyroid neoplasia. (22) Elevated concentrations of calcitonin have been found in the thyroid tumor and in the peripheral blood of involved animals. This syndrome in bulls bears a remarkable resemblance to one of the multiple endocrine adenomatoses (MEA type 2) seen in humans (see below). (23)

(c) Human Calcitonin Immunoassay

The immunoassay for human calcitonin has been used to evaluate calcitonin secretion in patients with medullary carcinoma of the thyroid, a tumor which can occur sporadically or as part of a multiple endocrine syndrome. (20) Excessive amounts of calcitonin are invariably found in such tumors. Furthermore, in most patients with this tumor, the basal concentration of calcitonin in the peripheral blood is markedly elevated above normal levels. The immunoassay can therefore be used to establish the diagnosis of medullary thyroid carcinoma, even in patients in whom the tumor is not yet clinically apparent. In a smaller group of patients with this tumor, however, basal concentrations of calcitonin are within normal limits. In these patients, provocative tests can be used to demonstrate the presence of the tumor. Calcium infusion has caused a marked increase in calcitonin secretions in all patients studied with this tumor (Fig. 5A). Glucagon

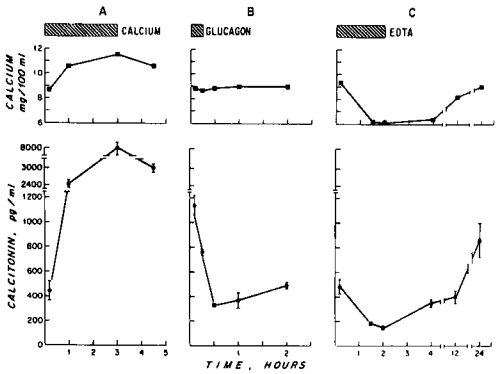

Fig. 5 Effect of calcium (A), glucagon (B) and EDTA (C) infusions on peripheral calcitonin concentrations in a patient with medullary thyroid carcinoma. Calcium resulted in a marked increase in calcitonin. Glucagon led to an unexpected decrease in calcitonin. EDTA induced hypocalcemia also resulted in a decrease in plasma calcitonin concentration.

adminstration also provokes calcitonin secretion in most patients with this tumor. (Glucagon also appears to increase the peripheral concentration of calcitonin in normal subjects). However, in some patients glucagon suppressed calcitonin release (Fig. 5B). Therefore, the administration of calcium seems to be a more reliable provocative test for establishing the diagnosis of medullary thyroid carcinoma in those patients with the tumor who have normal basal peripheral levels of calcitonin. It is notable that EDTA induced hypocalcemia will also suppress calcitonin secretion in patients with this tumor. (Fig. 5C) Therefore, medullary thyroid carcinomas, like normal parafollicular cells, are fully responsive to changes in blood calcium; hypercalcemia stimulates and hypocalcemia inhibits the release of calcitonin.

In addition to usefulness to establish a diagnosis of medullary carcinoma of the thyroid, the site and extent of the tumor can also be defined by application of the immunoassay. This can be accomplished by demonstrating a localized increase in the concentration of calcitonin in blood samples taken during catheterization from the venous drainage of the tumor. The role of calcitonin in other appropriate human diseases is also being evaluated. In preliminary studies, the peripheral concentration of calcitonin has not been elevated in osteopetrosis and chronic hypercalcemic states.

(d) Immunoassay for Salmon Calcitonin

The immunoassay for salmon calcitonin is being applied to the study of the clinical pharmacology of this peptide. It has been demonstrated in man and other mammals that salmon calcitonin exerts more potent biological effects on a molar

basis than any of the other calcitonins isolated. The reasons for this increased potency of the salmon peptide have not been established. We have been testing the hypothesis that the increased potency of salmon calcitonin in mammals, as compared to porcine and even human calcitonin, may be due to a more prolonged half-life of the salmon peptide once administered. Preliminary evaluation of the metabolic fate of salmon calcitonin by immunoassay suggests that a prolonged half-life may indeed play a role in the increased potency of the salmon peptide. The metabolic fate of calcitonin after termination of an infusion of the peptide in the dog is represented by a multi-exponential function. The initial half-life is approximately 18 minutes. In an experiment with porcine calcitonin, a similar multi-exponential function was described by the decreasing concentrations of the peptide. However, the initial half-life of porcine calcitonin was almost ten-fold shorter than that of salmon calcitonin. This prolonged half-life of salmon calcitonin may contribute to its greater biological potency.

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A Comparison of the Immunochemical and Physical Properties of Porcine (PTC), Bovine (BTC), and Human (HTC) Thyrocalcitonin

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Manuscript not received

Structure-Activity Relationships of Calcitonin Peptides

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Manuscript not received

HUMAN CALCITONIN M

ASPECTS OF SYNTHESIS AND CHARACTERIZATION

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This contribution will describe some points of interest which we have encountered in our synthesis of human calcitonin M in the CIBA laboratories by a conventional fragment condensation approach.

Considering the large number of papers given today on solid phase synthesis of peptides, and after Dr. Merrifield's very pertinent remarks about the present state of this method, it might be appropriate to explain the reflections which led us to plan our calcitonin synthesis on a conservative fragment condensation principle. One of the reasons was certainly that we had more experience with peptide chemistry in solution, than with synthesis on a solid resin [1]. On the other hand, our experience with the latter technique, especially in analyzing each step of a model synthesis, helped us to recognize some of the difficulties inherent in the solid phase method. In the particular case of human calcitonin, it was difficult to think of a good combination of protecting groups (as for instance for histidine and cysteine) which would be compatible with the presence of methionine and with the stability of the whole molecule under the conditions of deprotection. Moreover, the two glutamines, the C-terminal prolinamide, and several additional sterically hindered positions were thought to be potential stumbling blocks. Finally, it would have been very difficult or perhaps impossible to purify the expected mixture from such a synthesis of a 32 amino acid peptide to the point of a pure compound on a larger scale.

According to our experiences, the classical fragment condensation produces pure end products, if all the intermediates are purified conscientiously, and thus an accumulation of by-products in the course of the synthesis is avoided. We have found that, in addition to crystallization and precipitation, especially solvent distribution in automatic countercurrent machines has proved to be extremely efficient for the purification of larger, protected peptides. In the present calcitonin synthesis, this method separated even diastereomeric impurities, which could not be detected by thin layer analytical methods.

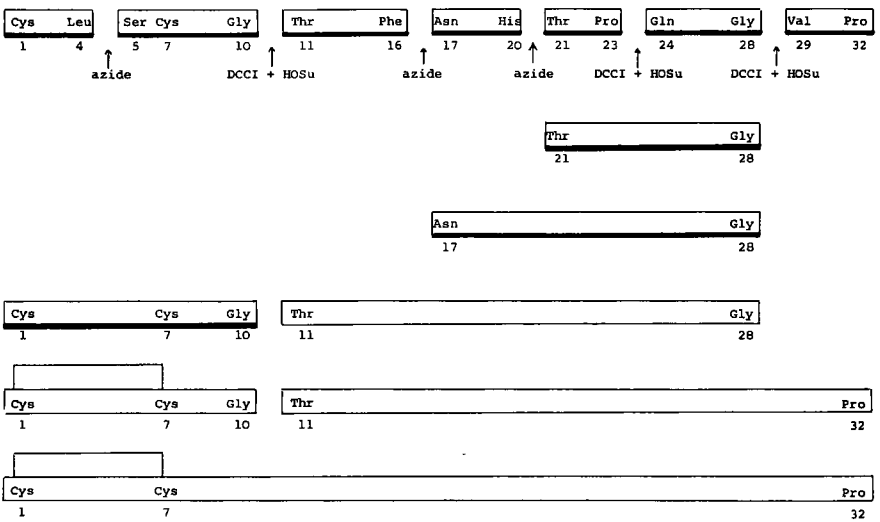

Fig. 1. Fragment coupling scheme used in the synthesis of calcitonin M

Fig. 1 represents very schematically our calcitonin M synthesis [2] [3] [4], starting from seven primary fragments which were mostly built up from their carboxyl ends by stepwise addition of N-proteced amino acid active esters. All the heavily underlined intermediates were obtained in the crystalline state. In the case of the 1 to 10 decapeptide, this was the derivative with trityl groups on the cysteine residues, before the disulphide bridge was formed. The two fragments 1 to 10 and 11 to 32 as well as the protected end product 1 to 32 were purified by Craig distribution.

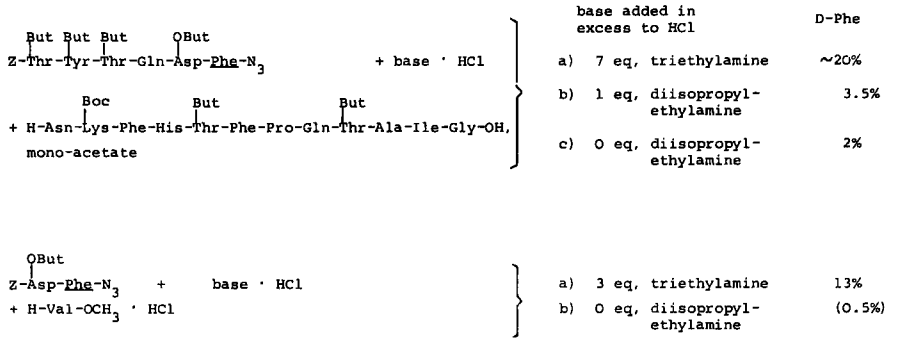

Fig. 2. Racemization during azide condensations

Concerning the condensation methods, Fig. 1 shows that we used the azide method for coupling the fragments in the three cases prone to undergo racemization. Up to now, the azide method has still had the widespread reputation of being free of the risk of racemization [5] [6]. One was inclined to think that the observations of azide racemization reported independently by Anderson and Weygand in 1966 [7] [8] were due to rather drastic conditions not occurring in normal peptide condensations. One of the currently used techniques of the azide method is reaction of the hydrazide in dimethyl formamide solution with tertiary butyl nitrite and hydrochloric acid, and neutralization of the latter with triethylamine. The amino component is then added directly to this azide solution, followed by further small additions of base in order to maintain the pH between 7 and 8. When our first sample of synthetic calcitonin M was analyzed by incubation of the total hydrolyzate with L-amino acid oxidase, we found small amounts of D-phenylalanine and D-histidine. By tracing backwards, it became evident that under our original conditions of azide coupling we had produced approximately 20 and 10% of D-phenylalanine and D-histidine, respectively, most of which was fortunately removed in the later purification steps of the synthesis. By changing the type and molar amount of the added base, we were then able largely to eliminate racemizations, as illustrated on Fig. 2 in the case of phenylalanine. The figures in the last column have been corrected for the amount of D-phenylalanine occurring under the conditions of total hydrolysis with 6 N HCl. It is quite remarkable that in the experiments b and c with one or zero additional equivalents of base, there was still some racemization, even in the presence of 3 equivalents of acid, namely the C-terminal carboxyl, one equivalent of acetic acid, and one of hydrazoic acid arising from the azide reaction. Purification of the protected intermediates 11 to 32 and 1 to 32 eliminated this residual amount of racemized material to a level which could no longer be detected with accuracy by our method of analysis,

i.e. below 1%. The second example shown in Fig. 2 represents a model tripeptide condensation with again considerable racemization in the presence of excessive base. In this connection, we should also remember the recent results of Kemp and coworkers [9], who demonstrated that even the most unsuspicious methods of peptide coupling produced traces of D-compounds, if analyzed by their extremely sensitive and accurate isotopic dilution procedure.

The following combination of protecting groups was used in our calcitonin M synthesis: The side chains of serine, threonine, and tyrosine were blocked as tertiary butyl ethers, the single aspartic acid by the tertiary butyl ester, and N^{ϵ} of lysine by a Boc group. This enabled us to use carbobenzoxy for N^{α} of most of the intermediate fragments, in connection with catalytic hydrogenation. In the case of the methionine-containing fragment 5 to 10, we took advantage of the Bpoc-group (Bpoc = 2-(p-biphenylyl)-isopropyloxycarbonyl) for N^{α}, which was developed two years ago in our laboratories by Sieber and Iselin [10] [11].

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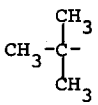
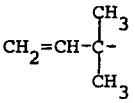
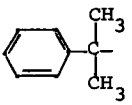
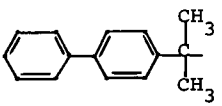
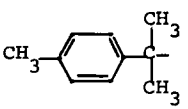
 (Boc-)	58 days	1
	14 h.	100
	2 h.	700
 (Bpoc-)	30 min.	3'000
	7 min.	11'000

Fig. 3. Removal of R-O-CO- from R-O-CO-NH-CH_2-$COOC_2H_5$ by 80% acetic acid

Fig. 3 shows a selection of urethane-type N-protecting groups with widely differing susceptibilities towards cleavage by acid. From these, and several others not listed here, the authors suggested using the Bpoc group in combination with Boc and the various tertiary butyl ethers and esters, for the following reasons: The relative rate of cleavage, as shown in Fig. 3 by comparison with the Boc group, is fast enough for a selective removal without attacking all the other acid-labile protecting groups. On the other hand, Bpoc derivatives are sufficiently stable to be used under the normal, weakly acid working up conditions in aqueous and organic solvent mixtures, as well as during the conversion of hydrazides into azides. For the preparation of Bpoc amino acids, we prefer to use the phenyl ester, a crystalline compound which is stable below 0°. Fig. 4 illustrates an example of the Bpoc application by its selective removal with 80% acetic acid from N^{α} of the methionine-containing peptide 5 to 10, in the presence of two tertiary butyl ethers and the S-trityl group.

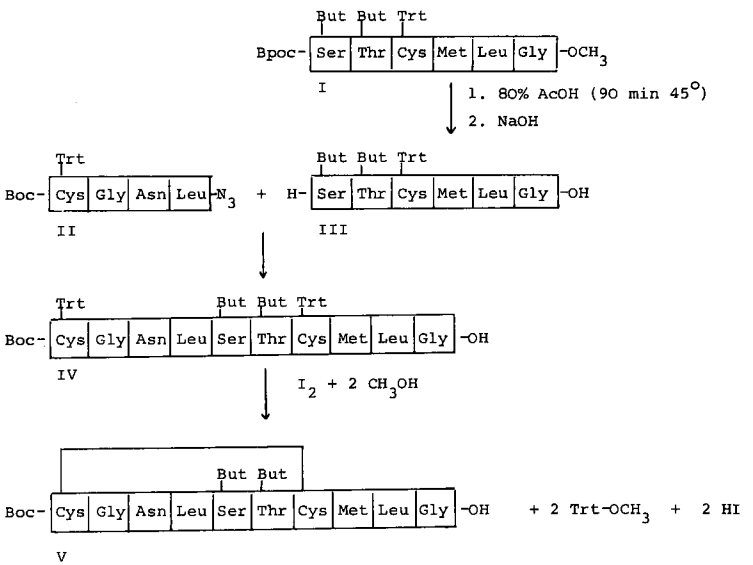


Fig. 4. Synthesis of the protected calcitonin M-(1-10)-decapeptide

The cystine ring was closed in the decapeptide (IV) by simultaneous cleavage of the two S-trityl groups and formation of the disulphide bridge with iodine and methanol. This general method for the formation of cystine peptides was described by Kamber and Rittel in 1968 [12]. The

reaction very probably proceeds through the intermediate formation of the sulfenyl iodides and produces trityl methyl ether as a by-product.

In the final deprotection of the fully assembled calcitonin molecule, altogether 10 acid-labile groups have to be removed, namely 7 tertiary butyl ethers, one tertiary butyl ester, and two Boc groups. The reaction is performed with concentrated hydrochloric acid for 5 to 10 minutes at zero degrees. Fig. 5 shows the two types of side reaction occurring under these conditions. The first one, observed to an extent of approximately 10%, is the well-known, alkali-reversible N to O migration of an acyl residue at the serine and threonine positions. The second, which is found only in traces, is the alkylation of the methionine

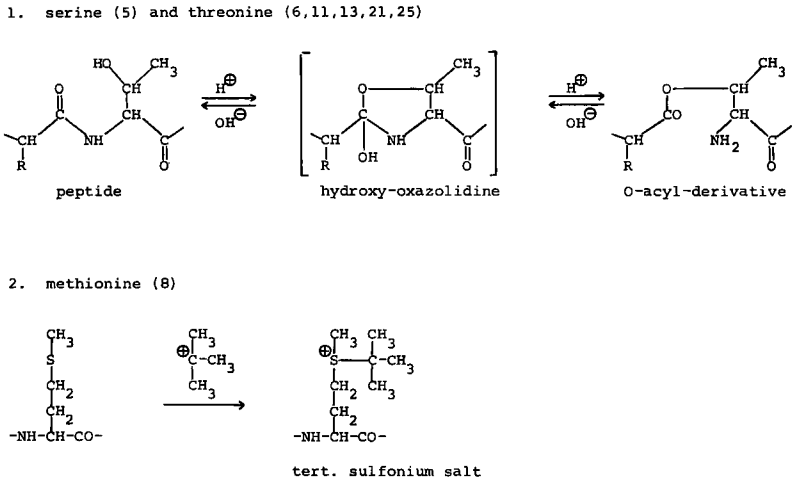


Fig. 5. Side reactions observed during acidolytic deprotection of calcitonin M

thioether by the tertiary butyl kations present in the reaction mixture. Both these by-products can easily be removed by Craig distribution. In Fig. 6, which is a cellulose thin-layer electrophoresis performed at pH 1.9 (90 min. at 16 V/cm), the first spot shows the calcitonin M as obtained directly from the deprotection reaction. The two types of by-product are not distinguishable from each other under these conditions, but they are well separated from the intact calcitonin owing to their additional positive charge. Spot no. 2 represents the pure product after Craig districation. The following 3 positions demonstrate the

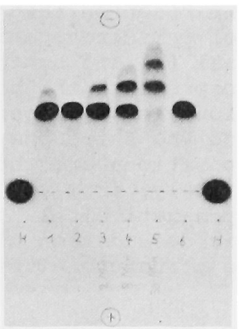


Fig. 6. Thin layer electrophoresis of calcitonin M and its acidolytic transformation products

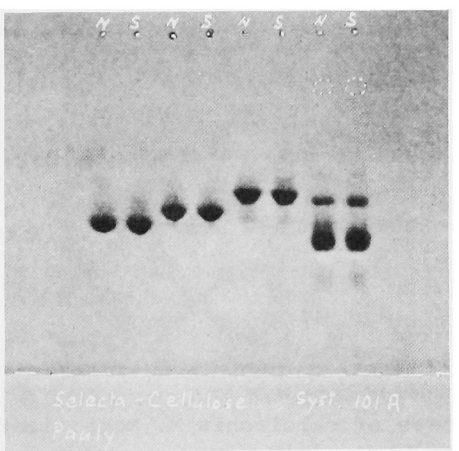


Fig. 7. Comparison of natural (N) and synthetic (S) calcitonin M on cellulose thin layer chromatography

effect of prolonged treatment with concentrated hydrochloric acid at room temperature (2 min., 8 min., and 30 min.). We observe the appearance of two and even three additional positive charges, which are found in molecules that have undergone the N to O acyl shift in more than one position.

The last spot demonstrates the complete reversibility of this rearrangement by a short treatment at pH 7.5.

Fig. 7 shows the comparison on thin layer chromatography (solvent system: n.butanol-pyridine-acetic acid-water 42:24:4:30) between natural (N) and synthetic (S) calcitonin M. The first pair of spots represents the hormone directly, the second pair after oxidation with hydrogen peroxide to the methionine sulphoxide, and the third one after performic acid oxidation. The last two positions are the mixtures obtained on tryptic degradation, namely, from top to bottom, the fragments 13 to 18 (Pauly-negative), 1 to 18, 19 to 32, and 1 to 12. A similar tryptic degradation mixture had been the starting material for the structure elucidation of human calcitonin M [13].

Comparison of the biological activities of synthetic and natural calcitonin M, as measured by the decrease of serum calcium in the rat, revealed identical dose-response curves, as well as an equal duration of action [14].

In conclusion, we should like to mention an observation that might be of interest to those who are engaged in physico-chemical studies with polypeptides. When calcitonin M is kept in a weakly acid solution for a prolonged time, the viscosity increases, and a slow precipitation of spherical,

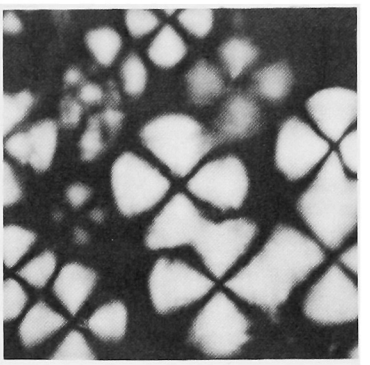


Fig. 8. Spherites of calcitonin M. Magnification 130.

gelatinous particles (spherites) occurs. These are shown in Fig. 8, in the dark field of a polarization microscope. We observe a pattern which is typical for centrosymmetrical particles. The clear viscous solution also exhibits double refraction, suggesting the presence of long, fibrous aggregations. In the electron microscope, as shown in Fig. 9, fibrils of approx. 80 Å diameter can be seen, which are often twisted together with a pitch of about 1500 Å [15].

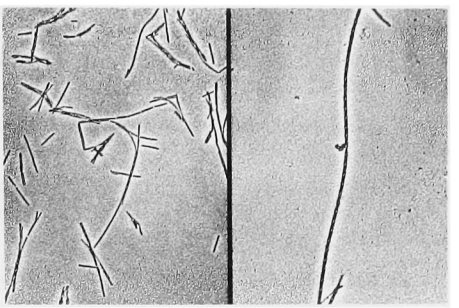


Fig. 9. Electron micrographs of calcitonin M fibrils. Magnification 21'000; Pt-shadowed.

Similar fibrils have already been shown to be formed by insulin [16] [17] and glucagon [18]. It looks as if the physico-chemical principle leading to fibril formation were of a rather common nature in the polypeptide field, although we do not know as yet the molecular conditions necessary for the occurrence of this phenomenon.

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15. We are indebted to Dr. W. Stäubli of our Biological Research Laboratories for the electron optical experiments with calcitonin M fibrils.

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AUTOMATED EDMAN DEGRADATION OF PEPTIDES

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A careful study by Edman (1,2) of the detailed chemistry and the reaction mechanism of the phenylisothiocyanate method established several years ago the optimum conditions for carrying out sequential degradation of proteins at almost 100% efficiency. In 1967 Edman and Begg applied this knowledge in designing and building an automated instrument, the protein sequenator, capable of carrying out continuous degradations of up to 60 cycles (3). A number of chemical and engineering problems, some obvious, some unexpected, were encountered and solved as described in their paper (3). The sequenator technique as applied to proteins has since become widely established.

A central and extremely novel feature of the original sequenator was the design of the reaction cell. The cell consisted of a cylindrical, continuously spinning glass cup in which the protein was distributed as a thin film over the wall. The protein in this dispersed state dissolves rapidly in the coupling and cleaving reagents. An additional advantage is the ease and efficiency of solvent extraction. The reaction cell is mounted in a bell jar which may be evacuated or filled with an inert gas as required during the cycles of degradation.

A considerable problem with this system, however, was encountered when attempts were made to use the volatile reagents employed in the manual degradation. These tended to evaporate from the cup to saturate the rather large vapor space surrounding it. This resulted in incomplete coupling and cleavage reactions and large accumulations within the system of poorly volatile salts derived from the tertiary amine used for coupling and the fluoro acid used for cleavage.

Edman and Begg solved this problem by substituting a non-volatile base, Quadrol, and a less volatile acid, heptafluorobutyric acid. With these alterations the instrument worked extremely well, as witnessed by the remarkable achievement of

60 cycles of degradation on myoglobin. However, as the authors point out (3) difficulties were expected, and have been encountered, in degradations on shorter peptides, due to loss of material in the prolonged solvent washes needed to remove the non-volatile Quadrol.

We have developed an alternate approach to the automated degradation of peptides, using a sequenator with a reaction cell of small vapor space (4). This limits the evaporation of reagents, so that virtually complete coupling and cleavage are obtained. A magnetic drive for the cup is substituted for a direct drive; this somewhat simplifies the construction of the reaction cell. Reagents and solvents lines are brought into the cup from above through a Kel-F plug which protrudes into the lumen of the cup, further reducing the vapor space. We have used this instrument at the prototype stage as reported earlier (4) for successful automated degradations of calcitonins and other peptides. It is now represented by the Beckman "Sequencer" Model 890. More recently (5) we have developed a series of different programs for rapid automated degradation of peptides; losses of peptide are reduced to a minimum by variations in solvent extractions. Recoveries of labile residues (serine and S-carboxymethyl cysteine) are improved by performing an extremely rapid cleavage step, since we have demonstrated that most of the destruction of these residues occurs in the anhydrous conditions used for cleavage. These and other details of the automated peptide methodology are being reported elsewhere. The present paper describes some applications of the technique carried out in our laboratory for sequence analysis of calcitonins and parathyroid hormones from various species. Some advances in identification procedures are also discussed.

Calcitonins

The general strategy for automated sequence analysis of calcitonin is shown in Figure 1. Since it is difficult to

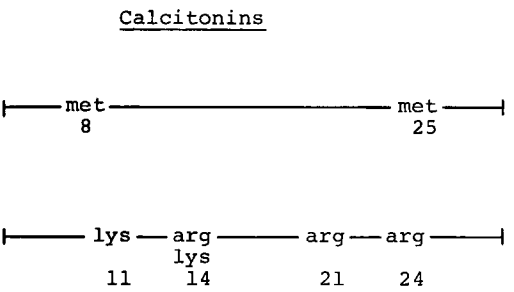

Fig. 1

achieve a complete degradation in a single run on the rather hydrophobic 32 residue calcitonins, the procedure we have used is to carry out an initial sequenator degradation using a suitable peptide program on about 0.1 - 0.3 micromoles of material. This generally allows the identification of the first 20-25 residues. The remainder of the sequence is obtained, again on a small amount of material, by specific cleavage at a residue near the carboxyl end of the molecule, and automated Edman degradation on the unfractionated peptide mixture. Figure 1 shows the distribution of cleavable amino acid residues in the calcitonins we have sequenced. It can be seen that suitable cleavages are possible at the methionine at position 25 (in the porcine, bovine and ovine hormones) or at the arginine at position 24 found in the various salmon calcitonins.

This approach is illustrated in detail for ovine calcitonin. Figure 2 shows the result of an initial degradation.

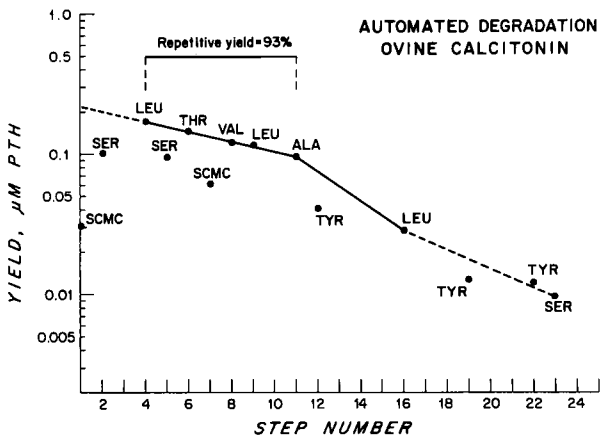

Fig. 2

The repetitive yields of the PTH amino acids were measured by gas chromatography. They fall progressively during the degradation owing to unavoidable extractive losses of the hydrophobic peptide. Residues for which yields are not illustrated in Figure 2 were identified by a combination of other gas chromatographic and thin layer chromatographic procedures. The arginine at position 21 was identified by the phenanthrenequinone reaction. The sequence obtained from this degradation was CYS-SER-ASN-LEU-SER-THR-CYS-VAL-LEU-SER-ALA-TYR-TRP-LYS-ASP-LEU-ASN-ASN-TYR-HIS-ARG-TYR-SER-GLY. The glycine at step 24 was obtained in low yield and no residue could be identified at step 25.

Figure 3 shows the method used to complete the ovine structure.

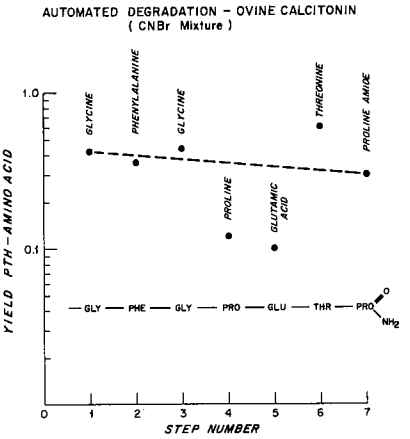

Fig. 3

As the composition of the ovine material had been determined, it was known that the remaining residues contained a single methionine, probably situated at position 25 as found in the bovine and porcine molecules. Hence, cyanogen bromide cleavage was performed and the resulting peptide mixture lyophilized and subjected to automated Edman degradation without any attempt at peptide fractionation. In addition to the PTH amino acids expected from residues 1-5 of the whole molecule, the sequence gly-phe-gly-pro-glu was obtained. At the sixth step PTH threonine was obtained in high yield, consistent with its origin from both peptide fragments. The residue remaining after the sixth Edman cycle was placed on the basic column of the amino-acid analyser and prolinamide was identified. The combined results from the automated degradations on the whole molecule and on the unfractionated cyanogen bromide mixture thus gave the complete sequence of ovine calcitonin.

Parathyroid Hormones

Bovine and porcine parathyroid hormones are 84 amino peptides whose isolation, characterization and sequence analysis are being reported elsewhere (6,7,8,9). Bovine parathyroid hormone exists in at least 3 different molecular forms differing in amino acid composition but separable by recently developed techniques (6). Human parathyroid hormone so far has been available only in small amount. However, its provisional amino acid composition resembles that of the porcine and bovine hormones (10).

The strategy for automated sequence analysis of parathyroid hormones is apparent from Figure 4. The whole molecule is of

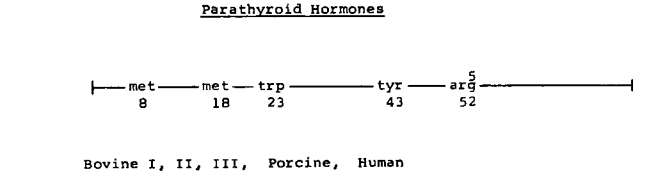

Fig. 4

sufficient size that initial degradations may be made with either the Quadrol system or the volatile peptide system. Runs of over 50 cycles have been accomplished on the bovine hormone. These degradations proceed past all the potentially cleavable residues shown in Figure 4. (There are no arginines carboxyl terminal to residue 52 in the major bovine hormone). Very strong confirmation of the sequences obtained was achieved for bovine parathyroid hormone by automated degradations with and without fragment separation after specific cleavages at positions indicated in the figure. Degradations on smaller fragments isolated from amino terminal, middle and carboxyl terminal portions of the bovine molecule were carried out using volatile reagents.

The structure for the first 34 residues of the molecule (Fig. 5) has been validated by synthesis of this fragment by

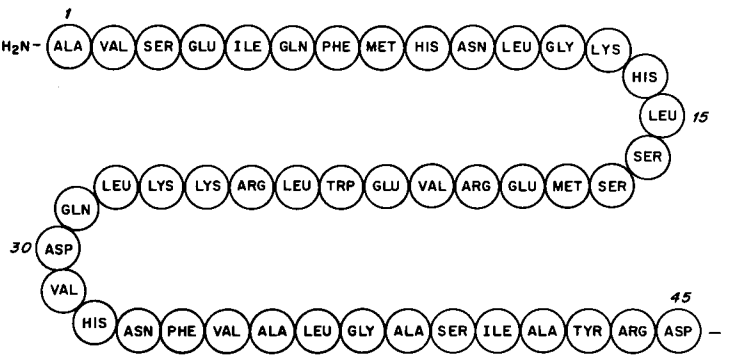

Fig. 5 Bovine Parathyroid Hormone: Sequence of active fragment

the solid phase procedure; the synthesis was initiated before the determination of the complete structure. This fragment was chosen for synthesis because earlier work had indicated that the first 45 residues contained the structural requirements for biological activity. (Fig. 5) A report of the

synthesis of the 1-34 fragment, which possesses all the osseous and renal activities of the whole 84 residue molecule, is being published elsewhere (11).

The strategy for sequence analysis of parathyroid hormone was established on the major bovine molecule, and is presently being applied in our laboratory to the other parathyroid hormones mentioned above.

Identification of PTH amino acids

Common to all variants of the Edman procedure is the problem of identification of the products. Recently we have developed a procedure using a new reagent, pentafluorophenyllisothiocyanate, for degradation. The resulting pentafluorophenylthiohydantoins have excellent gas chromatographic properties (Figure 6), and may have several advantages over

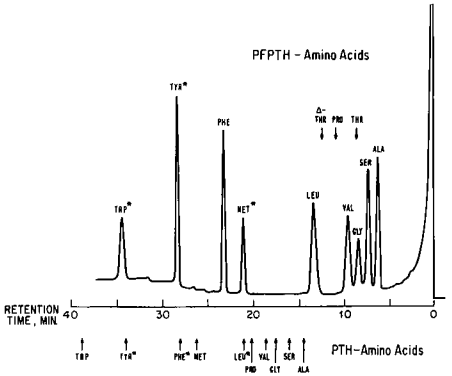

Fig. 6 Gas chromatography of fluorinated PTH derivatives

the previously used phenyl derivatives. One of these is their potential for detection at high sensitivity by electron capture techniques. Their full evaluation is in progress.

We have also developed a new modification of the Edman procedure in which two (or more) different isothiocyanate reagents are used in alternation during the degradation. For example, phenylisothiocyanate may be used at steps 1, 3, 5, etc. and pentafluorophenylisothiocyanate or methylisothiocyanate at steps 2, 4, 6 etc. This is particularly useful in the analysis of complex mixtures, or of peptides with repeating residues, since confusion due to incomplete extraction of the thiazolinones from earlier cycles is eliminated. (Some incomplete extraction of thiazolinones is inevitable when working at high sensitivity, since the solvent extractions must be reduced greatly to avoid losses of peptide). Naturally to use this technique one must be able to identify at least two sets of thiohydantoin derivatives simultaneously. We have developed a two column gas chromatographic procedure for simultaneous identification of phenyl and pentafluorophenyl derivatives. This is illustrated in Figure 5. The elution

positions of the phenyl derivatives is shown in comparison to the fluorinated thiohydantoins on a DC 560 column with a temperature program. Almost all derivatives have distinct elution times. Pairs of derivatives which overlap, indicated by an asterisk, are easily separated by further derivatization and reinjection under similar conditions. We have used this approach already in the simultaneous sequence analysis of a mixture of four tryptic peptides from two molecular variants (II and III) of salmon calcitonin.

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Gas Chromatographic Analysis of Amino Acid Phenylthiohydantoins

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Manuscript not received

QUANTITATIVE SEQUENCE ANALYSIS OF POLYPEPTIDE MIXTURES

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INTRODUCTION

In recent years synthesis of peptides has been carried out by techniques in which amino acid residues are added in a sequential fashion to the growing polypeptide chain.[1] If the reaction coupling the amino acid residues to the chain is less than complete, the free reactive groups resulting may couple to an amino acid residue later in the sequence, introducing heterogeneity. Heterogeneity of this type may be important in the synthesis of macromolecules such as enzymes.[2,3]

Heterogeneity of amino acid sequence is also a common feature of proteins. There is strong evidence that new protein species may be formed by the process of linear gene duplication.[4] The duplicated gene is free to undergo point mutation, perhaps in the process developing new functions. Since the three dimensional structure of the mutated protein imposes a constraint on the types of amino acid exchanges that can be effected; often

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Supported by Grants RO1-AI-08614 from the Public Health Service, the National Science Foundation and the American Heart Association

there is no change in charge of the molecule, or if a change in charge occurs at one locus, it may be balanced by a second mutation which balances the overall charge.[5] From the point of view of structural analysis, macromolecules are obtained which show little or no change in overall structure and charge and may be very difficult to separate from each other. The immunoglobulins are examples of such molecules.

Table I shows the structure at the N-terminal end of a number of human myeloma protein K chains which have been arranged into their respective sub-classes (Data of Dr. Leroy Hood). As it seems likely that such structures are representative of normal γ globulin mixtures, it may be very difficult to isolate specific antibody sequences from complex mixtures.

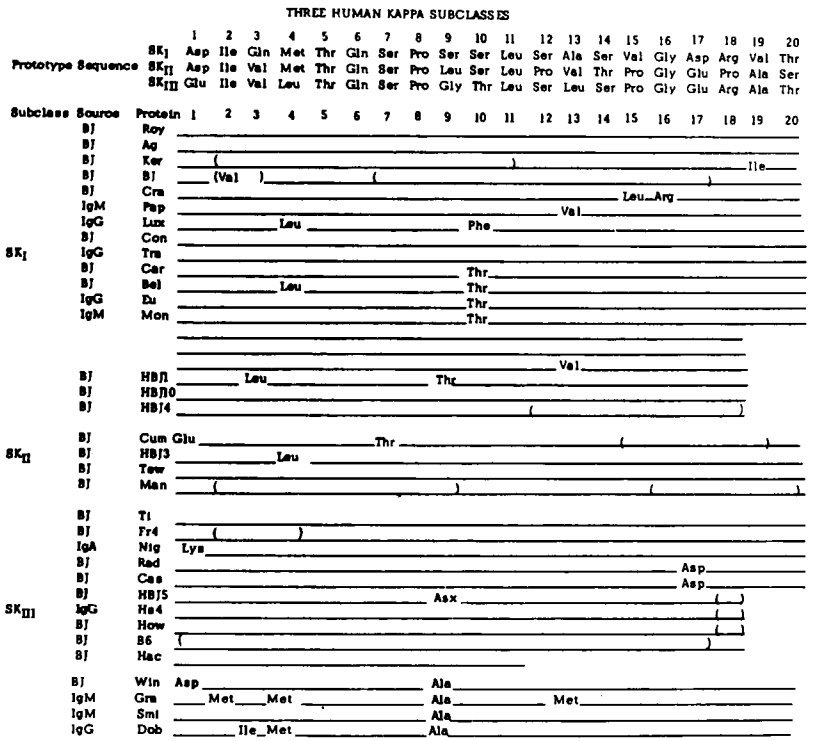

Table I. Subclasses of human immunoglobulin Kappa light chains. The archetypes of each sub-class are given in the top three lines. In the sequences given below, variant amino acids occurring at each locus are given.

A method has been devised which is capable of carrying out quantitative sequence analysis on mixtures of peptides. It consists of a modified Edman procedure using the volatile reagent, methyl isothiocyanate. This reagent is coupled to the N-termini of a polypeptide mixture and subsequently cyclized with acid. The released methyl thiohydantoin (MTH) amino acid derivatives are diluted into a standard mixture of ^{15}N labeled MTH amino acids whose $^{14}N/^{15}N$ ratio has been previously determined. ^{14}N MTH amino acids from the N-termini of the polypeptide mixture dilute the standard mixture and the new $^{14}N/^{15}N$ ratios are determined on a single-focusing mass spectrometer. The complexity of the mass ion and fragmentation ion pattern of the twenty ^{14}N and twenty ^{15}N MTH amino acids is resolved using two techniques. The mass spectrometer is run at low ionizing voltage (10 eV) which retains the mass ions but reduces the complexity of the fragmentation ion pattern. In addition, use is made of the differential volatility of the MTH amino acid derivatives. The sample containing MTH amino acid mixtures is placed in the solid sample inlet of the CEC 21-490 mass spectrometer, at a pressure of 10^{-7} torr. The temperature is raised between 80-220° C and the MTH derivatives are distilled sequentially into the ion beam. The mass ions, fragmentation patterns and distillation temperature give unequivocal identification and quantitation of all MTH amino acid derivatives in a complex mixture.

METHODOLOGY

Edman Reaction. Methyl isothiocyanate is employed as the Edman reagent. The methylthiocarbamate adduct is cyclized with trifluoroacetic acid. Excess methyl isothiocyanate is removed at 10^{-3} torr and the MTH amino acid derivatives extracted with tetrahydrofuran. Figure I shows in summary the conditions employed. Details of reagent preparation and procedure are given elsewhere. [6,7]

Two different procedures have been employed in quantitating amino acid residues. Originally, standard mixtures containing 95% atom excess ^{15}N labeled amino acid MTH derivatives, in quantities approximately equimolecular to the N-terminal amino acids to be detected, were added to the reaction mixture at the beginning of each degradative cycle. It was determined later that ^{15}N amino acids could replace the MTH derivatives without loss of accuracy. Since the addition of equimolecular quantities of twenty

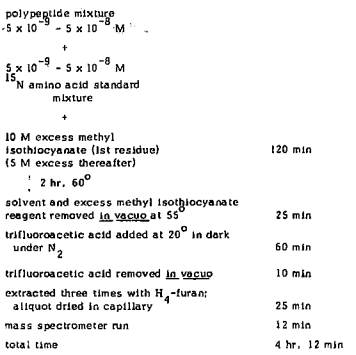

Figure I. Flow Sheet for the Sequential Degradation Procedure

^{15}N amino acids is relatively expensive, we now run each sequence analysis in duplicate. The first run determines the amino acids found at each locus. In the duplicate determination, only those ^{15}N amino acids present at each locus are added for quantitation.

Identification and Isotope Ratio Assay on the Mass Spectrometer. A CEC 21-490 single-focusing mass spectrometer fitted with a variable temperature probe, and an electrical detection system is used. A sample is placed in the solid inlet probe and the temperature is gradually increased. When the ion current begins to rise, a note is made of the indicated temperature and spectra at 10 eV and 80 eV are taken rapidly (30-40 secs) over the range m/e 90-280. Each amino acid residue may be identified unequivocably by a combination of the following criteria:

(a) The M+ ion.. All amino acid MTH derivatives give mass ions with the exception of serine and S-methylcysteine.

(b) The fragmentation patterns are characteristic for each amino acid MTH and determine unequivocably the nature of residues having the same mass ion; i.e. leucine MTH and isoleucine MTH. The relative peak heights at 10 eV and 80 eV serve as further criteria of identity.

(c) Temperature of volatilization of the MTH derivatives is used in conjunction with the parameters mentioned above to characterize the residue. Table II sets out in detail the characteristics of the major ion peaks and their temperatures of volatilization.

Table II. Mass Spectroscopic Identification of Amino Acid Methylthiohydantoin Derivatives.[a]

Amino Acid Methylthiohydantoin Derivative	m/e	Relative Abundance 80 eV	Relative Abundance 10 eV	Temp Range (°C)
Valine	172*	65	100	70-80
	130	100	50	
Leucine	186*	70	100	80-90
	143	30	30	
	130	100	72	
Isoleucine	186*	47	44	80-90
	130	100	100	
Glycine	130*	100	100	90-100
	102	32	46	
Threonine	174*	4	2	90-100
	156	76	20	
	130	100	100	
Serine	160*	0?	0?	90-100
	142	100	100	
Alanine	144*	100	100	90-110
Proline	170*	100	100	100-110
Methionine	204*	100	100	110-125
	156	16	16	
	143	43	78	
	130	48	91	
S-Methylcysteine	190	50	50	120-130
	142	100	100	
Phenylalanine	220*	60	30	125-140
	129	6	6	
	91	100	100	
Glutamine	201*	75	100	125-150
	184	100	88	
	156	20	8	
	142	80	44	
	130	17	2	
Glutamic acid	202*	90	100	140-160
	184	65	60	
	156	20	8	
	142	100	30	
	130	5	2	
Tyrosine	236*	20	40	150-170
	152		3	
	130	30	20	
	107	100	100	
Aspartic acid	188*	100	100	160-180
	170	3	3	
	142	80	28	
Asparagine	187*	100	100	170-190
	170	48	30	
	142	80	60	
Tryptophan	259*	20	6	170-190
	130	100	100	
Lysine	274*	3	4	190-210
	243	80	100	
	212	10	60	
	201	35	34	
	184	40	37	
	143	100	25	
	130	30	6	
Arginine	229*	10	9	190-210
	212	70	13	
	170	94	100	
	142	100	17	
	130	88	71	
Histidine	210*	100	100	220-230
	130	25	18	

(a) m/e Ratios and relative abundances at 80 and 10 eV are given for the M+ ions (*) and for those fragmentation ion peaks useful for identification. The temperature ranges given are those for the maximal ion current for each compound.

RESULTS

The quantitative sequencing method has been used both to explore the quantitative aspects of the Edman reaction and to use this reaction for sequence analysis of protein and peptide mixtures.

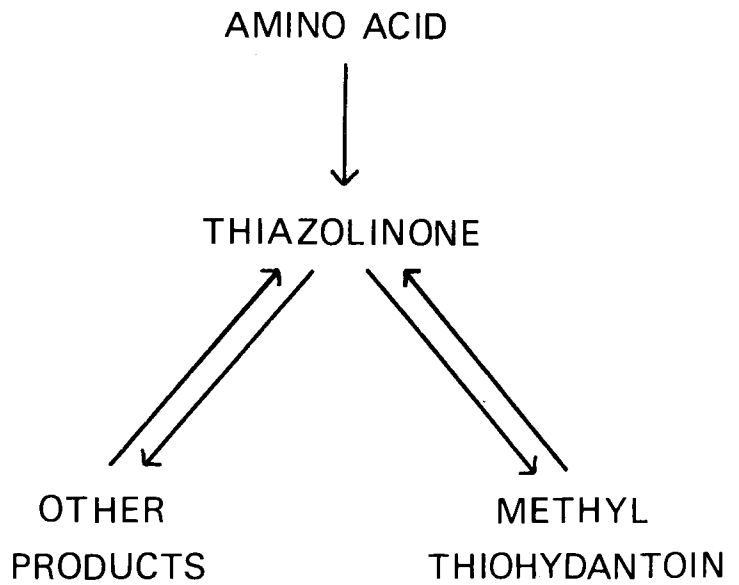

Figure II. The Reaction Sequence of the Edman Reaction

Figure II shows the reaction sequence leading to the formation of the MTH amino acid derivatives. It was suggested [8] that if conversion of the thiazolinone intermediates to products other than thiohydantoin derivatives is quantitatively important, it may be difficult to use the reaction for quantitative purposes since it would be necessary to know the equilibria of all the reactions involved. The importance of such alternative pathways was tested on insulin. The A and B chains of reduced ∝ carboxymethylated bovine insulin (Mann Biochemicals) were sequenced together. ^{15}N isotope dilution was introduced in the form both of the MTH derivative and of the amino acid. If diversion of the label to compounds other than the MTH derivatives is a quantitatively important process, the recoveries of N terminal amino acids using ^{15}N MTH as isotope dilution agent should have been systematically below the value found in experiments in which ^{15}N amino acids were used as isotopic label. (See Figure II.) In fact, no systematic differences were encountered (Figure III) suggesting that the alternative pathways are not important enough to interfere with the quantitation of procedure.

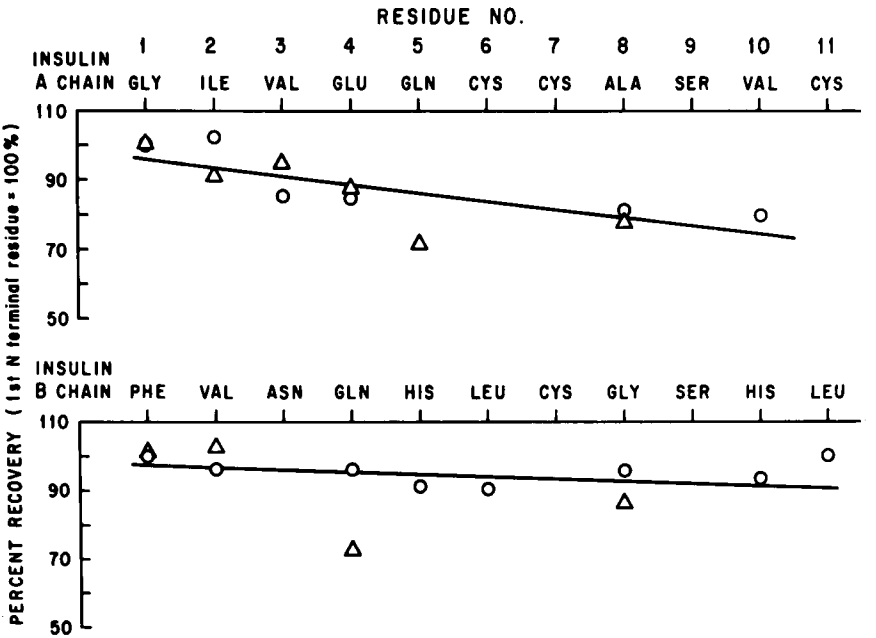

Figure III. Recovery of methylthiohydantoin amino acids on simultaneous sequential degradation of the A and B chains of insulin: (— △ —) recovery values using ^{15}N methylthiohydantoin derivatives in the isotope procedure; (— O —) recovery values using ^{15}N amino acids. Average loss in reactive N-terminal residues equals 2.5% per residue (A chain); 0.9% per residue (B chain).

Figure III also shows that the rate of loss of insulin A and B is quite different, being about 2.5% per residue for the A chain and about 0.9% per residue for the B chain. It seems likely that such differences under the same reaction conditions must be due to loss of peptide chains in the extracting solvent, tetrahydrofuran; the A chain being presumably more soluble than the B chain.

Quantitative Analysis of Complex Mixtures. Table III sets out the result of the analysis of an "unknown" mixture of polypeptides. The quantities of each amino acid residue found at each locus is set out in decreasing order of quantity in Section "A" of the table.

Table III. Simultaneous Analysis of a Mixture Containing Four Reduced and Carboxymethylated Polypeptide Chains.

	Amino Acid Residue										
	1	2	3	4	5	6	7	8	9	10	Conclusions
Section A[a] (μmoles)	Lys, 7.0 Gly, 1.3 Phe, 1.2	Glu, 4.7 Val, 3.2 Ile, 1.0	Thr, 4.7 Phe, 2.2 Val, 1.0 Asn, 1.0	Ala, 4.7 Gly, 2.2 Gln, 0.9 Glu, 0.8	Ala, 4.6 Arg, 2.1 His, 1.0 Gln, 0.8	Ala, 4.3 Leu, 1.0 CM-Cys, N/M	Lys, 3.8 Glu, 1.6 CM-Cys, N/M	Phe, 4.6 Leu, 1.7 Ala, 0.8 Gly, 0.8	Glu, 3.3 Ala, 2.3 Ser, 1.5	Arg, 3.4 Ala, 1.5 Val, 0.8 His, N/M	4 Polypeptide chains present
Sequence 1 (μmoles)	Lys, 4.7	Glu, 4.7	Thr, 4.7	Ala, 4.7	Ala, 4.6	Ala, 4.3	Lys, 3.8	Phe, 4.6	Glu, 3.3	Arg, 3.4	Major sequence (47%)
Section B[b] (μmoles)	Lys, 2.3 Gly, 1.3 Phe, 1.2	Val, 3.2 Ile, 1.0	Phe, 2.2 Val, 1.0 Asn, 1.0	Gly, 2.2 Gln, 0.9 Glu, 0.8	Arg, 2.1 His, 1.0 Gln, 0.8	CM-Cys, N/M Ile, 1.0	Glu, 1.6 CM-Cys, N/M	Leu, 1.7 Ala, 0.8 Gly, 0.8	Ala, 2.3 Ser, 1.5	Ala, 1.5 Val, 0.8 His, N/M	
Sequence 2 (μmoles)	Lys, 2.3	Val, 2.2	Phe, 2.2	Gly, 2.2	Arg, 2.1	CM-Cys, N/M	Glu, 1.6	Leu, 1.7	Ala, 2.3	Ala, 1.5	Second sequence (25%)
Section C[c] (μmoles)	Gly, 1.3 Phe, 1.2	Val, 1.1 Ile, 1.0	Val, 1.0 Asn, 1.0	Gln, 0.9 Glu, 0.8	His, 1.0 Gln, 0.8	Ile, 1.0 CM-Cys, N/M	CM-Cys, N/M	Ala, 0.8 Gly, 0.8	Ser, 1.5	Val, 0.8 His, N/M	
Unresolved sequences 3 and 4 (μmoles)	Gly, 1.3 Phe, 1.2	Val, 1.1 Ile, 1.0	Val, 1.0 Asn, 1.0	Gln, 0.9 Glu, 0.8	His, 1.0 Gln, 0.8	Ile, 1.0 Cys, N/M	CM-Cys, N/M CM-Cys, N/M	Ala, 0.8 Gly, 0.8	Ser, 0.8 Ser, 0.8	Val, 0.8 His, N/M	Unresolved sequences 14% each sequence
Section D[d]											
Ribonuclease (5 μmoles)	Lys	Glu	Thr	Ala	Ala	Ala	Lys	Phe	Glu	Arg	
Lysozyme (2.5 μmoles)	Lys	Val	Phe	Gly	Arg	Cys	Glu	Leu	Ala	Ala	
Insulin A (1.4 μmoles)	Gly	Ile	Val	Glu	Gln	Cys	Cys	Ala	Ser	Val	
Insulin B (1.4 μmoles)	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	

(a) The number of micromoles of recovered amino acid methylthiohydantoin derivative at each locus are given. The inferred major sequence (47% of total moles of protein present) is given as sequence 1. N/M= residue identified but not quantitated. (b) Micromoles of amino acid methylthiohydantoin at each locus after subtraction of sequence 1. The second (25%) sequence inferred is given. For ambiguity at residue 9, please see text. (c) The residues at each locus of the two minor sequences (14%) present in equimolar proportions. (d) Composition of test mixture. The composition of each protein added was checked by amino acid analysis.

At some loci three amino acids are found (Loci 1, 2, 6, 7 and 9). At other loci (3, 4, 5, 8 and 10) four amino acids are found. Therefore, there must

be at least four different polypeptide sequences in the mixture. Those loci where all four amino acids are different, the molar quantity of each amino acid must be the same as that of the polypeptide of which it is a member. The most abundant amino acids at loci 3, 4, 5, 8 and 10 decrease in value from 4.7 μm at residue 3, to 3.4 μm at residue 10, due to sequential handling and extraction losses. We can therefore extrapolate values for the most abundant amino acids at residues 1, 2, 6, 7 and 9. In each case there is only one amino acid present in the necessary abundance at each locus. An unambiguous major sequence may therefore be constructed and this is given as Sequence #1. It represents 47% of the total moles of protein present.

This sequence is subtracted from the amino acids initially present, the remainder is set out in Section "B", and the same analysis is repeated and Sequence #2 is obtained. (25% of the total moles of protein present.) This sequence shows a single ambiguity at locus #9. S-methylcysteine at loci 5 and 6 and histidine at locus 10 were not measured quantitatively. Nevertheless, inspection shows that it is possible to assign these residues unambiguously to their correct polypeptides.

When the second sequence is subtracted, it is clear that the remainder (Section "C") corresponds to two further sequences present in approximately equimolecular amounts each totaling about 14% of the protein present. Since the same quantity of each sequence is present it is not possible to assign the residues to either one chain or the other.

The labor involved in sequencing simultaneously four peptides is less than that required for the sequence determination of a single polypeptide using conventional detection methods.

APPLICATIONS OF THE METHOD - PRESENT AND FUTURE

The method has so far been used to answer the following kinds of questions:

(a) Sub-units of Yeast Enolase. It has been found that yeast enolase is composed of two sub-units of equal size.[9] The whole molecule, however, binds two magnesium ions which have different affinities for

the enzyme molecule. The more tightly bound magnesium ion induces conformational change in the enzyme, the more loosely bound magnesium ion is probably involved in substrate catalysis.[10] This has raised the question whether there may be differences between the sub-units. A careful quantitative analysis revealed that a unique sequence Ala-Gly-Lys-Val-Gly-Asp-Thr-Gl^{n} was present in twice the molar concentration of the protein, and that no other amino acids were present in quantities greater than 5×10^{-9} moles. Thus residues 1-8 of the two sub-units of enolase are identical.[11]

(b) Protein Fractionation Without Separation of Peptides. Hen's egg lysozyme was digested with cyanogen bromide. The three resulting peptides 1-12, 13-105, 106-129 were simultaneously analyzed for amino acid sequence for ten residues. It was possible to keep track of the three peptides due to the very different loss rates for the three peptides. Over 20% of the total sequence of the whole protein could be obtained in a single run from a single sample. Similar studies have been performed on the light chain of a human myeloma γ G immunoglobulin (PAS).

Where a proteolytic agent splits a protein very efficiently, so that equimolecular quantities of each fragment peptide are obtained, two principles may be followed to change the proportions of the peptides. Unfractionated peptides may be added back. This serves to increase the amount of the N-terminal peptide relative to the other peptides and allows its identification. (Enrichment technique.) An incomplete Sephadex separation may also serve to change the proportions of the peptides relative to each other. A combination of these techniques will usually overcome difficulties due to the presence of equimolecular quantities of fragment peptides.

(c) Differentiation and the Dynamics of Cell Populations. When an animal is immunized with a protein to which a strong immunological determinant (hapten) has been covalently attached, the antibodies elicited against the hapten form a complex population in which individual

members of the population differ from each other both in structure and in affinity for the hapten.[12]

If the average intrinsic binding constants for the whole population is measured early and late in the immune response, it can be demonstrated that the cells producing antibodies have undergone a population change. Early in the response the average K^A may be as much as four orders of magnitude lower than the average K^A late in the response.[13] The kinetics of the cell population changes may be followed by tracing the appearance and disappearance of the major antibody sequences in the population during the differentiation of the immune response. Such studies have been undertaken.[14]

(d) Applications to Peptide Synthesis. Weigand has used isotope dilution methods to estimate quantities of "failure sequences" in the solid state synthetic technique.[15] The quantitative sequence analysis technique can be carried out on an automatic sequenator.[16] We have used a Bio Cal ES300 machine for this purpose. One of us (R.L.) has designed and built an automatic interface unit between the ES300 and a CEC 21-490 single focusing mass spectrometer. Computer handling of mass spectrometer output is in use in many laboratories. It should therefore be possible to construct fully automatic feed-back systems, which continuously monitor the attachment of the last amino acid residue to the growing polypeptide chain. From such data, the length of time of the coupling reaction, or perhaps the concentration of reagents could be continuously adjusted until the addition reaction has gone to completion. Alternatively, such systems could be used to stop reactions at a stage where addition is incomplete.

A GENERAL CRITIQUE OF THE QUANTITATIVE SEQUENCE ANALYSIS METHOD

The method is intended to work with polypeptide mixtures. It requires that the individual peptides constituting the mixtures shall not be present in equimolar or near-equimolar concentrations. The usual methods of protein isolation produce situations where predominant and minor peptide

constituents are present in mixtures. Most agents which cleave proteins do not work with 100% efficiency and such mixtures may be used with this method without modification. For equimolecular peptide mixtures the enrichment methods or partial peptide separation methods mentioned above, may be used.

In order to construct sequences, it is necessary to compute the rate of loss of N termini available to the Edman reagent. This can be done only at those loci where the amino acids of all sequences differ. Table III shows that when four completely different polypeptides are analyzed together, in five out of the first ten loci, the same amino acid occurs in more than one sequence. If the number of polypeptides is increased, the number of loci at which there are common amino acids will be expected to increase also. It will be necessary to sequence more residues of the peptide mixture in order to find enough loci at which all amino acids are different and which may be used to calculate the slope of the "loss" line. In essence the occurrence of the same residues at the same locus in more than one sequence constitutes a series of simultaneous equations with more than one variable in each. The computer should help in the solution of such equations. With present techniques it seems probable that between 6-8 peptides constitutes the maximum number which can be sequenced at once.

At present the optimal sample size is between 5×10^{-8} - 5×10^{-7} moles although it is possible to measure samples present in quantities as low as 1×10^{-9} moles. Introduction of chemical ionization methods, improvements in mass spectrometer solid sample inlet geometry and heat control, should reduce the size of the sample required. In general the method is considerably less tedious than the present methods of sequencing, and it produces accurate quantitative results. Since the basis of the method is the Edman reaction, the method shares the difficulties of this method. (Reviewed elsewhere in this volume.) The cost of a single focusing mass spectrometer is in the same range as the cost of an amino acid analyser. And the cost of isotopes is low relative to the cost of labor required for the manual identification of amino acid residues.

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METHYLATION AS A ROUTE TO PEPTIDE INTERMEDIATES OF N-METHYLAMINO ACIDS AND ε-N-METHYLLYSINES

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N-Methylamino acids are generally less reactive than amino acids, consequently the preparation of their derivatives often presents problems. What I am going to describe is a completely new approach to the synthesis of such derivatives. Our approach differs from available procedures in that the derivatives are not prepared from the methylamino acid, but instead a derivative of the parent amino acid is used as starting material and this is converted to the corresponding N-methylamino acid derivative. The reaction is described in Fig. 1. It involves the methylation of an N-acyl- or N-alkyloxycarbonylamino acid with sodium hydride and methyl iodide to give the N-protected N-methylamino acid methyl ester, followed by selective or complete deprotection as desired (1). A summary of the background literature on the alkylation of amides appears in Fig. 2. We found only two references in the literature to the use of sodium hydride for the alkylation of amides and urethanes (2,3).

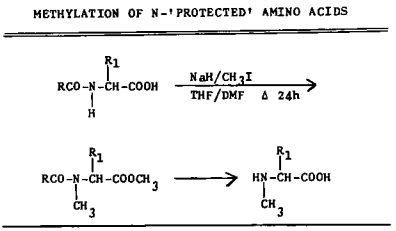


Fig. 1

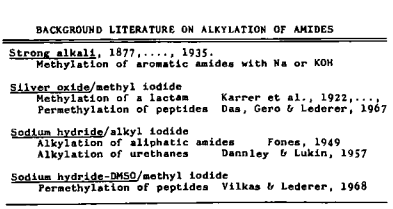


Fig. 2

Also, in line with our previous interests in the ε-N-methyllysines, I shall describe a new synthesis of ε-N-methyllysine and ε-N-trimethyllysine derivatives. And finally I shall present some data, obtained in experiments with model compounds, which gives a comparison of the efficiency of our methylating technique with that of the other two techniques commonly used for the permethylation of peptides for sequential analysis by mass spectrometry.

When working with N-methylamino acids, one encounters the obstacle that they are difficult to determine because the color yield of their reaction with ninhydrin is very low. We have found that the ninhydrin color constants obtained with an amino acid analyzer for N- and α-C-methylamino acids increase dramatically, from 5 to 30 times, when the eluting buffer flow rate is decreased to half-normal (4), as indicated in Fig. 3.

CHROMATOGRAPHIC DATA FOR DIFFERENT BUFFER FLOW RATES*

	Constant (H x W)/c		Elution time (min)	
	68 ml/h	34 ml/h	68 ml/h	34 ml/h
Gly	23.4	38.9	33	67
MeGly	3.0	36.3	30	59.5
Phe	22.7	40.6	79	157
MePhe	0.66	23.5	61	107
Ala	24.1	42.7	32.5	61
MeAla	0.36	7.5	28	54
α-C-Me-Ala+	1.8	10.2	33	62
Leu	23.9	40.5	51	102
MeLeu	0.74	15.7	34	63
α-C-Me-Leu	1.95	11.2	46.5	92.5
EtLeu	0.03	1.0	32	60.5

* Beckman Analyzer. 0.9 x 50 cm column of AA-15 resin, 0.35 N sodium citrate, pH 4.25, 57°.
+ α-Aminoisobutyric acid.

Fig. 3

METHYLATION* OF N-'PROTECTED'-LEUCINES

Derivative	Yield** (%)		
	MeLeu	Leu	Total
Cbz	96	1.1	97
Boc	86	3.0	89
Bz	96	0.1	96
Ac	88	0.7	89
For	95	4.0	99
Tos	61	4.0	65
Pht	46	26.3	81***
Trt	11	84.6	96

* MeI/NaH/RLeu (8:3:1) THF/DMF (10:1) 80° 24h
** Yields determined with amino acid analyzer after suitable deprotection
*** Includes 9% α-C-methyl-leucine (NaOH; HCl deprotection).

Fig. 4

Our initial methylating experiments were carried out with leucine derivatives on an analytical scale. The results appear in Fig. 4. The conditions given are those which were found to give the highest yields. The dimethylformamide is not at all essential to the reaction, but it increases the yield by 3-5%. Note in the first five cases the high yield of N-methylleucine and the correspondingly low recovery of leucine. We believe the low total recovery for the tosyl derivative experiment was due to difficulty with deprotection. The phthalyl and trityl derivatives were tested to see if these could be used for protection against N-methylation. The phthalyl group provided some protection, but also promoted α-C-methylation, and moreover must have been partly cleaved by the reagent since substantial N-methylation did take place. The results indicated that the trityl group could be used for protection against N-methylation.

Results for alkylations with other alkyl iodides appear in Fig. 5. It is seen that ethylation and propylation were complete for a glycine derivative, but incomplete for a leucine derivative and poly-leucine. The reaction of sodium hydride/methyl iodide with other functional groups is described in Fig. 6. When the starting material was an ethyl ester, a

EXTENT OF N-ALKYLATION WITH VARIOUS IODIDES

Derivative	Alkyl iodide	RI/NaH/X*	Residual amino acid (%)
Cbz-Gly	Me	8:3:1	0.3
	Et	32:3:1	0.9
	Pr	32:3:1	1.1
Cbz-Leu	Me	8:3:1	1.1
	Et	8:3:1	12.3
	Pr	8:3:1	41.1
	Pr	32:3:1	43.3
Poly-Leu	Me	8:3:1	2.4**
	Et	8:3:1	36.4

* THF/DMF (10:1) 80° 24h
** 91% methyl-leucine detected.

Fig. 5

REACTION OF SODIUM HYDRIDE - METHYL IODIDE WITH OTHER FUNCTIONAL GROUPS

	—COOH	⟶	-COOMe
Tyr	—C_6H_4-OH	⟶	-C_6H_4-OMe
Lys	—$\overset{+}{N}H_3$ (ε)	⟶	-$\overset{+}{N}Me_3$
Asn	—CO-NH_2	$\overset{*}{\longrightarrow}$	-CO-NMe_2
PhCOOH	—COOEt	$\overset{*}{\longrightarrow}$	-COOMe
Tyr(Cbz)	—C_6H_4-OCbz	⟶	-C_6H_4-OMe

* Evidence from n.m.r. only.

Fig. 6

substantial but incomplete ester interchange took place (results of Mrs. B.A. Stoochnoff). Experiments with Cbz-Ser and Cbz-Thr indicated a breakdown of the molecule under the standard reaction conditions.

The use of the methylation reaction on a preparative scale is described in Fig. 7 and 8. It is important to note

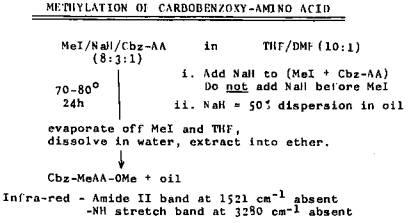


Fig. 7

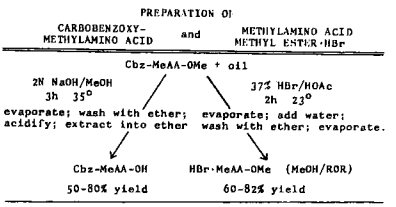


Fig. 8

that if the sodium hydride is added to the Cbz-AA before the methyl iodide, the yield of product is substantially lower. The esters obtained contained less than 0.1% of unmethylated amino acid ester, and the L-MeLeu-OMe, before crystallization, contained less than 1% of the D-isomer as judged by the method of Manning and Moore (4,5). Other derivatives prepared in the same manner are shown in Fig. 9.

YIELDS* (%) OF METHYLAMINO ACID DERIVATIVES

	Carbobenzoxy	Ester·HBr	Acetyl	Benzoyl
MeAla	65	82	7	57
MeVal	60	61	73	
MeLeu	50	65	55	74
MeIle	55**	56	37	
MePhe	82**	81	55	
MeTyr(Me)		71		

* Recrystallized. Having satisfactory C H N analysis.

** Oil

Fig. 9

ALKYLATION OF α-N-TRITYL-ε-N-CARBOBENZOXY-LYSINE METHYL ESTER

Products (%)	Methyl	Ethyl	Propyl
ε-N-alkyl-lysine	103	90	-
lysine	0.6	1.2	20
Total	104	91	-

Cbz
RI/NaH/Trt-Lys-OMe (8:5:1) 80° 24h

Fig. 10

Having established the protecting action of the trityl group against methylation, use was made of this in investigating the alkylation of an ε-N-Cbz-amino group (Fig. 10), and the methylation of a free amino group (Fig. 12). Both the methylation and ethylation of an ε-N-Cbz-amino group went to completion, but propylation did not. From this was developed a synthesis of a very useful peptide intermediate of ε-N-methyllysine, the methyl ester of the ε-N-Cbz derivative (Fig. 11) which also provides a convenient source for ε-N-methyllysine. In an analogous manner, the corresponding ε-N-ethyllysine ethyl ester derivative has been obtained in 20% yield (results of Mr. G. Moore). Methylation of a free amino group gave the trimethylamino group which provided a useful derivative of and a new synthesis of ε-N-trimethyllysine (Fig. 12). The starting materials for these studies were

TRITYL GROUP FOR N-PROTECTION.
A NEW SYNTHESIS OF ε-N-METHYL-L-LYSINE

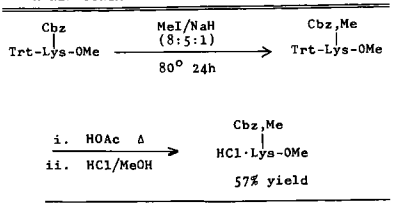
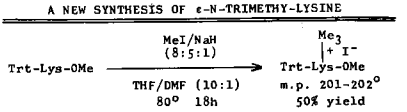


Fig. 11

METHYLATION OF A FREE AMINO GROUP.
A NEW SYNTHESIS OF ε-N-TRIMETHY-LYSINE



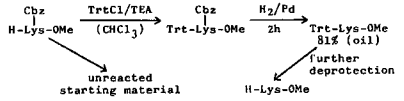
i. Amino acid analysis after deprotection indicated quantitative conversion to ε-N-trimethyl-lysine

ii. Satisfactory n.m.r. for ester of trimethyl-trityl-lysine

Fig. 12

obtained from Lys(Cbz)-OMe, prepared using boron trifluoride/methanol (6), as described in Fig. 13.

PREPARATION OF α-N-TRITYL-LYSINE ESTER
for studying products of methylation of free amino group



Both readily removed by washing a chloroform solution with water after treating with N NaOH/THF at 25° for 0.5h. This saponifies all esters except those of α-N-trityl amino acids.

Fig. 13

COMPARISON OF DIMETHYLFORMAMIDE AND DIMETHYLSULFOXIDE AS SOLVENTS

Products (%)	Ac-Leu		Cbz-Leu		
	DMF	DMSO	DMF	DMSO	
MeLeu	88	42	96	18	32
Leu	2.9	36.8	1.1	0.9	0.6
Total	91	79	97	19	33

Reagents, 8:3:1, 80° 24h
Solvent, THF/DMF (10:1) or THF/DMSO (10:1)

Fig. 14

There is presently considerable interest in the determination of the sequence of peptides by mass spectrometry. Ordinary peptides are not volatile enough for analysis, so they are converted to a derivative prior to analysis. One of the approaches to this is the permethylation of the peptide bonds, as introduced by Dr. Lederer's group (7), which has the additional effect of simplifying the fragmentation pattern such that the major peaks observed on the spectra are the sequence-determining peaks. Methylation has been carried out with methyl iodide and silver oxide (8) or methylsulfinyl carbanion (9), which is prepared from sodium hydride and dimethylsulfoxide. We have carried out the methylation of two model compounds, Ac-Leu and Cbz-Leu, using our standard conditions, and after replacing the dimethylformamide with dimethylsulfoxide (Fig. 14), and then using the reagents and conditions described in the literature for the permethylation of peptides (Fig. 15 and 16). The results of all these experiments can be summarized as follows: using sodium hydride, the methylation reaction goes to near completion, and all of the starting material is accounted for if the solvent is THF/DMF but not THF/DMSO; using silver oxide, the reaction is incomplete but all of the starting material is accounted for; using methylsulfinyl carbanion, the reaction is incomplete and not all of the starting material can be accounted for. In the latter case, there obviously must be some reactions taking place besides N-methylation. On the basis of these and other results, we

have suggested the use of sodium hydride/methyl iodide for the permethylation of peptides for sequential analysis by mass spectrometry (10).

COMPARISON OF METHYLATING REAGENTS
I Methylation of Carbobenzoxy-leucine

Products (%)	Sodium hydride*	Silver oxide**	Methylsulfinyl carbanion***
	8:3:1	16:4:1	8:4:1
MeLeu	96	66	23
Leu	1.1	27.1	27.7
Total	97	93	51

* Method of Coggins and Benoiton
** Method of Thomas, Das, Gero and Lederer (1968)
*** Method of Vilkas and Lederer (1968)

Fig. 15

COMPARISON OF METHYLATING REAGENTS
II Methylation of Acetyl-leucine

Products (%)	Sodium hydride*	Silver oxide**			Methylsulfinyl carbanion***	
	8:3:1	16:2:1	32:4:1	32:8:1	8:2:1	8:4:1
MeLeu	88	43	41	85	35	35
Leu	2.9	60	50	11	28	0
Total	91	103	91	96	63	35

* Method of Coggins and Benoiton
** Method of Thomas, Das, Gero and Lederer (1968)
*** Method of Vilkas and Lederer (1968)

Fig. 16

We have also carried out experiments on the methylation of some 'N-protected' di- and tripeptide esters. As evidenced by infra-red analysis (see Fig. 7), the methylation proceeded to completion. However, we were never able to obtain a satisfactory recovery of N-methylamino acids after hydrolysis of the peptides. Though we have no evidence to this effect, this difficulty could be accounted for on the basis of the known resistance to hydrolysis of peptide bonds of N-methylamino acids and/or the known propensity of N-methylamino acid dipeptides to form diketopiperazines (11). Some typical results appear in Fig. 17. It should be noted additionally

PRODUCTS FROM METHYLATION* OF Cbz-Gly-Gly-Leu-OMe

Hydrolysis time (h)**	Yield (%)						
	MeGly	MeAla	Gly	Total	MeLeu	Leu	Total
48***	0	0	114	114	0	111	111
24	21	4	0.2	25	45	0.4	46
72	22	4	0.2	25	48	0.3	48
Ac-Leu-Gly-OMe	26	13	0.2	39	57	0.6	58

* Reagents 8:3:1; THF/DMF (1:1) 80° 24h
** 6N HCl/110° in an evacuated sealed tube
*** Control hydrolysis of starting material.

Fig. 17

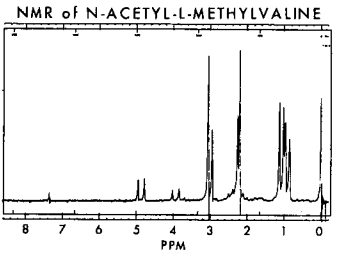


Fig. 18

that one of the products of the methylation of the glycine peptides was N-methylalanine which indicates that the glycine residue not only underwent N-methylation, but also α-C-methylation. We looked for but could find no evidence of carbon alkylation of the leucine residue in these experiments. Both glycine and aspartic acid residues in peptides have been shown to undergo some carbon alkylation during the permethylation of peptides (12). We have investigated the methylation of Cbz-Asn (Fig. 6) under our standard conditions (Fig. 4), and on the basis of n.m.r. analysis, no carbon alkylation of this compound occurred. In another experiment carried out in tetrahydrofuran only as solvent, we found no evidence of the carbon alkylation of Cbz-Gly.

An additional interesting application of the N-protecting action of the trityl group was shown when Trt-Leu-Ala-OMe was

methylated. After hydrolysis, the following were obtained (yield, %): Leu, 90; MeLeu, 0; Ala, 7; MeAla, 79. This in principle therefore provides a synthetic route to a C-terminal methylamino acid dipeptide from the parent amino acid dipeptide.

At many stages of our work, n.m.r. was used as a tool for investigating reactions and characterizing products. A very interesting phenomenon was encountered with the N-acyl-N-methylamino acids. In Fig. 18 is shown the n.m.r. curve for Ac-MeVal in $CDCl_3$. Note, from right to left, the quartet for the side-chain peaks, the doublet for the acetyl protons, the doublet for the N-methyl protons, followed by two well separated doublets for the α-CH proton. N.m.r. curves characterized by double resonances for the α-CH and N-methyl protons were obtained (in $CDCl_3$) for all six N-acetyl-N-methylamino acids (Fig. 9) and (in trifluoroacetic acid) for the two N-benzoyl-N-methylamino acids (Fig. 9). In some but not all cases, two resonances were observed for the N-acetyl and side-chain protons. In no case were doublets observed for the unmethylated derivatives. This phenomenon has previously been reported for Ac-MeAla-OMe (13). These curves provide evidence for the existence of two conformers resulting from restricted rotation about the amide bond.

(NLB is an Associate of the Medical Research Council. Financial support of this work by the Medical Research Council of Canada is gratefully acknowledged).

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PREPARATION AND MASS SPECTRA OF "AZULENE-PEPTIDES" AND THEIR USE FOR THE ANALYSIS OF SYNTHETIC PEPTIDES

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During the past few years mass spectrometry has become a powerful tool to solve analytical problems in the field of peptide chemistry. Methods for the determination of the amino acid sequence of natural peptides and proteins have been worked out in many laboratories (1). These methods can be applied as well for the analysis of synthetic peptides obtained by conventional (1,a) or solid phase techniques (2) especially with respect to the search for unknown impurities such as sideproducts of the synthesis containing failure sequences. The largest peptide derivative which could be evaporated and analyzed in a mass spectrometer so far had a chain length of 22 amino acids (3), but the sequence of only 6 amino acids could be determined in this spectrum. The upper limit for the direct examination of an amino acid sequence in one mass spectrum seems to be 12 amino acid residues at the present time (4).

If a peptide larger than such a chain length is to be analyzed it has to be cleaved prior to the mass spectrometric investigation by means of partial chemical or enzymatic hydrolysis. Subsequent to the cleavage and before the mass spectra can be recorded, the different fragments must be separated by methods such as ion-exchange chromatography, electrophoresis or gel filtration and derivatized using esterification, N-acylation or even permethylation (route A).

In an alternative way (route B) derivatization of the mixture of fragments is carried out immediately after the hydrolysis step and the peptide derivatives are separated subsequently by methods different from those being used in route A. The separation of N-trifluoroacetyl-peptide methyl esters has widely been accomplished by gas chromatography (5). Quite recently N-adamantoyl-peptide methyl esters could be separated by thin-layer chromatography prior to the determination of their mass spectra (6).

In an attempt to investigate the advantages of a novel N-acyl group which can be used in route B, we have synthesized a series of so-called "azulene-peptides" and their respective methyl esters and have studied their properties. The azulene-group was attractive because of the apparent ease of separation of even similar peptide derivatives and their behaviour in the mass spectrometer. The new N-acyl group is a chromophoric moiety and is derived from

1,4-dimethyl-7-isopropyl-azulene (Guaiazulene, I). This hydrocarbon can be converted to [1-methyl-7-isopropyl-azulyl-(4)]-acetic acid (III) via its anion (II) using a method described by Hafner and his coworkers (7). We could successfully couple this carboxylic acid (III) with proline methyl ester by means of dicyclohexylcarbodiimide in methylene chloride to yield the blue coloured N-[1-methyl-7-isopropyl-azulyl-(4)]-acetyl-proline methyl ester (IV) and by subsequent saponification N-[1-methyl-7-isopropyl-azulyl-(4)]-acetyl-proline (V) was obtained (FIG. 1). Both compounds III and V could then be attached to a tetrapeptide methyl ester (VI) using again the DCCD method (dimethylformamide as solvent) with the formation of the corresponding azulene-peptide methyl esters (VII and VIII) as shown in FIG. 1. The abbreviation MIAA- is suggested for the azulene moiety [1-methyl-7-isopropyl-azulyl-(4)]-acetyl- and is used in the formulas.

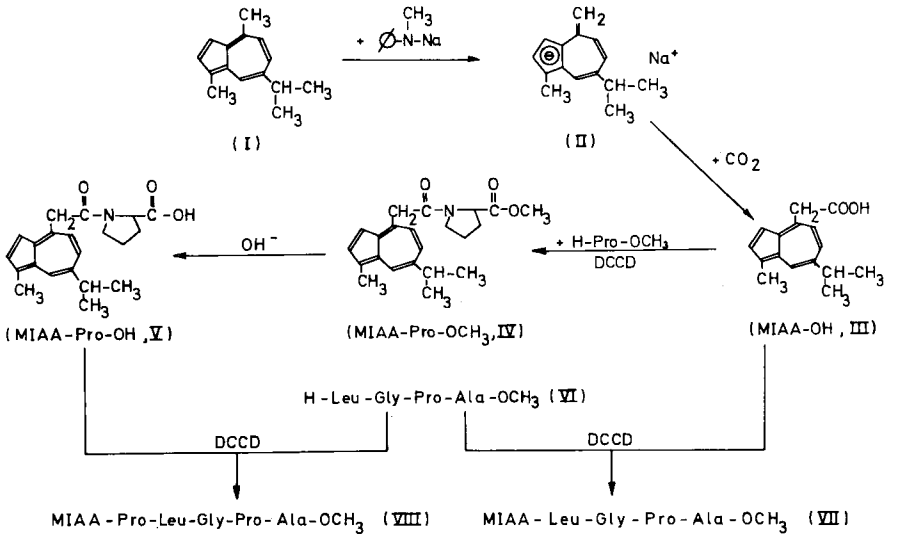

FIG. 1

Synthesis of azulene-peptide methyl esters using the dicyclohexylcarbodiimide method.

The formation of active N-hydroxysuccinimide esters of III and V could also be achieved (IX and X) and these active esters reacted with salts of amino acids and peptides in aqueous dioxane solutions with formation of N-MIAA- amino acids and N-MIAA- peptides as examplified in FIG. 2. Due to the hydrophobic properties of the azulene group the reaction products could be extracted directly from the acidified aqueous solutions (subsequent to the removal of dioxane) by means of organic solvents such as benzene, ethyl acetate or n-butanol. This fact is of importance since it provides the possibility of a first separation of several peptides by means of a fractionated extraction with solvents of different polarity, possibly in combination with a simultaneous change of pH.

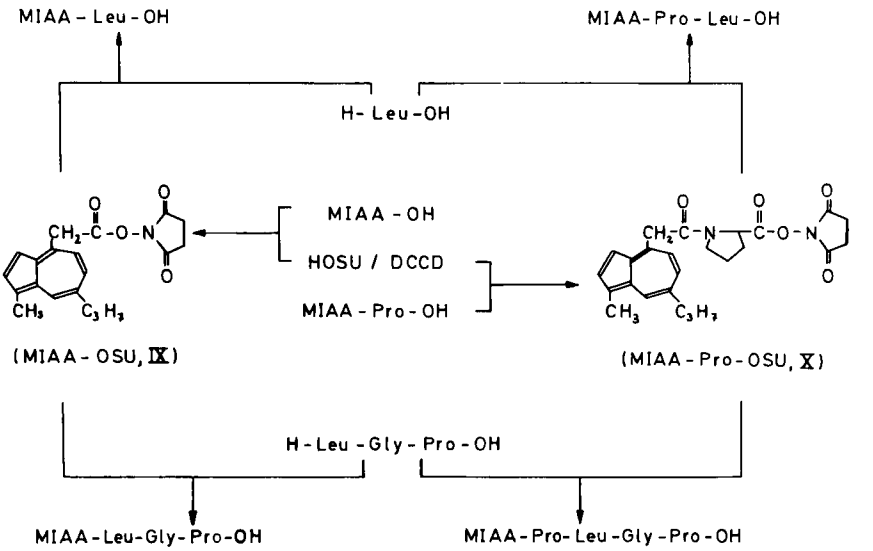

FIG. 2

Synthesis of azulene-peptides using the N-hydroxysuccinimide method.

All azulene-peptides which were prepared by the N-hydroxysuccinimide method could easily be esterified by means of O-alkyl-N,N'-dicyclohexylisourea using a method which was first applied by Vowinkel (8) for the esterification of carboxylic acids. The reaction of dicyclohexylcarbodiimide with methanol in the presence of catalytic amounts of copper-(I)-chloride yields O-methyl-N,N'-dicyclohexylisourea. This product can be isolated and reacts smoothly with N-MIAA-peptides with formation of N-MIAA-peptide methyl esters and dicyclohexylurea (FIG. 3). Excess reagent can simply be removed by reaction with acetic acid. This method of esterification can be recommended in general for the derivatization of peptides. In contrast to an acid-catalyzed esterification, the cleavage of peptide bonds under these conditions is not very likely and furthermore, unlike the diazomethane method, esters other than methyl can be prepared by the use of different alcohols. This is particularly important since esters of chlorinated or fluorinated alcohols can be obtained by this method.

The spectral properties of the azulene-group were not changed by the synthetic procedures described so far. As is shown in FIG. 4 the absorption spectrum of MIAA-OH was almost identical with the spectrum of an azulene-pentapeptide methyl ester and intensively blue coloured peptide derivatives could be isolated.

N-MIAA-peptide methyl esters were rather volative in the high vacuum of a mass spectrometer at temperatures between 80° and 220° C. Excellent mass spectra were obtained up to a chain length of 6 amino acid residues in the case of MIAA-Pro-Leu-Gly-Pro-Ala-Ala-OMe (FIG.6).

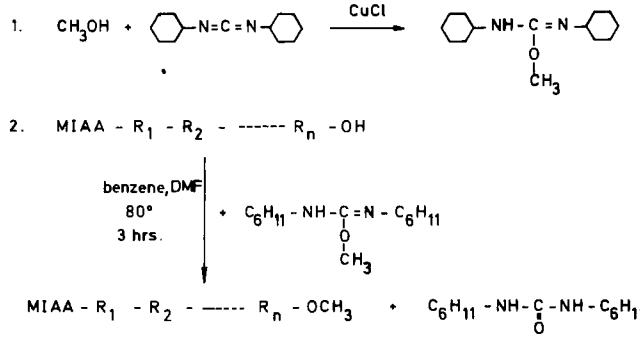

FIG. 3

Esterification of azulene-peptides by means of O-methyl-N,N'-dicyclohexylisourea.

Two typical spectra are shown in FIG. 5 and it can be seen that very intense molecular ions appeared in the order of 60-70 % relative abundance. The predominant peak in all spectra was observed at m/e 224 due to the formation of the ion $[C_{16}H_{16}O]^+$ by fission of the carbon-nitrogen bond indicated as b_o in FIG. 5 and subsequent loss of one hydrogen. This base peak was always accompanied by rather intense peaks of two other fragments of the azulene-moiety at m/e 209 $[C_{15}H_{13}O]^+$ and m/e 198 $[C_{15}H_{18}]^+$. These fragments obviously were formed by loss of one methyl group from the ion of m/e 224 and

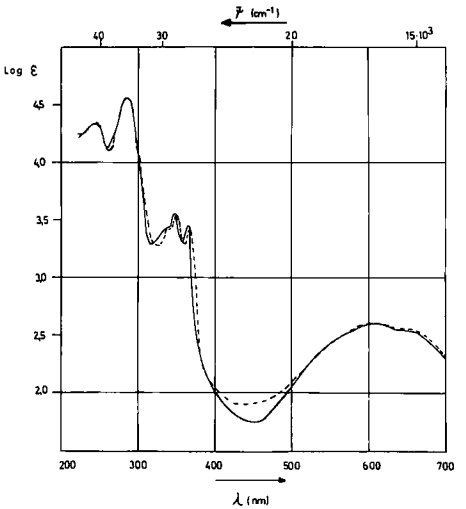

FIG. 4

Absorption spectra of MIAA-OH (———) and MIAA-Pro-Leu-Gly-Pro-Ala-OMe (- - - -) in methanol.

by fission of the carbon-carbon bond indicated as a_0 with subsequent addition of one hydrogen. The triplet of peaks at the mass to charge ratios 224, 209 and 198 was very characteristic for the spectra of all N-MIAA-peptide derivatives and allowed a good interpretation of these spectra especially with respect to the search for sequence ions. The two series of sequence ions a_1, a_2 ... and b_1, b_2 ... are found in all spectra but with rather low intensity compared to the abundance of $[M]^+$.

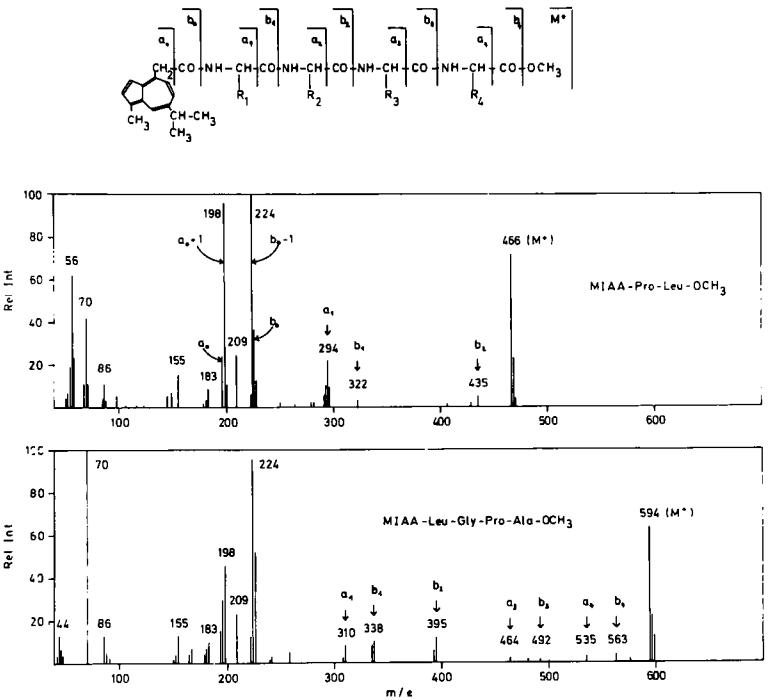

FIG. 5

Mass spectra of MIAA-Pro-Leu-OMe and MIAA-Leu-Gly-Pro-Ala-OMe.

AEI mass spectrometer MS 902; direct inlet system;
temp. ion source 170° C; 70 eV.

The analysis of a mixture of different N-MIAA-peptide methyl esters prior to mass spectrometry is also facilitated by the special properties of the azulene-group.
Firstly the hydrophobic properties of the MIAA-moiety, being in contrast to the more or less hydrophilic properties of the peptide chain, guarantee a good separation by means of thin-layer chromatography in mixtures of organic solvents (with or without addition of water).
Secondly, the absorption properties make it possible to recognize visually the different blue spots on a thin-layer plate. The spots can be removed from the plate and the blue derivatives are eluted

separately with methanol. A quantitative determination of two or more components in such a mixture can then be carried out by a simple photometric measurement of the methanolic solutions at 610 nm (ε_{610} = 485). Amounts in the order of 1-2 % of a contaminating peptide can be detected by this method. Finally, after removal of methanol, the different compounds are inserted into the direct inlet system of the mass spectrometer and their fragmentation patterns can be recorded.

The following group of quite similar azulene-peptide methyl esters could be separated by thin-layer chromatography using the solvent system n-heptane/tert.-butanol/acetic acid (5:1:1, V/V) and were then analyzed in the mass spectrometer separately: MIAA-Pro-Leu-OMe, MIAA-Pro-Leu-Pro-OMe, MIAA-Pro-Leu-Pro-Leu-OMe and MIAA-Pro-Leu-Gly-Pro-OMe.
The mass spectra of a second group of similar peptide derivatives, obtained after the separation of the compounds in a similar solvent system, are shown in FIG. 6.

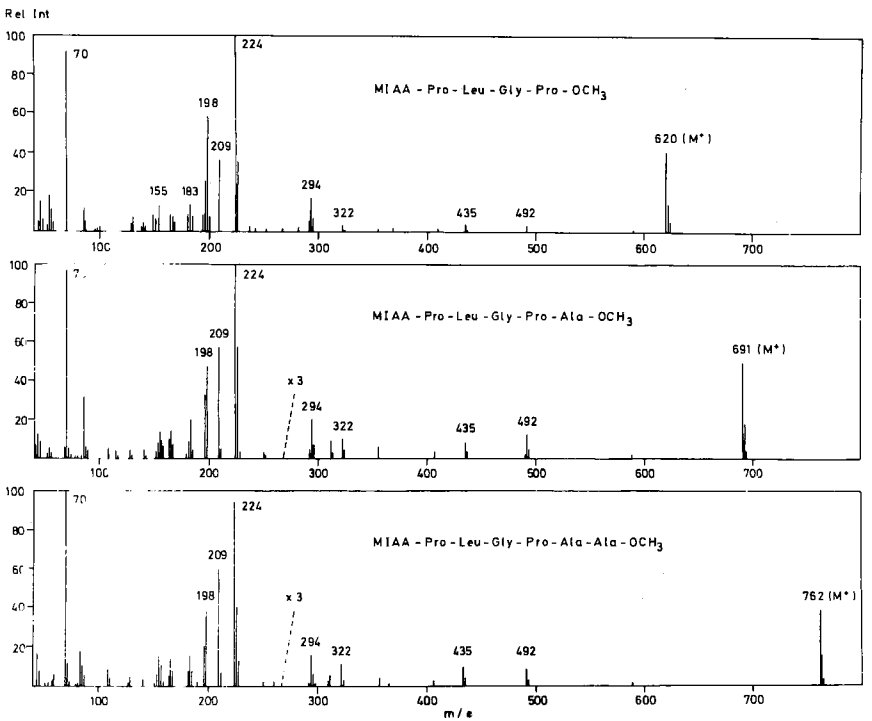

FIG. 6

Mass spectra of MIAA-Pro-Leu-Gly-Pro-OMe, MIAA-Pro-Leu-Gly-Pro-Ala-OMe and MIAA-Pro-Leu-Gly-Pro-Ala-Ala-OMe, recorded subsequent to separation by thin-layer chromatography on silica gel G in the solvent system n-heptane/tert.-butanol/acetic acid (3:1:1, V/V).

The possibility of separating quite similar peptides in the manner described is of importance with respect to one of the final goals of these investigations; namely to prove the presence or absence of failure sequences in synthetic peptides. It is clear, that the opportunity to detect a fragment containing a failure sequence (X_{wrong}) besides the fragment with the correct sequence ($X_{correct}$) in the mixture of cleavage products of a longchain peptide depends on the sensitivity of the separation method. If the separation of X_{wrong} and $X_{correct}$ cannot be achieved prior to the mass spectrometric analysis, one will probably miss the detection of the failure sequence in the mass spectrum of fragment X. This is especially true if the wrong sequence is present in a low percentage, because under these circumstances the spectrum of X_{wrong} disappears in the background of the spectrum of $X_{correct}$.

The judgement, if a spectrum of one t.l.-spot of an azulene compound contains the spectrum of an eventually unseparated impurity, is possible if the esterification step is carried out a second time using a different alcohol. In this case any non-fragment peak must shift to another m/e-value (according to the different molecular weights of the two esters) should it be a true molecular ion. If such a shift is not observed, the peak can be considered a fragment ion and the presence of an unseparated byproduct can be excluded. The use of a chlorinated alcohol also enables such a judgement since only a real molecular ion (but not a fragment ion) can exhibit the typical isotope-ratio ($Cl^{35}:Cl^{37}=3:1$) of a chlorine containing ion.

It was of importance to prove also that N-MIAA-Peptides can be cleaved into shorter fragments by means of proteolytic enzymes. The azulene-moiety did not inhibit an enzymatic hydrolysis with carboxypeptidase B, subtilisin or collagenase; again due to the hydrophobic properties of the MIAA-group the resulting N-terminal fragments of such cleavages could be extracted directly from the aqueous incubation mixtures. In one example a mixture of MIAA-Pro-Leu-Gly-Pro-Arg-OH and MIAA-Pro-Leu-Gly-Pro-Ala-Arg-OH was digested with carboxypeptidase B (20 hrs., 37° C, 0,05 m Tris-buffer and 0,01 m Ca-acetate, pH 8,0; ratio enzym : substrate = 1 : 100). After acidification, a mixture of MIAA-Pro-Leu-Gly-Pro-OH and MIAA-Pro-Leu-Gly-Pro-Ala-OH could be extracted with ethyl acetate and was esterified. The two methyl esters were separated by means of t.l.c. and were then analyzed in the mass spectrometer as previously described.

In summary, a new approach to the mass spectrometric analysis of synthetic peptides has been initiated. Derivatives of a novel [1-methyl-7-isopropyl-azulyl-(4)]-acetyl group (MIAA-) have been synthesized and methods which allow the attachment of this group to amino acids and peptides (and derivatives) in non-aqueous as well as in aqueous solutions have been developed. The special properties of the azulene-moiety facilitate the separation of azulene-peptide derivatives by both extraction and thin-layer chromatography. Subsequent to the separation a precise quantitative and qualitative determination of the azulene-peptide derivatives is possible by photometric measurement and subsequent mass spectrometric analysis.

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THE ELUCIDATION OF THE PRIMARY STRUCTURE OF OLIGOPEPTIDES OF BIOLOGICAL IMPORTANCE VIA MASS SPECTROMETRY

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INTRODUCTION

Mass spectrometry has proven to be a valuable tool to the organic chemist during the past decade. This technique has simplified molecular weight determinations and composition and has facilitated the elucidation of the primary structure of the compound studied. Nucleosides (1), steroids (2), fatty acids (3), peptides (4), alkaloids, sugars, etc., have all been studied. The technique is limited by the requirements that the sample have volatility (or can be appropriately derivatized), and that the molecular weight be lower than 1-2,000 amu (5). In the past few years, samples of biological origin have been successfully analyzed by means of combination gas-chromatography-mass spectrometry instruments (6), and in some cases, with high resolution mass spectrometry.

The study of proteins and peptides is amenable to mass spectrometry (7). Enzymatic cleavage of proteins to oligopeptides of a proper molecular weight and containing up to ca. twenty amino acids is a necessary first step. Although mass spectrometry is not as sensitive as the Edman degradation technique or the microdansylation technique (8), more complete sequence information is obtained from a single mass spectrum (9). Indeed, if the N-terminal amino acid is blocked, then all other standard techniques are not applicable, whereas mass spectrometry could still provide sequence information.

Therefore, the technique to be outlined here is of general interest to the peptide chemist. Peptides isolated from biological sources can be sequenced. Molecular weights of intermediates, or confirmation of sequence of synthetic peptides, can be established.

This paper will review the field of mass spectrometry for the newcomer, cover the various derivatives necessary for mass spectrometry, illustrate the technique with the application to biological problems, and introduce a new technique on the horizon of mass spectrometry-chemical ionization.

REVIEW OF MASS SPECTROMETRY

This section introduces the field of mass spectrometry to the uninitiated and provides background information necessary for the peptide chemist in order to (1) understand the required manipulations of sample and data, (2) interpret the output and (3) see the limitations of the system.

Low Resolution Mass Spectrometry. A mass spectrometer is an instrument which vaporizes a sample, bombards the sample molecules with an electron beam to produce positive ions, accelerates the ions with a large negative potential, and analyzes the ion beam according to mass with a magnetic field (10). The ion current output is collected, amplified, and recorded. The equation relating the mass-to-charge ratio (m/e), magnetic field strength (H), radius of deflection (r) of the ion beam in the accelerating voltage (V) is given in equation (1).

$$m/e = \frac{H^2 r^2}{2V} \qquad (1)$$

A schematic representation of the instrumentation is given in Figure 1.

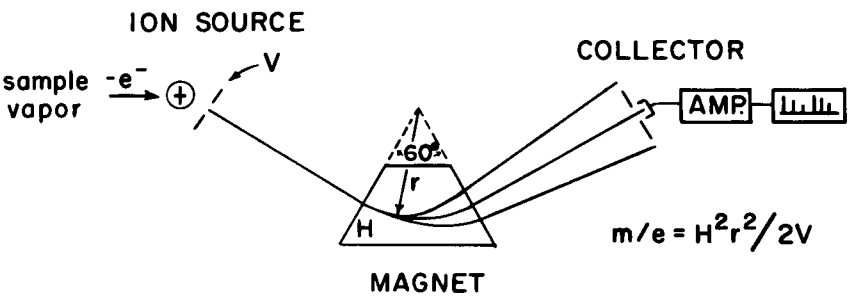


Figure 1. Schematic of a Low Resolution Mass Spectrometer

The output of such an instrument is shown in Figure 2, which is the mass spectrum of glycine. A mass spectrum is a plot of mass vs. abundance. In the case of low resolution mass spectrometry, the masses are nominal masses.

High Resolution Mass Spectrometry. The number of possibilities of elemental compositions for each nominal mass is very high. In order to find a unique composition, or at least restrict the number to a few, the mass must be determined more accurately to within a few millimass units (mMU) (11). A schematic representation of the instrumentation required to do this is given in Figure 3. The output is a list of seven digit accurate masses which is converted to a list of elemental compositions by a digital computer. Such a list of masses found for a peptide will be seen below.

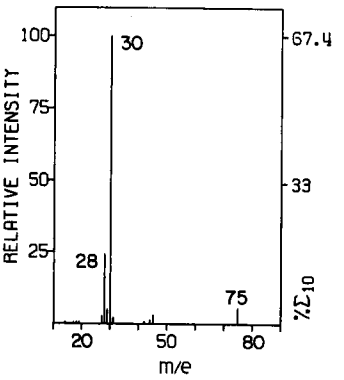


Figure 2. Mass Spectrum of Glycine

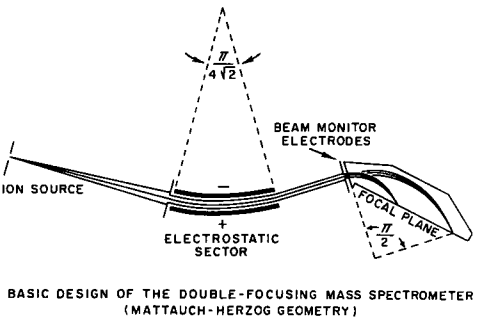


Figure 3. Schematic of a High Resolution Mass Spectrometer (Mattauch-Herzog Geometry)

Fragmentation Processes. The output obtained in the form of a mass spectrum must be interpreted in terms of what is known about the ion chemistry of the particular class of compound being investigated. Very generally, the following processes occur in a hypothetical molecule ABCD following electron impact: (10)

$$ABCD + e^- \longrightarrow ABCD^+ + 2e^- \quad \text{Ionization} \quad (2)$$

$$ABCD^+ \longrightarrow A^+ + BCD\cdot \quad (3)$$

$$\longrightarrow AB^+ + CD\cdot \quad (4)$$

$$AB^+ \longrightarrow B^+ + A\cdot \quad (5)$$

$$AB^+ \longrightarrow A^+ + B\cdot \quad \text{Fragmentation} \quad (6)$$

$$\longrightarrow CD^+ + AB\cdot \quad \text{...etc.} \quad (7)$$

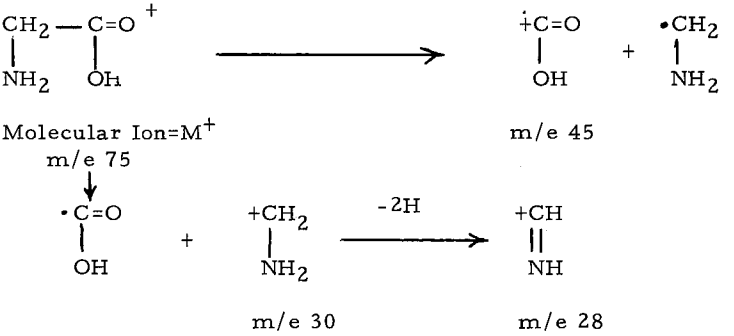
$$\longrightarrow AD^+ + BC\cdot \quad \text{Rearrangement} \quad (8)$$

$$ABCD^+ + ABCD \rightarrow [ABCD\ ABCD]^+ \rightarrow ABCDA^+ + BCD\cdot \quad (9)$$

Ion-Molecule Reaction

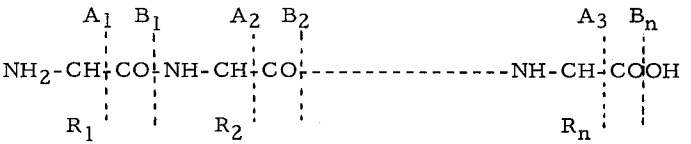
As the ion transit time from the source to the collector is a few microseconds, those processes initiated by electron impact and having a kinetic rate of a few microseconds will be recorded. Processes will occur faster and slower than this time, but will not be recorded.

As an extremely simple case of interpretation of a mass spectrum, the following is an explanation of the genesis of the ions 75, 45, 30, and 28 in the mass spectrum of glycine:



Thus we see a competition between m/e 45 and 30 for the positive charge. From Figure 2, we see that the free electron pair of nitrogen can stabilize a positive charge more effectively than the slightly positively polarized carbonyl carbon. Incidentally, it should be further pointed out that only the ions are recorded in a spectrum, not the radicals.

Peptides undergo a general fragmentation pattern upon electron impact as follows (12, 13, 14):



Thus, the sequence determining ions $A_{1,\ldots,n}$ and $B_{1,\ldots,n}$ are normally obtained in a single mass spectrum, and it is a simple matter for a computer to search out the significant peaks by considering all of the possible R-values and determine the sequence.

DERIVATIZATION TECHNIQUES

The primary requirement that mass spectrometry imposes on any analysis is the volatility. The sample must minimally provide a sample pressure of ca. 10^{-4} torr @ 300°C. In some instances the sample cannot provide the necessary vapor pressure (or decomposes), so that appropriate derivatives must be formed. The following are reasons for derivatizing peptides for mass spectrometry:

1) decrease the zwitterionic character,
2) increase the volatility which is usually lowered by interchain hydrogen bonding,
3) form appropriate derivatives of those amino acids containing functional groups in their side chains, and
4) if possible, stabilize particular fragmentation pathways containing the maximum sequence information.

The N-terminus is usually acylated. This usually provides enough volatility without esterifying the C-terminus. However, if the C-terminus must be esterified, diazomethane is preferred over methanolic HCl due to the possibility of peptide bond cleavage.

O,N permethylation techniques have been successfully applied to peptides (15). In one reaction, the four criteria above could be met in certain types of peptides. Originally, AgO was employed as a catalyst, and peptides were permethylated on the milligram scale (15a). Sodium hydride plus N,N dimethylacetamide was used as a catalyst in the elucidation of feline gastrin at the microgram level (9). Most recently, a methylsulfinylcarbanion catalyst has been employed (15b,c,d).

Cysteine-containing peptides can be treated with Raney nickel (16). Arginine causes a particular difficulty, and can be either handled by enzymatic cleavage or by chemical conversion to a more volatile derivative (17).

Trimethylsilyl derivatives are currently in vogue with other types of compounds and do provide peptides with good gas chromatographic properties (18). However the complexity of the mass spectra of these derivatives usually masks the sequential information (19).

APPLICATION TO BIOLOGICAL SAMPLES

The techniques described above have been applied to two peptides of biological origin - the pentadecapeptide proposed to be a "memory word" called scotophobin and the TSH-releasing hormone, TRF.

Scotophobin was extracted from rats trained to have a fear of the dark. The sample was acylated with trifluoroacetic anhydride (20). A molecular ion was not observed in the mass spectrum of the derivatized sample, but Fig. 4 shows the series of di- and tripeptide fragments which were found by high resolution mass spectrometry. These fragments overlapped sufficiently to permit us to propose the pentadecapeptide sequence. This peptide is being synthesized and will be tested for biological activity and mass spectrometric fragmentation (21).

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
SER	ASP	ASN												
		ASN	ASN											
			ASN	GLU										
				GLU	GLN									
					GLN	GLY								
						GLY	LYS							
						GLY	LYS	SER						
								SER	ALA					
									ALA	GLU				
										GLU	GLN			
											GLN	GLY		
											GLN	GLY	GLY	
												GLY	GLY	TYR-NH_2
SER	ASP	ASN	ASN	GLU	GLN	GLY	LYS	SER	ALA	GLU	GLN	GLY	GLY	TYR-NH_2
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Figure 4. Peptide fragments found from scotophobin

The structure of ovine TRF was elucidated by mass spectrometry (22). As this tripeptide has an N-terminal pyroglutamyl residue, the microdansylation and Edman degradation techniques were not applicable. Mass spectra of the natural TRF and a series of related, synthetic tripeptides (all treated with diazomethane) were obtained. The mass spectrum of derivatized PCA-His-Pro-NH_2 was quite similar to the spectrum of derivatized natural TRF. (see Figure 5).

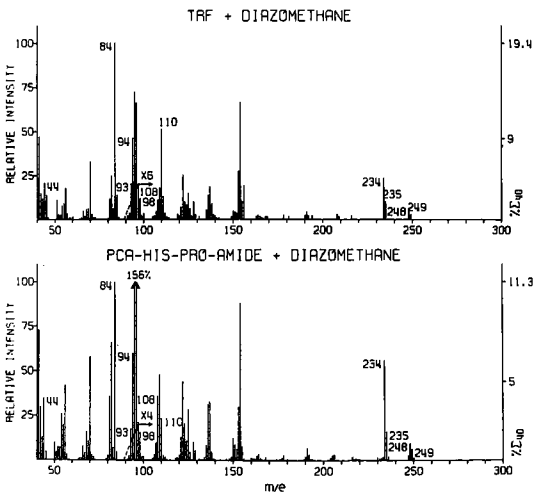
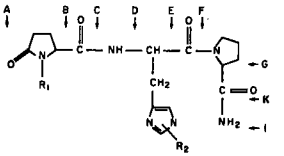


Figure 5. Mass spectra of diazomethane treated natural (top) and synthetic (bottom) TRF

This fact, plus other corroborative evidence permitted the structural assignment of TRF. Figure 6 shows the various fragmentation points of TRF along with the accurate mass, error, and elemental composition of each fragment determined by high resolution mass spectrometry.



Fragmentation points	Measured mass	Error mMU	Elemental Composition
A – B	84.0463	+1.5	C_4H_6NO
A – B, R_1 = CH_3	98.0576	-3.0	C_5H_8NO
D – E	94.0535	+0.4	$C_5H_6N_2$
D – E, R_2 = CH_3	108.0673	-1.4	$C_6H_8N_2$
F – K, minus 4H	93.0211	-0.4	C_5H_3NO
F – I, minus 2H	110.0480	0.0	$C_5H_6N_2O$
G – I	44.0120	-1.6	CH_2NO
A – E, R_1 or R_2 = CH_3, minus H or C – K	234.1112	-0.4	$C_{11}H_{14}N_4O_2$
D – I, minus H			
C – I, minus 2H	248.1147	0.0	$C_{11}H_{14}N_5O_2$
A – E, R_1 = R_2 = CH_3 or D – I, R_2 = CH_3 or C – K, R_2 = CH_3, minus H	249.1327	-2.4	$C_{12}H_{17}N_4O_2$

Figure 6. High resolution mass spectral data of TRF

CHEMICAL IONIZATION

In a conventional ion source, a beam of electrons with 70 electron volts of energy bombards the vaporized sample. This amount of energy is sufficient to initiate those reactions given in equations one through nine. In some cases, the amount of energy available, or the propensity of the particular type of molecule towards fragmentation, is such that few molecular ions are collected. In the case of peptides, various sequence-determining ions may be induced towards further fragmentation and thus, be found in lower abundance.

A different method of producing ions has been applied recently to peptides, alkaloids and other types of compounds. Chemical ionization is a technique whereby ionization occurs by a reaction of the molecule of interest with a set of ions which serve as ionizing reagents (23). The amounts of energy in this form of ionization are lower than usual and the mass spectra are quite simple.

Various hydrocarbon gases have been employed, but the gaseous ion chemistry of methane has been extensively studied so that the ions found in these reactions will be listed below. These ionic reactions are the result of an electron beam of a few hundred volts of energy bombarding methane at about 1.00 torr of pressure:

Primary Ions: CH_5^+, CH_4^+, CH_3^+, CH_2^+, CH^+, C^+, H_2^+, H^+
Secondary Ions: $C_2H_5^+$, $C_2H_4^+$, $C_2H_3^+$
Tertiary Ions: $C_3H_7^+$, $C_3H_5^+$, polymers

The three most intense ions in the plasma generated by this method are CH_5^+ (47%), $C_2H_5^+$ (41%), and $C_3H_5^+$ (6%).

Space will not permit much more discussion of this interesting technique, other than this brief, necessary introduction. Suffice it to say that various peptides have been investigated with this technique and very promising results have been obtained on underivatized peptides. As one example, synthetic and natural TRF chemical ionization mass spectra

were obtained on the underivatized material and abundant ions in the molecular ion region were found for the first time (24). In Fig. 7, m/e 363 is the molecular weight plus one hydrogen transferred from the Brønsted acid, CH_5^+.

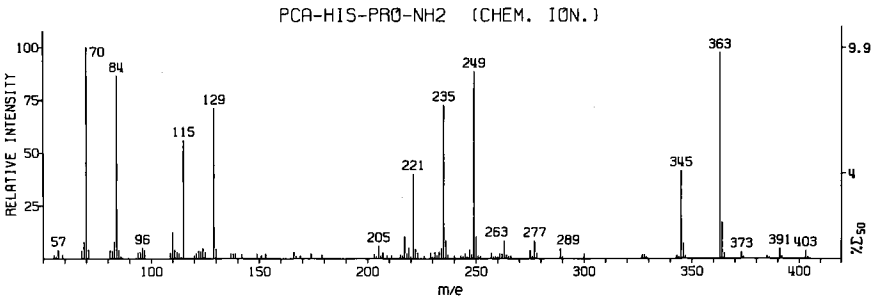


Figure 7. Chemical ionization mass spectrum of underivatized TRF.

The other ions in the molecular ion region, initially confusing but actually of great diagnostic value, are listed here:

403	Mol. Wt. + 41	($C_3H_5^+$, allyl)
391	Mol. Wt. + 29	($C_2H_5^+$, ethyl)
373	Mol. Wt. $-H_2O$+29	

We feel that this type of ionization will offer many advantages over the conventional electron impact source with some types of compounds. As with many other new techniques, however, it will not replace the older methods, but will furnish a powerful supplemental type of information.

CONCLUSIONS

The two biological examples given above clearly illustrate how useful mass spectrometry is in elucidating the structure of peptides.

Work is in progress in improving various aspects of the technique-derivatives for volatilizing peptides, a reduction in the number of preliminary chemical steps, understanding of basic fragmentation pathways, design of the probe, ion production, photoplates for ion detection in high resolution work (25), computers to acquire, reduce, and analyze the data (26).

The advances which are possible in this work, coupled with the increasing number of peptides being extracted from biological sources (and being synthesized), should enable mass spectrometry to play a more prominent and mutually useful role in peptide chemistry.

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A SIMPLE METHOD FOR THE DETERMINATION OF THE N-TERMINAL RESIDUES OF PEPTIDES, DEMONSTRATED ON TRYPTIC FRAGMENTS OF CONCANAVALIN A

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Numerous methods (1) have been devised for the determination of the N-terminal residues in peptides and proteins. Some of these, e.g., dinitrophenylation (2), Edman degradation (3), and dansylation (4), are quite reliable and have found wide application in sequence analysis. Justification for proposing this additional approach lies in its simplicity, and in the fact that its execution does not require a sacrifice of valuable material.

For the separation and purification of peptides from partial hydrolysates of proteins, e.g., from tryptic digests, preparative paper chromatography, thin layer chromatography and paper electrophoresis are frequently used. The bands containing the separated peptides are usually located by spraying a part of the paper (or thin layer) with ninhydrin. The peptides are then recovered from the untreated portion of each band. The ninhydrin stained part is sacrificed in this procedure. It occurred to us that one can put such stained areas to good use.

Ninhydrin oxidatively deaminates those amino acids in the peptides which have a free amino group. Apart from lysine only the N-terminal residue is subject to deamination. Therefore, hydrolysis of the peptide eluted from the ninhydrin stained area should give a mixture of amino acids identical to that from the unstained part of the band, except that the amount of the N-terminal amino acid is greatly reduced. A comparison of the results of quantitative amino acid analyses of the peptides from these two areas could be used for the identification of the N-terminal acid. The decomposition of lysine should be kept in mind but it need not represent a serious limitation.

This method was tested on tryptic fragments of concanavalin A (5). Details of the degradation of this protein will be discussed elsewhere. Peptides A and B (Table I) were obtained from the C-terminal tryptic fragment, a nonapeptide for which the following sequence was determined by conventional methods: Leu-Leu-Gly-Leu-Phe-Pro-Asp-Ala-Asn. Peptide A (Leu-Leu-Gly-Leu-Phe-Pro) resulted from mild acid hydrolysis (6) of this fragment, while peptide B (Gly-Leu-Phe-Pro-Asp-Ala-Asn) was obtained

by digestion of the nonapeptide with leucine aminopeptidase for a limited time.

Table I

The C-terminal Tryptic Peptide of Concanavalin A

Leu-Leu-Gly-Leu-Phe-Pro-Asp-Ala-Asn

	Peptide A (0.03N HCl) Leu-Leu-Gly-Leu-Phe-Pro				Peptide B (LAP) Gly-Leu-Phe-Pro-Asp-Ala-Asn					
	Leu	Gly	Phe	Pro	Gly	Leu	Phe	Pro	Asp	Ala
Unstained	2.95	1.00	0.96	0.63	1.15	1.01	1.00	1.03	2.01	1.09
Stained	1.93	1.00	1.06	0.65	0.14	0.92	1.00	1.01	1.88	1.01

The sequences of two additional tryptic peptides are shown in Table II. The entire sequence of peptide C (Asx-Glx-Lys) could be deduced by

Table II

Tryptic Peptides from Concanavalin A

	Peptide C Asx-Glx-Lys			Peptide D Trp-Asn-Met-Gln-Asn-Gly-Lys				
	Asp	Glu	Lys	Asp	Met	Glu	Gly	Lys
Unstained	0.99	1.00	1.00	2.14	0.93	0.94	1.00	1.11
Stained	0.25	1.00	0.20	2.07	0.27	0.99	1.00	0.25

applying the here-proposed method: because it is a tryptic fragment, lysine had to be assumed to be its C-terminal residue, while the decrease in the amount of aspartic acid on staining revealed Asx as the N-terminus. In the case of the tryptic peptide D (Trp-Asn-Met-Gln-Asn-Gly-Lys), also elucidated through conventional methods of sequence analysis, the N-terminal tryptophane was not determined by quantitative amino acid analysis, but the assignment of this residue as N-terminal was confirmed by the fact that only lysine and methionine were found in reduced amounts in the stained portion. From independent evidence (CNBr cleavage) (7) it was known that methionine is not terminal. Its partial destruction by ninhydrin is being studied.

A number of synthetic peptides have also been used to test this method. A synthetic hexapeptide amide corresponding to the C-terminal sequence of secretin (8) showed (Table III) a large reduction in the quantity of leucine recovered after ninhydrin treatment. Leucine was the N-terminal residue. In a synthetic peptide corresponding to a sequence from cholecystokinin (9), with lysine at its N-terminus, a significant reduction occurred (Table IV) in this constituent only. It is noteworthy that the seryl residue of the peptide was not altered in any discernible manner. A series of synthetic tetrapeptides related to the C-terminal sequence of oxytocin, but each with a different N-terminal residue,

Table III

The C-terminal Hexapeptide of Secretin

Leu-Leu-Gln-Gly-Leu-Val-NH_2

	Leu	Glu	Gly	Val
Unstained	2.54	0.96	1.02	1.00
Stained	1.88	1.09	0.79	1.00

Table IV

A Synthetic Hexapeptide

With Lysine at its N-terminus

Lys	Ser	Pro	Gly	Ala	Arg
0.24	0.96	1.00	1.09	0.91	1.02

suffered distinct losses (Table V) in the corresponding amino acid after ninhydrin treatment.

Table V

Synthetic Tetrapeptides with Various N-terminal Residues After Ninhydrin Treatment

"A"-Pro-Leu-Gly-NH_2

	"A"	Pro	Leu	Gly
"A"=Asn	0.11	0.98	0.96	1.00
"A"=Leu	--	0.72	1.02	1.00
"A"=Phe	0	1.14	1.02	1.00
"A"=Pro	--	1.01	1.01	1.00

Nearly all of the examples given above are the results of experiments performed on peptides purified on silica gel thin layer plates. The application of this method to peptides isolated by paper chromatography has at times given unsatisfactory results, apparently on occasion leading to complete destruction of the peptide chain. The reasons for this have not yet been established.

The possibility of an extension of this method by subsequent cleavage of the oxidatively deaminated residue, e.g., with alkaline hydrogen peroxide (10) has not been overlooked. Yet such a procedure may not be warranted in view of the already available well established methods of sequential degradation (11). On the other hand, the proposed determination of N-terminal residues can be applied routinely for the characterization of peptides.

We wish to thank Drs. M. Léon and M. Young for a purified sample of concanavalin A and for their suggestion to study its chemistry, also Dr. N. C. Chaturvedi for a sample of the hexapeptide corresponding to the N-terminal sequence of cholecystokinin, and to Dr. John T. Sheehan of the Squibb Institute for Medical Research, New Brunswick, New Jersey, for the tetrapeptides related to oxytocin. Mr. Gregory C. Coleman's participation in these studies is sincerely appreciated. This work was supported in part by a grant from the U. S. Public Health Service (NIH 1R01 AM 12473). One of us (D. F. D.) is a NASA research fellow.

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DETERMINATION OF THE STEREOCHEMICAL PURITY OF AMINO ACID RESIDUES IN SYNTHETIC PEPTIDES

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Since the ultimate stereochemical purity of a synthetic peptide can be only as high as that of the amino acid derivatives used in the synthesis, it is essential to have a sensitive method for determination of the stereochemical purity of the starting materials. For example, if each L-amino acid used for the synthesis of an L-polypeptide containing 100 residues has 1% of the D-enantiomer, then the highest possible yield of pure product is 36%. Also for the peptide chemist there is need for a method which can establish the stereochemical purity of large completed peptides with a sensitivity of the order of 1%. In this study we will describe a method which can establish the stereochemical purity of the starting amino acids to 99.99% and which can be used to detect about 1% of a D-amino acid residue in a completed peptide.

The method represents a combination of the technique of ion exchange chromatography (1) together with a simple procedure for converting the D- and L-isomers of amino acids into diastereomers that can be separated (2) on an ordinary amino acid analyzer (Fig. 1).

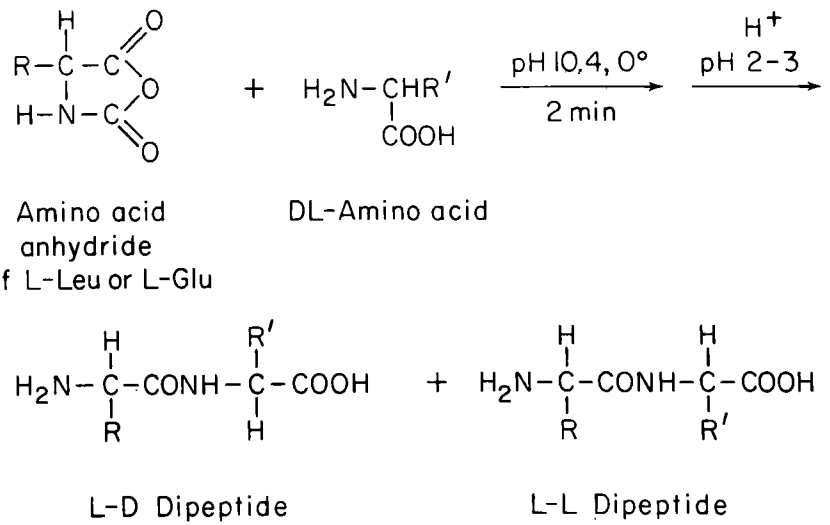

Fig. 1 - Reaction of an L-amino acid N-carboxyanhydride with a DL-amino acid.

Preparation of Diastereomeric Dipeptides. The procedure of choice has been the method of Hirschmann and his associates (3). In this procedure, blocking groups are not required for the amino acids. So it is quite simple to prepare the L-D and L-L dipeptides from a mixture containing the D- and L-isomers of a given amino acid. The reaction with the N-carboxy-anhydride (NCA) takes place in two minutes at pH 10.4, and gives a yield of about 90%; Hirschmann and his colleagues established that no racemization takes place during the synthesis (4). For analytical purposes, we have scaled-down their procedure about 1000-fold.

Separation of Diastereomeric Dipeptides. The reaction mixture can be applied directly to an amino acid analyzer; the resulting dipeptides are readily separable by ion exchange chromatography. For example, shown in Fig. 2 is the separation of L-Leu-D-Asp and L-Leu-L-Asp on the 62 cm column of an amino acid analyzer; the eluent is pH 3.25 sodium citrate.

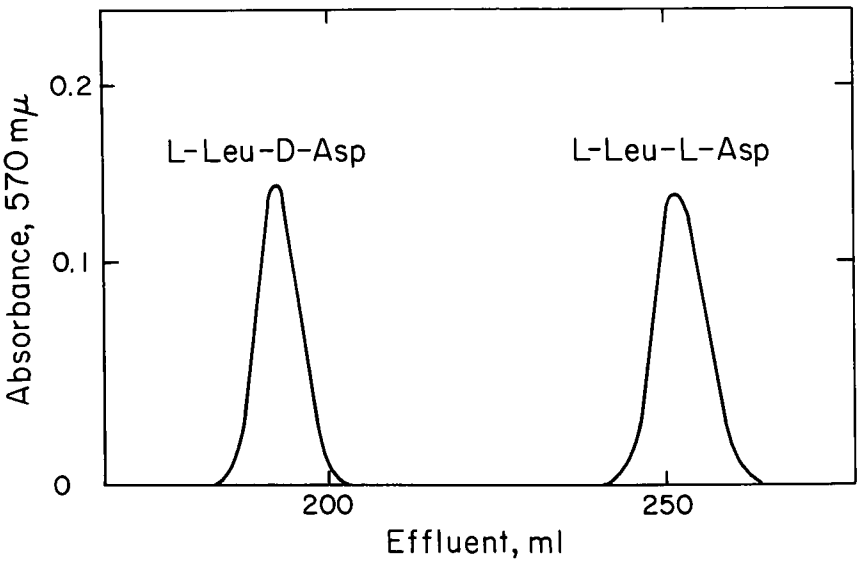

Fig. 2 - Separation of L-Leu-D-Asp and L-Leu-L-Asp on the amino acid analyzer at pH 3.25, 52°. A 0.9 x 62 cm column packed with Spinco AA-15 resin was used.

Using L-leucine NCA, the dipeptides have been prepared for the acidic and neutral D- and L-amino acids and conditions have been found for the separation of each pair. For the most part, the buffers for routine amino acid analysis are used (5).

The L-leucyl dipeptides of the aromatic and basic amino acids, however, are rather strongly retarded on the column. Therefore, the NCA of L-glutamic acid, prepared as described by Hirschmann et al. (6), has been used to obtain the dipeptide pairs of those amino acids; the extra carboxyl group

increases the rate of elution of the dipeptides from the sulfonated resin. The dipeptide pairs corresponding to all 19 of the amino acids that are commonly found in peptides have been successfully separated.

Sensitivity of the Method. Because each pair of diastereomeric dipeptides is so well resolved, the column can be heavily loaded to measure very small amounts of one diastereomer; the sensitivity of the method is illustrated by the data in Table I.

TABLE I

Analysis of Known Mixtures of D- and L-Alanine

	D-Alanine, %	
Expt	Added	Found
1	0.003	0.003
2[a]	0.009	0.008

[a] One-half the theoretical amount of L-leucine NCA was added so that the reaction would proceed to only 50% completion.

In experiment 1 a known amount of D-alanine was added to pure L-alanine to make a solution that contained .003% of the D-isomer. The diastereomeric dipeptides were prepared and then separated on the amino acid analyzer. The added D-alanine was quantitatively recovered. It was possible to detect 3 parts of D-isomer in 100,000 parts of L. From this result we can also say that the L-leucine NCA which was used for the coupling was stereochemically pure to this degree. In experiment 2, one-half of the theoretical amount of NCA was used; the added D-alanine was also recovered. This result shows that there is no detectable stereoselectivity during the coupling of an L-amino acid NCA with a D- or L-amino acid.

In general, as little as .01% of a stereochemical impurity can be detected in the amino acids used as starting materials for peptide synthesis.

Analysis of Peptides and Proteins. In peptide synthesis, it is most important to know if the product, the peptide synthesized, has any racemic residues as a consequence of the synthesis, and if so, which ones they are. Most chemical methods which peptide chemists use to detect racemization are useful to evaluate methods of coupling of fragments in model peptides, but they cannot be readily adapted to the examination of each residue of a large synthetic polypeptide. Enzymatic methods generally do not have the required accuracy or sensitivity. The method that we have described above can also be used for the evaluation of the stereochemical purity of large synthetic peptides. However, whereas the analysis is relatively simple for a single amino acid, there may be a problem with overlapping dipeptides derived from the amino acids in an acid hydrolysate of a peptide. One must either devise an elution system that will avoid overlaps or isolate the

given amino acid by a preliminary chromatogram and apply the derivatization to that sample. In general, in work with peptides we have operated on a scale that would give us a precision of 0.5% of D-isomer.

For the study of the stereochemical purity of a synthetic peptide, bovine insulin A chain synthesized by the solid phase method was chosen (7). The synthetic peptide was hydrolyzed in 6 N HCl at 110° for 22 hours. This acid hydrolysis step always produces some racemization (2,8) and the question arises as to whether the small amount of D-amino acid in the hydrolysate was present in the synthetic peptide or whether it was formed by racemization during the hydrolysis. To illustrate the problem, Table II shows our results for alanine and leucine of the insulin A chain.

TABLE II

Racemization of Amino Acid Residues during Acid Hydrolysis of Natural and Synthetic Insulin A Chain

Source	% D-Isomer in Acid Hydrolysate	
	Alanine	Leucine
Free L-Amino acid	1.4	1.3
Natural A Chain	1.5	4.0
Synthetic blocked A Chain	1.6	5.5
Synthetic deblocked A Chain (deblocked with Na/NH_3)	6.0	5.5

For correction of the racemization which occurs during acid hydrolysis of a peptide, heating of the free L-amino acid under the same conditions is sometimes indicative, as we found with alanine in insulin. Within the precision of these particular analyses, the per cent of D-alanine in a hydrolysate of natural insulin was the same as that in the amino acid control. However, with leucine this was not the case. The leucine residues of natural insulin A chain were racemized more than free L-leucine during acid hydrolysis. This result suggests that the extent of racemization during acid hydrolysis is probably influenced in some manner by the neighboring amino acid residues in the peptide and hence can vary from one sequence to another (2). For this reason, the natural peptide is a useful control.

About the same amounts of D-leucine and D-alanine were found in acid hydrolysates of the natural A chain and the synthetic blocked A chain. We therefore conclude that these residues in the synthetic peptide are stereochemically pure within these limits.

When the other amino acid residues of this blocked synthetic peptide were examined they were also found to be stereochemically pure. However, after deblocking the peptide with sodium/liquid ammonia we found that the amount of D-leucine did not change but that some D-alanine (a net of 4%) was formed.

In analogous experiments with synthetic oxytocin, which does not initially contain alanine, we have found that 0.07 mole of alanine per mole of peptide is formed during gentle deblocking with sodium/liquid ammonia and that 50-60% of the alanine is the D-isomer. In all of these instances, we attribute the presence of D-alanine in the deblocked product to reductive cleavage of benzyl mercaptide from the blocked cysteine residues to give intermediates which produce largely racemic alanine upon reduction. The cysteine derivatives which we have used in the synthesis were prepared in our laboratory by a method which did not involve sodium/liquid ammonia, and they contained no detectable alanine prior to use.

Since the natural insulin A chain was available, we could readily determine the increased amount of racemization of amino acid residues during acid hydrolysis. But what do we use as a control with a synthetic peptide when we do not have the natural peptide? In addition there is the fundamental problem of the determination of the configuration of the amino acid residues in naturally-occurring peptides, particularly in antibiotics that may possess unusual linkages. What control can we use to examine the possibility of increased racemization of amino acid residues during the hydrolysis in these instances?

Hydrolysis with Tritiated HCl. Bruce Merrifield suggested that perhaps the incorporation of tritium into the amino acid during hydrolysis of the peptide in tritiated HCl would be a practical way for measurement of the amount of racemization that takes place during acid hydrolysis. The amino acid would incorporate some tritium on the α-carbon atom during protonation in the process of racemization. Denkewalter et al. (4) utilized a similar approach to check the degree of racemization during peptide synthesis. The idea of using tritium in conjunction with the amino acid analyzer was appealing since scintillation flow cells are now available that can be attached to the columns of the amino acid analyzer.

The first tests were on L-alanine shown in Fig. 3 (9).

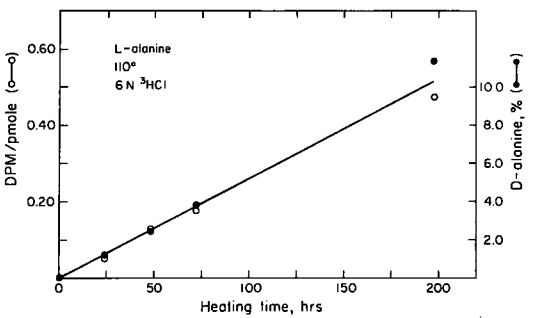

Fig. 3 - Correlation between the amount of tritium incorporated and the amount of D-alanine formed during heating of L-alanine in 6 N ^{3}HCl at 110°.

The specific radioactivity of the amino acid and the amount of D-alanine measured chromatographically were determined for several heating times. A linear relationship between these two measurements was found. There is 3 to 4 times less tritium incorporated into the alanine than one would predict from the tritium content of the initial tritiated HCl. We attribute this to an isotope effect. However, we need not correct for this isotope effect if we simply use the empirical relationship between observed incorporation and per cent D-isomer as shown in this Figure. We have used this graph as a standard in all of our experiments to find the per cent of D-isomer formed during hydrolysis of a peptide in tritiated HCl; we standardize each new batch of tritiated water in terms of disintegrations per minute per ml. In another laboratory, with different equipment and hydrolysis times, the slope of the line may be slightly different from the one shown here.

The next question was to find out for which amino acids this method was applicable. To answer this question we heated several free L-amino acids in the same amount of tritiated HCl that we had used for the experiment with L-alanine. The specific radioactivity of the amino acid was determined and the amount of D-isomer was calculated with the graph for alanine as a standard; this value is shown under the heading "^{3}H-incorporation" in Table III.

TABLE III

RACEMIZATION OF FREE L-AMINO ACIDS UNDER THE CONDITIONS OF ACID HYDROLYSIS. COMPARISON OF THE TWO METHODS OF ANALYSIS.[a]

L-Amino acid	% D-isomer as determined by	
	^{3}H-incorporation[b]	L-D dipeptide[c]
Alanine	1.4	1.0
Valine	0.2	0.7
Isoleucine	0.4	1.0[d]
Leucine	0.9	1.3
Serine	0.6	0.4
Threonine	0.9	0.5
Lysine	1.8	3.0
Arginine	1.4	1.6
Methionine	2.7	2.2
Proline	2.3	2.2

[a] Hydrolysis was carried out for 22 hr at 110°. [b] The specific radioactivity of the amino acid was converted into the amount of D-isomer using Fig. 3 as a standard graph; average of 2 or 3 determinations, max. dev. from mean: 0.3%. [c] Determined by the method of Manning and Moore (2); max. dev. from mean: 0.5%. [d] Epimerization of L-isoleucine at the α-carbon atom affords D-alloisoleucine which can be separated on the amino acid analyzer.

The amount of D-isomer was also determined by chromatographic analysis of the diastereomeric dipeptides and these results are shown in the column labeled "L-D dipeptide". The amino acids included in this Table are those for which the amount of D-isomer, as calculated by the two methods, agreed to within about 1% which is the accuracy of the methods combined. Thus, with this degree of precision, tritiated HCl may be used for measurement of the racemization of these amino acid residues during the hydrolysis of a peptide (9).

This technique is not applicable to either glutamic acid or aspartic acid where the methylene hydrogens adjacent to the ω-carboxyl groups are exchangeable. For these two amino acids we established the location of the exchangeable hydrogen atoms by nuclear magnetic resonance spectroscopy of samples that had been heated for 22 hr. in 6 N deuterium chloride (9). With phenylalanine the β-hydrogen atoms are slowly exchangeable during acid hydrolysis,and with tyrosine (10) the hydrogens ortho to the ring hydroxyl are completely exchangeable. The C-2 hydrogen atoms on the imidazole ring of histidine are partially exchanged in strong acid (11), and cystine is largely racemized during this treatment (12). Thus the tritium method is applicable to 10 of the 16 optically-active amino acids that are usually found in an acid hydrolysate of a peptide.

Application to Synthetic and Natural Peptides. We have applied these methods to L-bradykinin synthesized by the solid phase method (16). The structure of this peptide is shown in Fig. 4.

Bradykinin

Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg

(all amino acids of the L-configuration)

$Arg_2, Pro_3, Gly, Phe_2, Ser$

Fig. 4 - Sequence and amino acid composition of L-bradykinin.

Merrifield and Stewart were interested in being sure of the optical purity of the synthetic peptide but it was resistant to digestion by the

aminopeptidases because of the Arg-Pro-Pro sequence. The results of our analysis are shown in Table IV.

TABLE IV

CORRECTION FOR RACEMIZATION OCCURRING DURING ACID HYDROLYSIS OF SYNTHETIC L-BRADYKININ[a]

L-Amino acid Residue	% D-isomer as determined by ^{3}H-incorporation	% D-isomer as determined by L-D dipeptide
Serine	0.1	0.3
Proline	2.8	2.3
Arginine	1.7	1.6

[a] Hydrolysis was carried out for 22 hr at 110°.

The column labeled L-D dipeptide represents the total amount of each D-isomer in the hydrolysate - that which was formed during the hydrolysis as well as that which may have been present in the original synthetic peptide. The values under the heading "^{3}H-incorporation" are the amounts of D-isomer formed by racemization during the acid hydrolysis. The difference between these two values is a measure of the amount of D-isomer in the original peptide. Since there was no difference, within experimental error, we conclude that these three residues in the synthetic peptide were stereochemically pure. The values for phenylalanine are not included in this table since the amount of racemization during acid hydrolysis cannot be determined for this amino acid with the tritium method because there is labeling in the side chain of the molecule. Phenylalanine had to be studied indirectly by using, as controls, phenylalanyl dipeptides corresponding to sequences in bradykinin (2). In this way we could show that the phenylalanyl residues in the synthetic peptide were stereochemically pure.

The approach that we used for phenylalanine is one that can be used for an amino acid residue for which the tritium method is not applicable.

As a test of our new method on a natural peptide, we chose bacitracin A. The structure of this peptide (Fig. 5) was worked out by Lyman Craig and his associates (13-15) and we are indebted to him for providing us with the samples for our analysis. As shown by Craig the first two residues of this peptide cyclize to form a thiazoline ring shown at the bottom of the Figure. The α-hydrogen atom of the amino-terminal isoleucyl residue is thereby labelized so that when the peptide is heated in acid, this isoleucyl residue is completely epimerized and about one-half of an equivalent of D-alloisoleucine is formed (13). This structure seemed to provide a good test of our tritium method since we should be able to measure the amount of racemization accurately and correlate it with the D-alloisoleucine which was formed. The results of our analysis are shown in Table V.

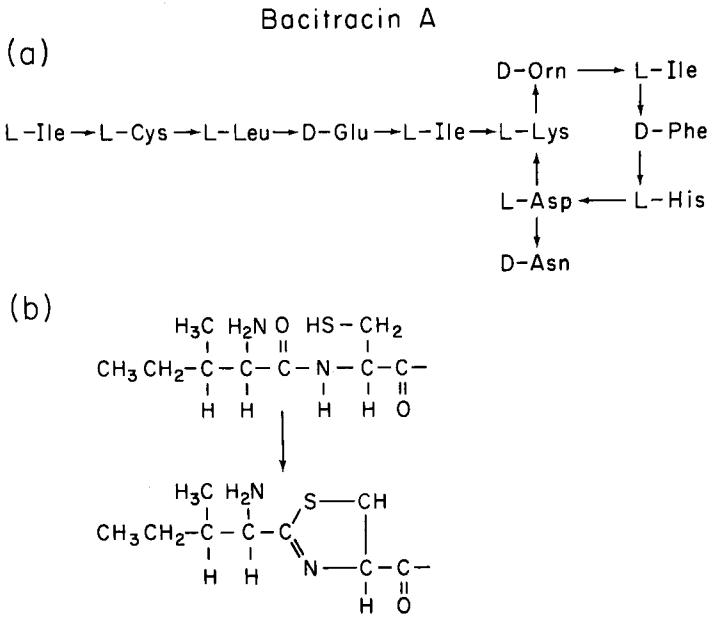

Fig. 5 - a) Sequence of bacitracin A.
b) Structure of the thiazoline formed between Ile-1 and Cys-2.

TABLE V

CORRECTION FOR RACEMIZATION OCCURRING DURING ACID HYDROLYSIS OF NATURAL BACITRACIN A[a]

L-Amino acid Residue	% D-isomer as determined by ^{3}H-incorporation	% D-isomer as determined by Chromatography
Isoleucine	17.4	15.7[b]
Leucine	5.8	6.8
Lysine	2.2	3.0

[a] Hydrolysis was carried out for 22 hr at 110°.
[b] Epimerization of L-isoleucine affords D-alloisoleucine which can be separated on the amino acid analyzer.

Since the peptide contains three L-isoleucine residues, the complete epimerization of one of them should give rise to 16.7% of the D-allo isomer. This is close to the value found chromatographically. The result from the amount of tritium incorporation shows about the same amount of D-isomer which indicates that the epimerization actually took place during the acid hydrolysis.

The amount of D-leucine found in the hydrolysate of bacitracin A is 5-6 times higher than that expected from the racemization of free L-leucine (9). The value from the tritium incorporation is about the same. These results, together with Craig's original observation on the low rotation of the leucine isolated from the hydrolysate (14), suggest that there may be some interaction of Leu-3 with some other residue in bacitracin A, possibly Cys-2.

The data indicate that the lysine residue of bacitracin A is not racemized any more than the corresponding free amino acid during acid hydrolysis.

In summary, a method for the determination of the optical isomers of amino acids has been described. The dipeptides are prepared in a matter of minutes with excellent yields. They can then be separated on the amino acid analyzer with standard resins and, in many cases, with the buffers already in use on the analyzer (2). The method can detect 0.01% of a stereochemical impurity in a sample of a given amino acid used as a starting material.

For analysis of the stereochemical purity of the residues in a synthetic peptide, hydrolysis with tritiated HCl can be used for determination of the amount of racemization during the hydrolysis for 10 of the 16 amino acids usually found in the hydrolysate of a peptide or a protein (9).

The two methods can be used to establish to better than 1% the configurations of most amino acid residues in either synthetic or natural peptides.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the National Institutes of Health. We wish to thank Miss Wanda Jones for her expert assistance.

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ON THE PROBLEM OF RACEMIZATION DURING THE SYNTHESIS OF SEQUENTIAL POLYPEPTIDES

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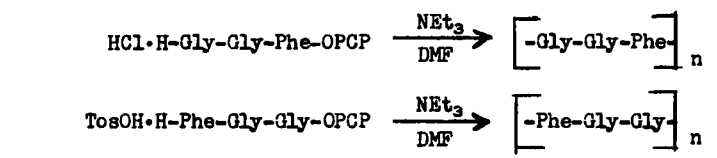
Sequential polypeptides are better protein models than polyamino acids or random copolymers. The optical purity of these polymers is essential when they are used to study secondary structures or biological properties. Racemization is a serious problem during the synthesis of these polymers since the inverted amino acid residue is permanently incorporated into the polypeptide chain. To illustrate this point, the probability of obtaining optically pure polypeptide containing 99 amino acids from a tripeptide active ester is about 18%, if 5% inversion occurs at its C-terminal amino acid residue. This probability is calculated from $(1 - p)^n$ given by Greenstein and Winitz (1), where p is the fraction of inverted amino acid residues, and n is the number of repeating tripeptide units.

Sequential polypeptides are usually synthesized from C-terminal activated oligopeptide intermediates as indicated by Scheme I, which shows the polycondensation of H-Gly-Gly-Phe-OPCP to poly-Gly-Gly-Phe and of H-Phe-Gly-Gly-OPCP to poly-Phe-Gly-Gly.

Scheme I



These high molecular weight polypeptides are identical except for N and C termini as indicated in Scheme II and therefore, the physical properties

Scheme II

G . G . P . G . G . P . G . G . P . G . G . P . G . G . P
P . G . G . P . G . G . P . G . G . P . G . G . P . G . G

G = Gly and P = Phe

including optical rotatory properties were considered to be practically identical, since the effect of the terminal groups was expected to be

negligible. Table I shows the physical data of these polypeptides prepared through different active esters.

Table I

Physical Data of Poly-Gly-Gly-Phe and Poly-Phe-Gly-Gly[a]

Prepared from tripeptide	$\bar{M}_w$	$[\alpha]_D^{23}$	$[R]_D^{23}$	$[\alpha]_{223}^{23}$
1. H-Gly-Gly-Phe-OPCP	20,000-25,000	31.5°	82.2°	3058°
2. H-Gly-Gly-Phe-OR[b]	14,000	30.8	80.5	3097
3. H-Gly-Gly-D-Phe-ONP	30,000	-31.3	-81.8	-3192
4. H-Phe-Gly-Gly-OPCP	12,500	32.9	85.8	3400
5. H-D-Phe-Gly-Gly-OPCP	24,000	-33.3	-86.8	-3461
6. H-Gly-Gly-D-Phe-ONP[c]	-	-19.8	-51.7	-3052
7. H-Gly-Gly-D-Phe-ONP[d]	11,000	-31.3	-81.8	-3131

a) The polymerizations were carried out in DMF in the presence of 2.5 equivalent of NEt_3 except for polymers No. 6 and 7. The concentrations in dichloroacetic acid for measurements of $[\alpha]_D^{23}$ for compounds 1 to 7 were: 1, 0.2; 2, 0.2; 3, 1.0; 4, 0.8; 5, 1.0; 6, 1.0; 7, 1.0%. Concentrations for measurements of $[\alpha]_{223}^{23}$ were: 1, 0.1; 2, 0.1; 3, 0.02; 4, 0.02; 5, 0.1; 6, 0.02; and 7, 0.02% in trifluoroethanol containing 0.5% lithium chloride. A mean residue weight of 261 was used to calculate $[R]_D^{23}$.

b) This compound is an ester of N-ethyl-3,5-dichlorosalicylicacid amide.

c) The polymerization was carried out according to DeTar (2) in dimethyl sulfoxide with the sodium salt of p-nitrophenol. For Poly-Gly-Gly-L-Phe a "molar rotation" of 67° (c 2 in dichloroacetic acid, corrected for 95% purity) was reported and this polymer was considered to be 96% optically pure, based on the rotation of the Phe, obtained by total hydrolysis.

d) Prepared in dimethylsulfoxide as the previous one, and the polymer was washed in dimethylformamide.

The optical purity of sequential polypeptides can be determined either by the total hydrolysis approach or by enzymatic methods. When the residue rotations in Table I reported for poly-Gly-Gly-Phe are compared with the residue rotation of 67° (see Table I footnote c), it indicates that the estimation of optical purity of a sequential polypeptide using the total hydrolysis approach should be taken cautiously. The enzymatic approach on the other hand is not applicable due to insolubility of such polymers in water.

Here we report an approach to estimate optical purity of sequential polypeptides. We previously stated that poly-Gly-Gly-Phe and poly-Phe-Gly-Gly should give practically identical specific rotations. However, Table I shows that the specific rotations of the two polypeptides are different.

The differences are considered to be due to some racemization of the phenylalanyl residues during the polycondensation of H-Gly-Gly-Phe-OPCP. On the other hand, H-Phe-Gly-Gly-OPCP has a C-terminal glycine and necessarily will yield optically pure poly-Phe-Gly-Gly. The latter polypeptide, therefore, can be used as a standard.

It was necessary to determine the sensitivity of the method. This was carried out by polymerizing D-Phe-Gly-Gly-OPCP with varying amounts of L-Phe-Gly-Gly-OPCP. The physical data of the copolymerized polypeptides so obtained are summarized in Table II. This table shows that 1.5% enantiomer of Phe can easily be detected at 223 mu using the Cary 60

Table II

Copolymerization of H-D-Phe-Gly-Gly-OPCP with Various Amounts of H-L-Phe-Gly-Gly-OPCP[a,b]

% of L-Tripeptide Active Ester	$\bar{M}_w$	$[\alpha]^{23}_{223}$	$[R']^{23}_{223}$	$[R]^{23}_{223}$
0.0	24,000	-3461°	-5088°	-9033°
1.5	12,000	-3366	-4948	-8785
2.5	8,000	-3322	-4883	-8670
5.0	10,000	-3202	-4707	-8357

a) The above rotations were taken in trifluoroethanol containing 0.5% lithium chloride at a concentration of 0.1%.
b) The residue weight of Phe was used to calculate $[R']^{23}_{223}$ and 261 was used to calculate $[R]^{23}_{223}$

spectropolarimeter. The data in Table II are plotted in Figure 1. This graph indicates that the decrease of $[\alpha]^{23}_{223}$ with increasing L-Phe in the

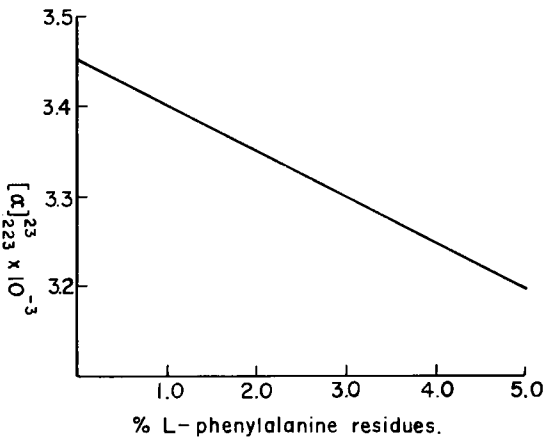


Figure 1.

D-polypeptide chain is linear. Using this plot it was estimated that all polypeptides prepared from Gly-Gly-Phe active esters contain 5-8% of the opposite enantiomer of Phe. It should be noted that in the Table all poly-Gly-Gly-Phe prepared by different active esters have nearly the same $[\alpha]^{23}_{D}$ value. Also the tripeptide active esters which gave these polypeptides have an N-terminal glycine. It is well known that glycine couples (3) faster than other hindered amino acids. Therefore poly-Val-Gly-Phe was prepared through p-nitro- and pentachlorophenyl esters of H-Val-Gly-Phe-OH.

The $[\alpha]^{23}_{225}$ values for poly-Val-Gly-Phe obtained from the p-nitrophenyl and pentachlorophenyl esters were -241.4° (c, 0.006% in trifluoroethanol

containing 0.5% LiCl) and -134° (c, 0.02 in trifluoroethanol containing 0.5% LiCl) respectively, using the Cary 60 spectropolarimeter. These results clearly indicate that a greater degree of racemization occurs with p-nitrophenyl ester than with pentachlorophenyl ester if the N-terminal amino acid is valine.

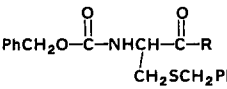
Racemization can occur through the well-known oxazolone intermediate. In addition, racemization can occur through direct α-hydrogen abstraction of an activated amino acid residue. The latter can seriously contribute to the total amount of racemization in the case of certain amino acids. This is supported by recent studies on cysteine active ester derivatives (3,4). It is well known that even N-carbobenzoxy cysteine derivatives racemize readily without the formation of oxazolone intermediates in the presence of a base.

At the First American Peptide Symposium we reported that the racemization of N-carbobenzoxy-S-benzylcysteine active esters in organic solvents and in the presence of a base occurs through α-hydrogen abstraction (3,5).

This observation led us to study the rate of racemization of many commonly used N-carbobenzoxy-S-benzyl cysteine active esters as well as the rate of coupling of the same active esters with H-Val-OCH_3. Table III shows the second-order racemization rate constants for the racemiza-

Table III

The Second-Order Racemization Rate Constants for the Reaction of N-benzyloxycarbonyl-S-benzyl-L-cysteine active esters with triethylamine[a,b]



Compound where R is:		$k_{rac} \times 10^4$ (M^{-1} sec^{-1})
(I)[c]	O—N-succinimide	48.8 ±2
(II)[c]	OC_6F_5	33.0 ±6
(III)[c]	$OC_6H_3-(NO_2)_2(2,4)$	29.6 ±2
(IV)[c]	$OC_6H_3-(NO_2)_2(2,6)$	29.0 ±2
(V)[d]	$OC_6H_2Cl_3(2,4,5)$	4.88 ±0.6
(VI)[c]	OC_6Cl_5	4.14 ±0.2
(VII)[c]	$OC_6H_4-pNO_2$	3.94 ±0.3
(VIII)[c]	$OC_6H_2Cl_3(2,4,6)$	0.80 ±0.05
(IX)[c]	OC_6Br_5	0.414 ±0.02
(X)[c]	$OC_6H_2Br_3(2,4,6)$	0.1718 ±0.001
(XI)[c]	OPh	0.0972 ±0.002
(XII)[c,e]	OEt	No racemization[f]
(XIII)	$NHCH_2CO_2Et$	" "

a) 23±1°, in tetrahydrofuran; b) the concentration range of triethylamine was 0.22—0.36M; c) the average of two experiments; d) the average of four experiments; e) 3·6 equiv. of triethylamine; f) up to 7 days.

tion of N-carbobenzoxy-S-benzyl-L-cysteine active esters with triethylamine. This Table shows that among the most frequently used active esters the fastest racemizing is N-hydroxysuccinimide followed by pentafluorophenyl, dinitrophenyl, pentachlorophenyl and p-nitrophenyl esters. The ethyl ester and amide derivative do not racemize under these conditions. It is interesting to compare the order of magnitude of the racemization rate constant to that of the second-order coupling rate constant shown in Table IV. It can be seen that the coupling rate is considerably faster than the racemization rate. It should also be noted that the order of these esters in Table IV is the same as that in Table III, with the exception of N-hydroxsuccinimide ester. This however does not mean that the "activity" of the ester is parallel with the rate of

racemization.

Table IV

The Second-Order Coupling Rate Constants for the Reaction of N-Carbobenzoxy-S-benzyl-L-cysteine Active Esters with Valine Methyl Ester[a]

Z-Cys-R BZL where R is:	$k_c \times 10^2$ $M^{-1}\ sec^{-1}$	90% Reaction Time (min.)
—OPFP[b,d]	40.4 ± 9	2.9
—ODNP (2,4)[b,d]	18.4 ± 3	6.3
—OSu[b,d]	5.44 ± 0.7	21
—ODNP (2,6)[b,e]	1.73 ± 0.2	67
—OPCP[e,f,c]	1.72 ± 0.2	62
—OTCP (2,4,5)[b,f]	0.298 ± 0.03	385
—ONP[b,f]	0.105 ± 0.01	1088
—OTCP (2,4,6)[b,f]	0.0626 ± 0.002	1856
—OTBP (2,4,6)[b,f]	0.0215 ± 0.006	5310

a) 23±1°, in tetrahydrofuran; b) the concentration of the active ester and valine methyl ester was 0.13M; c) the concentration of this ester and valine methyl ester was 0.0845M; d) the average of four experiments; e) the average of three experiments; f) the average of two ex-experiments;

An important conclusion can be drawn from these data, namely a fast coupling active ester which racemizes relatively slowly is considered to be the best choice for the preparation of sequential polypeptides and in general for the synthesis of all peptides. Table V shows the ratios of the coupling rate constant to the racemization rate constant.

Table V

Ratio of Coupling and Racemization Rates[a]

Z-Cys-R BZL where R is:	$\frac{k_c}{k_2}$
—OPFP	245
—ODNP (2,4)	124
—OPCP	83
—OTBP (2,4,6)	25
—OSu	22
—OTCP (2,4,6)	16
—OTCP (2,4,5)	12
—ODNP (2,6)	12
—ONP	5.3

a) The value for k_2 was one-half of k_{rac} given in Table III (see ref.3).

These ratios indicate numerically the best choice of an active ester for peptide synthesis. The larger this number the smaller the amount of racemization to be expected during coupling.

Next, the effect of the side chain of amino acids on the rate of racemization and coupling was investigated. It is known from Anderson(6), Liberek (7) and others that several amino acid active ester derivatives racemize in the presence of tertiary base, and systematic investigation is now under way to clarify this point. Results with Glu and Asp are indicated by Table VI, VII and VIII.

Table VI

The Second-order Coupling Rate Constants for the Reaction of N-Carbobenzoxy-γ-methyl-L-glutamic Acid Active Esters and N-Carbobenzoxy-β-methyl-L-aspartic Acid Active Esters with VALINE Methyl Ester Respectively[a,b].

Z-Glu-R (OMe) where R is:	$k_c \times 10^{2}$ [(c)] M^{-1} Sec^{-1}	90% Reaction Time (min.)
OPFP	14.9108 ± 2.0	7.8
OSU	2.9746 ± 0.3	40
OPCP	.1475 ± .0617	791
OTCP (2,4,5)	.1005 ± .0489	1161
ONP	.0452 ± .0042	2578
Z-Asp-R (OMe) where R is:	**$k_c \times 10^{2}$ M^{-1} Sec^{-1}**	**90% Reaction Time (min.)**
OPFP	14.7 ± .9	7.9
OPCP	.737 ± .07	158
OTCP (2,4,5)	.262 ± .01	446
ONP	.0718 ± .008	1538

Table VI shows the second-order coupling rate constants which were determined under the same conditions employed for the corresponding cysteine active esters (3). It is seen that the order of magnitude of the rate constants for both amino acid active ester derivatives are comparable to that of the corresponding cysteine active esters. On the other hand, the data in Table VII indicate that the racemization is about 13 to 23 times slower for aspartic acid active esters, and 50 to 100 times slower for glutamic acid active esters than the corresponding cysteine active esters.

Table VIII shows the ratios of the coupling rates to racemization rates. These ratios are so much larger for glutamic acid active ester derivatives than the corresponding ratios of the cysteine esters, that racemization with these esters of glutamic acid would be considerably lower than for either cysteine or aspartic acid active esters.

In order to understand the details of the mechanism for α-hydrogen abstraction, the base catalyzed racemization and deuterium exchange of N-carbobenzoxy-S-benzyl-cysteine pentachlorophenyl ester was studied in chloroform in the presence of triethylamine and mono deutero methanol.

Table VII

The Second-Order Racemization Rate Constants for the Reaction of N-Carbobenzoxy - γ - methyl-L-glutamic Acid Active Esters and N-Carbobenzoxy - β - methyl-L-aspartic Acid Active Esters with Triethylamine Respectively[a,b].

Z-Glu-R (OMe) where R is:	$k_{rac} \times 10^{6}$ [(c)] M^{-1} Sec^{-1}
OSU	44.09 ± 6.5
OPFP	34.71 ± 3.5
ONP	3.20 ± .30
TCP (2, 4, 5)	2.35 ± .77
PCP	1.96 ± .57
Z-Asp-R (OMe) where R is:	$k_{rac} \times 10^{6}$ M^{-1} Sec^{-1}
OPFP	243.7 ± 8.9
OTCP (2,4,5)	35.2 ± .27
ONP	27.0 ± 2.2
OPCP	17.6 ± .92

Table VIII

Ratio of Coupling and Racemization Rates

Z-Glu-R (OMe) where R is:	$\frac{k_c}{k_2}$
OPFP	8593
OPCP	1503
OSU	1349
OTCP (2, 4, 5)	856
ONP	283
Z-Asp-R (OMe) where R is:	$\frac{k_c}{k_2}$
OPFP	1207
OPCP	837
OTCP (2, 4, 5)	149
ONP	53

Table IX shows the kinetic data which were obtained by measuring the

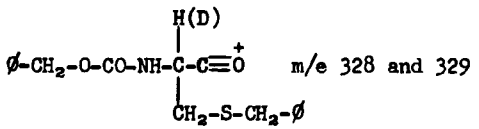
Table IX

Kinetic Data for Triethylamine[a] Catalyzed Racemization and Deuterium Exchange of N-carbobenzoxy-S-benzyl-L-cysteine Pentachlorophenyl Ester[b] in the Presence of Methanol-O-d[c] in Chloroform.

Time (min)	$[\alpha]_D^{22.5}$ (c 2, DMF)	%D Calculated for m/e 328 ion
30	−34.4	-
60	−28.1	-
90	−26.8	-
120	−19.0	3.8
180	−13.8	-
240	−10.3	-
360	− 4.1	12.9
615	-	21.3
780	-	29.2
1470	-	39.1
2040	-	47.8
2880	-	59.9

a) The triethylamine concentration was 0.31 M.
b) The active ester concentration was 0.0435 M.
c) The concentration of methanol-O-d was 2.3 M.

rotations of the reisolated samples, and by calculating the deuterium content of each sample, using the acylium ion

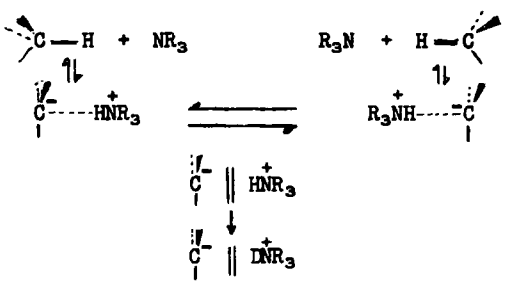


m/e 328 and 329

in the mass spectrum.

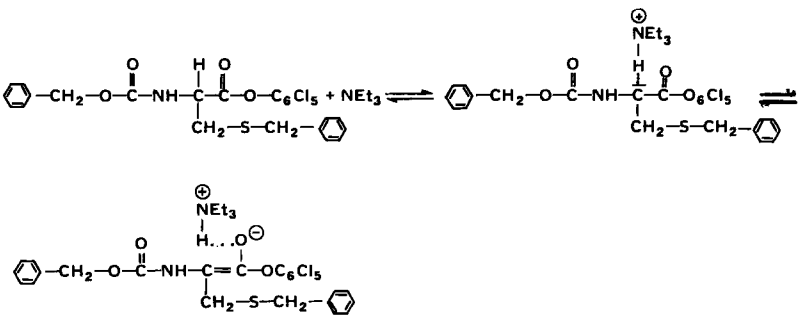
From these data the ratio of the rate of exchange (k_e) to the rate of racemization (k_α) was calculated and was found to be 0.063 and 0.049 in two kinetic runs. This ratio was not affected significantly by the presence of 0.001M triethylamine hydrochloride, and according to Cram (8), indicates isoracemization. Scheme III shows, according to Cram (8), the mechanism for isoracemization: the active ester forms a contact ion pair

Scheme III



with the base, which racemizes much faster through a "conducted tour mechanism", then forms a solvent separated ion pair, which will exchange deuterium with the medium. This is the first time that isoracemization has been observed, according to our best knowledge, for an amino acid derivative, or in general for an asymetric carbon α to a carbonyl group. Scheme IV shows the conducted tour mechanism.

Scheme IV



N-carbobenzoxyphenylalanine pentachlorophenyl ester was racemized under similar conditions described for the cysteine derivative. The ratio of the k_e/k_{ex} was found to be 0.03, which seems to indicate that isoracemization is probably a general mechanism for amino acid active esters in organic solvents which are frequently used in peptide synthesis, and in the presence of triethylamine.

Acknowledgement: This work was supported by Grants from the National Institutes of Health (GM 06579 and 08795). We wish to thank Dr. Roger L. Foltz of Battelle Memorial Institute, Columbus, Ohio, for the mass spectra.

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STUDIES ON DIPEPTIDE CONFORMATION AND ON PEPTIDES WITH SEQUENCES OF ALTERNATING L AND D RESIDUES WITH SPECIAL REFERENCE TO ANTIBIOTIC AND ION TRANSPORT PEPTIDES

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1. INTRODUCTION. This paper is concerned with two completely different aspects related to peptide conformation:

(a) The first deals with the theory of dipeptide conformation with special reference to NMR data, in which it is shown that the dihedral angle ϕ can be determined quite accurately from the corresponding NMR coupling constants $J(NH\text{-}C^{\alpha}H)$, a possibility which will be of great interest in relation to NMR studies on the structures of simple peptides, particularly of cyclic peptides containing a small number of residues.

(b) The second part deals with the conformation of peptides having sequences of alternating L and D residues--sequences of the type LDLDLD..., or of the type LDDLLDDL.... The presence of such repeating sequences containing combinations of L and D residues is shown to lead to special types of conformations which cannot be taken up by the usual peptide chains in proteins having only L residues. Therefore, the conformation of such peptides is of great interest in relation to their biological activity. In fact, almost all peptides having antibiotic properties contain D residues, and have either isolated LD combinations, or sequences of repeating LD pairs. This first study of the conformations of sequences of peptide units having alternating L and D residues will therefore be of great interest in relation to the understanding of the action of antibiotics.

It is known that some of the important ion-transport peptides, such as enniatin and valinomycin, have a cyclic structure, with repeating sequences containing LD or DL pairs. The structures of these two cyclic peptides have therefore been specially studied and the preliminary indications are that the nature of the alternating sequence of L and D residues plays a dominant part in the ion-transport property of these peptides. A more detailed study of enniatin has led to a

preliminary theory of how this peptide is able to capture and release ions.

2. VARIATION OF NMR COUPLING CONSTANT WITH DIHEDRAL ANGLE ϕ. The range of allowed conformations for a pair of linked peptide units, whose conformation is defined by the dihedral angles (ϕ,ψ) is well known (see for example Ref. 1). For the sake of ready reference, Fig. 1 gives the definition of these dihedral

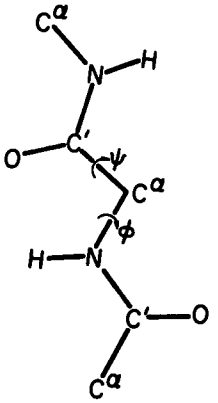


Fig. 1. Schematic diagram of a dipeptide unit backbone, showing the dihedral angles ϕ and ψ.

angles and Fig. 2 gives the range of allowed conformations for an alanyl residue, which has a β-carbon atom, and for a glycyl residue without one. In what follows in this section, we shall be dealing with the so-called dipeptide unit, whose formula is given by CH_3-CO-NH-C^{α}HR-CO-NH-CH_3, in which the side chain R of the middle α-carbon atom may be any one of the twenty different side chains that are found in the naturally occurring amino acids. We shall denote the compound by the name of this side chain; for example, "alanyl dipeptide unit", and so on. It is well known that the conformation of a long peptide chain or polypeptide chain can be described in terms of the local conformation (ϕ,ψ) at the various α-carbon atoms. We shall therefore discuss in particular the information provided by NMR data regarding these dihedral angles at an α-carbon atom.

The dihedral angle ϕ produces a twist about the bond N-C^{α} and thereby produces an angle between the projections of the bonds NH and C^{α}H in a plane perpendicular to the bond N-C^{α}. If we call this angle θ, then there is a simple relation between ϕ and θ, given by

$$\theta = |240^{\circ} - \phi|, \qquad (1)$$

as is shown in Fig. 3. Hence, if the angle θ can be determined, then ϕ can be determined, except for a certain ambiguity. It is well known that the angle θ can be connected to the coupling constant $J(NH\text{-}C^{\alpha}H)$, which we shall simply refer to as J in this paper, by the relation

$$J = a + b\cos\theta + c\cos 2\theta. \qquad (2)$$

This expression can be recast in the form

$$J = A\cos^2\theta + B\cos\theta + C\sin^2\theta. \qquad (3)$$

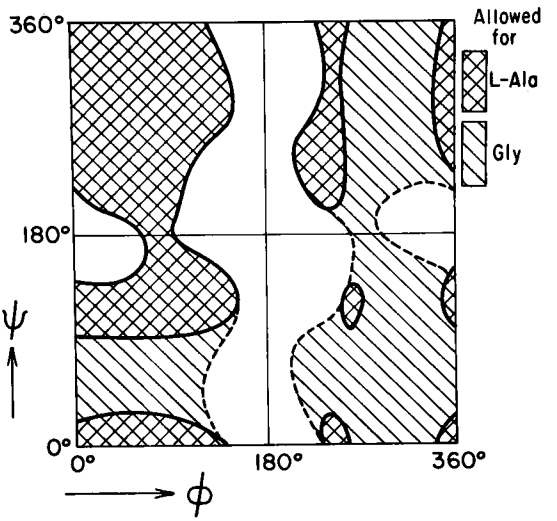


Fig. 2. Conformational map showing the regions of the (φ,ψ)-map allowed for (a) glycyl and (b) L-alanyl dipeptide units. Note that the allowed regions of the Gly map are symmetric about the centre of the diagram, while it is not so for Ala because the α-carbon atom is asymmetric. The allowed regions correspond to the potential energy being less than 2 kcal/mole.

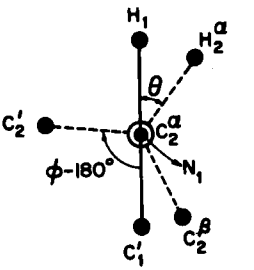


Fig. 3. Relation between the dihedral angle φ at an α-carbon atom and the corresponding angle θ relevant for the NMR coupling constant J(NH-C$^{\alpha}$H).

If the constants A, B, C can be determined for the coupling constant between the NH proton and C$^{\alpha}$H proton, then the angle θ can be determined from a measurement of J for any particular example, and hence the dihedral angle φ can be deduced from θ using Eq. 1. The best values of A, B, and C were determined in this investigation by measuring the mean value of J for a number of dipeptides derived from various amino acids and other related compounds having the NH-C$^{\alpha}$H bond. These dipeptides were prepared and studied in solution in dimethyl formamide so that an internal hydrogen bond was not formed in the molecule. Under these conditions, it is obvious that all the allowed conformations (φ,ψ) will be taken up by the molecule and that each conformation will have a probability corresponding to its energy V(φ,ψ), the relationship between the probability P and the energy V being given by the well-known Boltzmann relation:

$$P(\phi,\psi) \propto e^{-V(\phi,\psi)/RT} \qquad (4)$$

The value of the energy V(φ,ψ) was calculated using standard conformational theory (see Ref. 1), and hence the mean value

of J was calculated from theory using Eq. 5.

$$J = A\langle\cos^2\theta\rangle + B\langle\cos\theta\rangle + C\langle\sin^2\theta\rangle, \qquad (5)$$

where the mean values were obtained by integrating over the (ϕ,ψ)-plane using the relevant probability values given by Eq. 4. The values thus obtained for $\langle\cos^2\theta\rangle$, $\langle\cos\theta\rangle$ and $\langle\sin^2\theta\rangle$ for a number of compounds are given in Table 1. Table 1 also lists the actual values of $\cos^2\theta$, $\cos\theta$ and $\sin^2\theta$ for a few other compounds in which the conformation is expected to be nearly rigid. The corresponding observed values of J are given in the last column of Table 1. A preliminary comparison between theory and experiment indicated that there is very good internal agreement between the first ten sets of data given in Table 1 and, therefore, these were used for obtaining the best fit between the theoretical expression (3) and the experimentally observed J values. It will be seen that there is excellent agreement between theory and experiment, the mean deviation between the two being less than 0.1 cps. (It may be mentioned that, when this study was started, it was felt that an agreement of between 0.5 and 1.0 cps would be highly satisfactory, and it is indeed remarkable that the theory fits the experimental observations so well, although the data themselves vary from 1 to 8 cps).

Formula (3), with values of the constants A, B and C given here, also yields values of J in reasonable agreement with observation for the NMR data of an analogue of ferrichrome, which has been studied by Llinas *et al.* (available as a preprint). The details are not given here.

There are rather appreciable differences between theory and experiment for valine and phenylalanine dipeptides. The case of valine would require further refinement in the

Table 1. Calculated* and Experimental Values of NH-$C^{\alpha}H$ Coupling Constants.

Compound	$\langle\cos^2\theta\rangle$	$\langle\cos\theta\rangle$	$\langle\sin^2\theta\rangle$	J_{cal}* (cps)	J_{exp}† (cps)
N-Methyl Acetamide	0.500	0.000	0.500	4.60	4.5
N-Ethyl Acetamide	0.604	-0.155	0.397	5.50	5.5
N-Isopropyl Acetamide	0.772	-0.787	0.228	7.68	7.7
Glycine dipeptide	0.647	-0.112	0.353	5.66	5.8
Alanine dipeptide	0.782	-0.858	0.218	7.86	7.8
Leucine dipeptide	0.835	-0.895	0.165	8.24	8.2
Dihydrouracil (Eq, 45°)	0.500	0.707	0.500	3.26	3.6#
Dihydrouracil (Ax, 75°)	0.067	0.259	0.933	1.60	1.5#
Cyclohexalactam (0°)	1.000	1.000	0.000	5.60	5.6
Glycyl diketopiperazine (60°)	0.250	0.500	0.750	2.20	2.2
Valine dipeptide	0.865	-0.920	0.136	8.46	8.0
Phenylalanine dipeptide	0.811	-0.881	0.189	8.08	8.8

* $J_{cal} = A\cos^2\theta + B\cos\theta + C\sin^2\theta$, with A=7.5, B=-1.9, C=1.7

† Most of the data are correct to ±0.2 cps, while some have errors of only ±0.1 cps.

These two data have half-weight in the least-squares calculations, and the values of θ used are not accurate.

calculations, but it appears that the variation from theory in the case of phenylalanine cannot be explained by simple refinements, but that important modifications will have to be made in the conformational theory itself, taking into account some aspects which have been neglected previously, such as a π-π interaction between a benzene ring and a peptide unit. This will be discussed separately elsewhere.

In conclusion, as a result of the present study, the NMR method can be used with great confidence in the solution of peptide structures, particularly those of cyclic peptides. (The whole of the study reported in this section was carried out in collaboration with the laboratory of Professor K.D. Kopple.)

3. CONFORMATION OF A SEQUENCE WITH SUCCESSIVE L AND D AMINO ACID RESIDUES. Most of the studies generally reported in conformational theory of peptides have dealt with L residues. Some workers, such as Shemyakin and coworkers (2,3), who have dealt with peptides having both L and D residues, have generally referred to the conformational map of the L-dipeptide unit for their theoretical interpretation. However, the case involving two successive L and D α-carbon atoms is very interesting and it leads to some entirely new consequences regarding the possible conformations of the peptide chain in the neighborhood. The essential nature of the resultant conformation can be derived, as in the case of the usual theories of peptide conformation, from the consequences arising from a dipeptide having either an L or a D side chain. The corresponding maps showing the allowed conformations for these two are shown superimposed in Fig. 4.* It will be seen from this figure that in general, what is allowed for an L side chain is disallowed for a side chain having a β-carbon atom in the D configuration. In fact, the only regions where β-carbon atoms in both L and D positions are allowed are shown cross-hatched in Fig. 4, and they form only a very small part of the total area of the (ϕ,ψ)-plane. The consequences of the results predicted by the map in Fig. 4 are discussed briefly in this section.

(a) The most interesting consequence is the fact that the bulk of the conformations allowed for a chain having mixed L and D residues are those that are not possible for a chain containing all L residues. Thus the conformations of such a mixed-residue chain will be entirely different from familiar conformations of the common all-L protein and peptide chains. This means that they can have very different biological activities; in particular, they can exhibit very specific inhibitory antibiotic behavior. The nature of the conformations likely to be preferred by certain typical antibiotics which have repeating sequences of alternating L and D residues and their associated properties are discussed in the next Section 4. In this section, we shall mention only some of the more general properties of the local conformations of an LD or a DL sequence.

(b) A sequence containing an L residue followed by a D

*The rules formulated by Edsall et al. (4) are adopted in this paper for notation and nomenclature. The absolute value of the dihedral angles are given, irrespective of whether the residue is L or D.

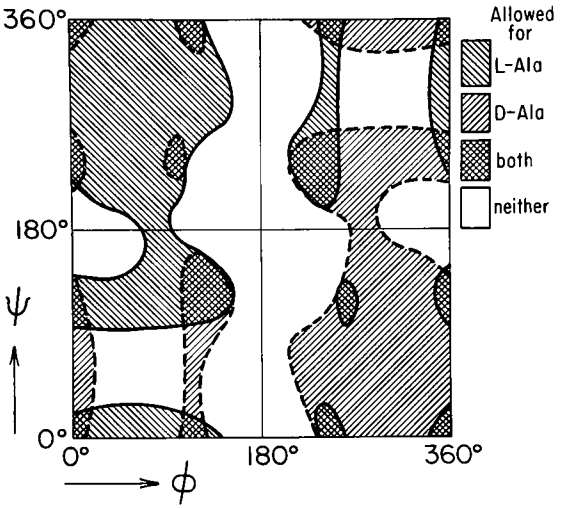


Fig. 4. Conformational map showing regions allowed for L-Ala and D-Ala dipeptide units. Note that the two regions are mostly exclusive of each other.

residue, or a D followed by an L residue, has the property of assuming a specific type of folding in which the three peptide units so linked produce a reversal in the chain direction. The importance of this bend produced by a combination of L and D residues was recognized only during this investigation. The possibility of such a reversal in chain direction occurring at two successive α-carbon atoms had been considered earlier in the authors' laboratory. Venkatachalam (5), showed that there are two types of such bends, which we shall designate as LL and LG, which are possible for the peptide units found in proteins (G stands for glycine, which does not have L or D side chains). Four combinations which can therefore be envisaged are shown in Fig. 5, namely, LL, DD, LD, and DL (LD and DL also correspond to LG and GL respectively, in which, for obvious reasons, G cannot be L.) An analysis of the known protein structures shows that the LL bend is very rare, while the LG bend is found not infrequently. It is particularly commonly found in cytochrome C (Dickerson, personal communication). Therefore, an attempt was made to find out the relative stabilities of the LL and LD bends. The LL and DD bends are equal in energy, and so are the LD and DL bends, since one of each pair is related to the other by reflection symmetry. Calculations show in fact that the LD bend is much more stable than the LL bend, both because it can have a lower minimum energy (-7.5 vs -6.5 kcal/mole, see Fig. 5) and also because a larger number of conformations have low energies for this type, as stated in Fig. 5. The values of (ϕ,ψ) given in Fig. 5 are those for the minimum energy conformation of each type of bend, and a range of approximately $\pm 20^{\circ}$ is possible for the low energy conformations.

If we now consider two LD bends being joined in a cyclic manner, with no other peptide units in between, the simplest structure would correspond to the sequence $\overline{\text{LDLLDL}}$ or $\overline{\text{(LDL)}_2}$.

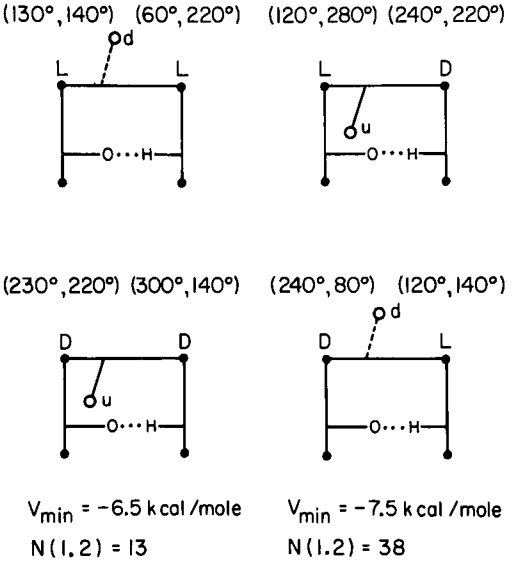


Fig. 5. Schematic diagrams of the LL, LD, DD and DL bends, showing the α-carbon atoms (•) which are nearly in the plane of the paper and the carbonyl oxygen (o) of the middle peptide unit. (u=above plane of paper, d= below). V_{min} is the lowest energy that this bend can have and N(1.2) is the relative number of conformations which have V less than V_{min} + 1.2 kcal/mole.

This can have two LD bends and a conformation similar to that of an antiparallel pleated sheet ($\phi \sim 30^\circ$ and $\psi \sim 330^\circ$) for the other two L α-carbon atoms. Surprisingly, this particular cyclic hexapeptide does not appear to have been observed for any of the natural antibiotics. But gramicidin S is only an extended form of this structure, in which two additional L residues are introduced in between the two bends in either half, namely having the sequence $\underline{\text{L(DLLLL)}_2\text{J}}$, with four internal hydrogen bonds between the two halves in the pleated sheet structure. In fact, this type of structure is the one considered to be most likely for gramicidin S from recent NMR data (6). It is interesting that the L-residue in the DL bend of gramicidin S is Pro, for which ϕ can only have values close to 120°, which is exactly the value needed for the DL bend. The bend would therefore be expected to be particularly stable in gramicidin S.

As mentioned earlier, several antibiotics have D residues in their sequence, and practically none are known which have a DD sequence. This is to be expected, since, from the conformational point of view, a DD sequence gives no advantage over an LL sequence. Hence, it is reasonable to assume that the isolated LD, or DL, sequence which leads to the special bent conformations discussed above, are the real seats of activity of the antibiotic. Antibiotics having more than one D residue (with L residues on either side) may have additional LD or DL bends, and the activity of the antibiotic should be sought for in these bends. The precise manner in which the LD bend confers antibiotic activity is discussed in Section 6.

(c) Since the conformation of an LD sequence leads to possible structures for the peptide chain which are quite different from those normally possible for the common protein chains, it is likely that, by careful trial and error, it would be possible to build a chain having proper sequences of either L or D

residues, which can assume a folded structure which imitate the "active site" of an enzyme--that is, the required groups may be brought close to each other in a similar relative disposition, as in the active site of the protein. It is suggested that two such groups can be brought into close proximity by bending the chain much more readily, and with a smaller number of residues, in a mixed DL peptide than in a peptide chain containing only L amino acid residues. Thus we now have the possibility of synthetically building analogues of enzyme structures using much smaller numbers of residues than are usually found in the common proteins. (We are grateful for discussions with Dr. B.B. Sarkar regarding this possibility).

(d) Fig. 4 contains a small number of regions in the (ϕ,ψ)-plane which are allowed for both D and L residues. It would therefore be possible for the backbone conformation to have (ϕ,ψ)-values in these regions even if two β-carbon atoms are attached to the α-carbon atom, as in the residue of α-aminoisobutyric acid, (abbreviated to AIB). This is a non-optically active amino acid and would therefore be able to assume the inverse conformations (ϕ,ψ) and $(-\phi,-\psi)$ with equal facility. The allowed regions of this amino acid contain the local conformations required for both the α-helix and for the 2.2_7 helix. In both cases, either the right-handed or the left-handed helices are permitted. The two structures will have equal energy for a homopolymer of AIB. However, if there are two different groups attached to the α-carbon atom, for example as in an α-methylserine residue, in which the two groups are CH_3 and CH_2OH, it is likely that one of the structures may be preferred to the other. For instance, it has been shown by Sarathy and Ramachandran (7) that L-serine can have an extra hydrogen bond for a left-handed α-helix. On the other hand, the left-handed helix is not a very stable structure for the usual L-amino acid residues, although it is not impossible. It is very likely that a polymer of α-methyl-L-serine may have a left-handed α-helix. Several such possibilities can be worked out, but these are not discussed here.

4. CONFORMATIONS OF REPEATED SEQUENCES OF ALTERNATING L AND D RESIDUES. (a) The types of stable conformations which occur when a pair of L and D amino acid residues is repeated in a chain are particularly interesting. Since, as already mentioned, the most stable conformation of a single LD pair is one that leads to an LD bend, a chain having the sequence LDLDLD... can have a series of bends corresponding to each pair of L and D residues. The resultant conformation of the backbone is shown schematically in Fig. 6. As will be seen from this, the chain takes the form of a ribbon, in which there are systematic hydrogen bonds NH···O in a direction parallel to the length of the ribbon, which is indicated by the long straight arrow in Fig. 6, although locally there are small twists at right angles to the length, shown by the series of small curved arrows. If this structure is actually built from space-filling atomic models, it will be seen that it is quite rigid. Calculations show that the ribbon is almost straight; in other words, for an LD pair the unit twist is close to 180° or, for a set of four residues composed of two pairs of L and D residues, the total twist is close to 360°. The repeat distance for four residues is approximately 8 Å, or the peptide

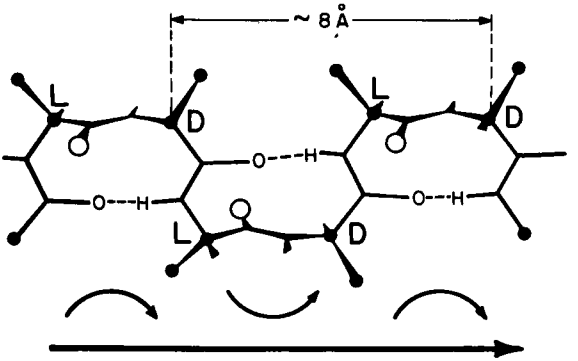


Fig. 6. Conformation of the LD-ribbon. The α-carbon atoms in the ribbon are nearly in the plane of the paper. (●=α- and β-carbon atoms, o=carbonyl oxygen atom).

units at right angles to the length of the ribbon are approximately situated at intervals of 4 Å along the length of the ribbon, and all these are connected together by NH···O hydrogen bonds. Alternate peptide units lying parallel to the length of the ribbon have non-hydrogen bonded NH and CO groups which can serve to attach the LD chain by hydrogen bonding to another molecule or biopolymer. In fact, as is well-known, the distance between the bases in a nucleic acid double helix is about 3.4 Å, so that it is quite possible for this ribbon structure to associate with neighboring bases in the nucleic acid structure by hydrogen bonding. If, in addition, the side chains attached to the α-carbon atoms in the neighborhood are mostly hydrophobic, then they would form a protective envelope surrounding the hydrogen-bonded portion and the resultant attachment of the LD peptide to the nucleic acid chain could be unaffected by disturbing influences of the medium.

If the most stable local conformation of an LD bend, namely (120°,280°), (240°,220°), is repeated, then the ribbon is found to have a unit twist of 160° for two units, or a left-handed twist of about 40° for a length of approximately 8 Å. However, a nearly straight ribbon, with a unit twist close to 180°, has a stablizing energy not materially different from the above minimum energy helix. On the other hand, a chain having the sequence LDLDLD... can also form a ribbon structure with a series of repeating DL bends. This also will be nearly straight, with the same values for the repeat and for hydrogen bond lengths as a corresponding LD ribbon, but with the opposite sense of twist. Thus, the structure formed with the most stable local DL bend, namely for (240°,80°), (120°,140°), will have a right-handed twist of nearly +40° for a repeat of four residues. The two ribbon structures, which we may denote by the terms LD-ribbon and DL-ribbon respectively, are equally probable for a long chain having alternating L and D residues, the energies of equivalent enantiomorphous structures being identical. For short peptides, one or the other may be favored, depending on the absolute configuration of the terminal residues. Among the various antibiotics that are known, gramicidin A has such a repeating LD sequence. and the ribbon structure may be expected to occur in this antibiotic.

Tests with model structures show that both the LD-ribbon

and the DL-ribbon can bind to DNA, forming at least two hydrogen bonds to the bases and two to phosphate groups. Thus, for example, the LD-ribbon can bind to two successive base pairs AT, CG by making hydrogen bonds with the C=O group of T and the NH_2 group of C in the major groove of the double helix. At the same time, two peptide NH groups on either side of the region binding to the bases can also be strongly hydrogen-bonded to phosphate oxygens in either chain of the DNA. The effect of such types of binding on the antibiotic activity of peptide chains having repeated LD sequences is discussed in Section 6.

(b) The sequence of residues LDLDLD... has in fact a slight twist for every pair of residues about a direction perpendicular to the length of the ribbon, but, as shown in Fig. 6, these twists are exactly equal and in opposite directions for successive pairs, so that the resultant ribbon is practically flat and straight, with only a slight twist possible about a direction parallel to the length (as discussed above). On the other hand, if we have the sequence LDDLLDDL..., then the situation is different, as is shown in Fig. 7, which is the structure of valinomycin. In this case, it is found that the perpendicular twist of every pair (particularly the successive ones of the type LD and DL) are in the same sense, so that there is a curving of the ribbon at right angles to its length, which actually closes up after twelve residues in valinomycin. A comparison of Fig. 7(b) with Fig. 6 shows the close similarity between the structures of the straight ribbon and the cyclic dodecapeptide. We shall comment on the ion-binding property of valinomycin in the next Section 5. However, because of the existence of a number of LD and DL bends, valinomycin would be expected to have antibiotic properties, of the type characteristic of peptides having such bends.

(c) In addition to the ribbon structure, the sequence LDLDLD... has also another type of conformation. This conformation is best understood by first examining the conformation of the cyclic hexapeptide $\overline{\text{LDLDLD}}$ as it exists in the naturally occurring peptide enniatin. Although, like valinomycin, enniatin is a depsipeptide, we shall discuss in this section the property of the analogous compound in which every residue is an amino acid residue containing the CO-NH group. An examination of cyclic structures having 4, 6, 8,..., residues shows that the number 6 is particularly favorable for binding Na^+ and K^+ ions and has a very good conformation at each of the α-carbon atoms, namely in the neighborhood of $(60^\circ, 300^\circ)$ for the L-, and $(300^\circ, 60^\circ)$ for the D-residues. As will be seen from Fig. 4, these values of (ϕ, ψ) correspond to low energies for both the L and D dipeptide units, and therefore the structure would be expected to be very stable, even though no internal hydrogen bonds are made. (In enniatin itself there are no proton-donating groups for forming hydrogen bonds). Hence, when a sequence of amino acid residues having alternating L and D configurations are linked together chemically, another residue cannot be added in a linear fashion at about the stage when five or six residues have been joined together, because the two ends would tend to cyclize. In fact, it was learnt during the Symposium that this actually occurred in a synthesis tried out by Dr. F.F. Richards (Yale) on a chain containing such alternating residues (all Ala) using the Merrifield technique (9). He found that the yield

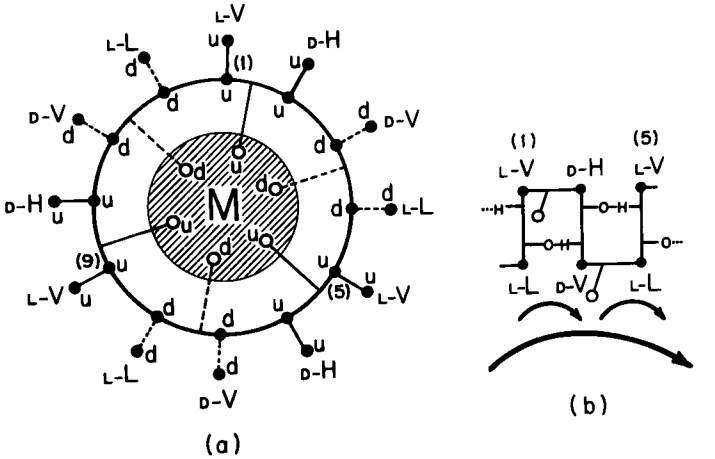
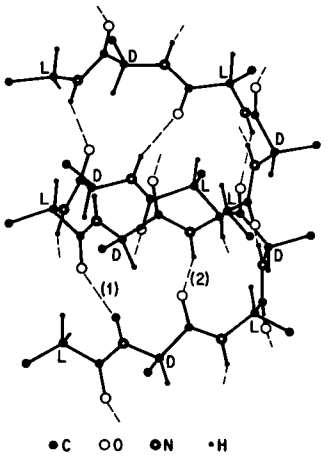


Fig. 7. Conformation of the sequence LDDLLDDL..., in valinomycin (●=α- and β-carbon atoms, o=carbonyl oxygens). (a) View down the axis of the cylindrical ribbon; u=above plane of paper, d=below; the centre of the metal ion M is in the plane. (b) One third of the chain is shown looking transversely. Note that the twists of neighboring LD and DL bends are in the same sense, and that the free oxygens are all on the inside of the cylinder.

was very poor when a sixth residue was added to the system, RESIN—LAla—DAla—LAla—DAla—LAla. Presumably, the chain folds back and the growing terminus buries itself into the resin, leading to this result.

We shall now consider the possibility of obtaining a helical structure from the cyclic hexapeptide structure. It is obvious that if the chain does not completely remain in a plane but winds either up or down, then, for a suitable value of the pitch of the helix, it would be possible to have hydrogen bonds between NH and CO groups. In fact it is found that there is a conformation in which every NH group, both of the L and D type of residues, is hydrogen-bonded to a CO group in a residue six units away from it (Fig. 8). This type of completely hydrogen-bonded helix is analogous to the classical α-helix of Pauling and Corey for a chain composed of all L-residues. However, there are signficant differences between the new LD-helix and the α-helix. Firstly, the successive NH···O hydrogen bonds are in directions alternately up and down the direction of progress of the helix in the new structure, while they are all in the same direction in the α-helix. Secondly, the two types of hydrogen bonds are not exactly equal, although both have satisfactory lengths and angles, as shown in Fig. 8. For the same reason, the strictly repeating unit in this helix is an LD pair, rather than a single residue. Therefore, in what follows, we shall use the number of LD pairs to denote the number of units (n) per turn of the helix, rather than the total number of residues per turn.

It is found that the helix can be either right-handed or left-handed, and that the two are mirror reflections of each



Helix Parameters

Number of units per turn (n) = 3.2

Unit height (h) = 1.55 Å

	ϕ	ψ
L-C^{α}	70°	304°
D-C^{α}	320°	52°

Hydrogen bonds

	Length	Angle
(1)	2.98 Å	17°
(2)	2.88	20

Fig. 8. Perspective diagram of a typical conformation of the right-handed LD-helix.

other. The dihedral angles at the L and D α-carbon atoms are both in the low energy region of the conformational map for a dipeptide unit. This, combined with the fact that every hydrogen bond can be made, would be expected to lend a great stability to this helix. However, the diameter of the helix is approximately 7.7 Å (for the C^{α} atoms), which is rather large, and it can therefore accommodate large alkali ions inside the helix (see also Section 6).

(d) It is quite likely that gramicidin A, which contains a repeating sequence of one LG and six LD pairs, may conceivably also occur in the form of this helix having about two turns, in which case it can accommodate ions inside the peptide ring. What is more, the exposed NH and CO groups on the top and bottom of such a two-turn helix might themselves be hydrogen-bonded with the correspondingly exposed groups of another helical fragment, so that such helical discs might stack themselves one over the other forming a cylinder by loose association via hydrogen bonds. At the same time, the cylindrical pack could enclose a large amount of inorganic ions within the central hole. It would be worthwhile examining whether such a phenomenon actually occurs with gramicidin A, or with any other suitable synthetic analogue.

Incidentally, the LD ribbon also has associating properties, and it would be possible for the ribbons to lie flat one on top of another, and be attached by NH•••OC hydrogen bonds of the peptide groups in the bends, which will be at right angles to the plane of the ribbon and of the right length to fasten the ribbons together. It would be interesting to examine if long peptide chains with repeating sequences of LD pairs would crystallize in this fashion.

(Dr. A.V. Lakshminarayanan materially assisted in making the calculations and building the models reported in this

section.)

5. ION-BINDING AND ION-TRANSPORT CYCLIC PEPTIDES. In this section, we shall consider two examples of cyclic peptides having repeating LD pairs which possess properties of transporting ions, namely enniatin and valinomycin. We shall discuss the structure of enniatin in particular detail and give a general picture of the method by which it binds ions and releases the ions under suitable conditions. The picture will then be extended to valinomycin.

(a) Enniatin. Enniatin is a cyclic depsipeptide containing six residues. Three modifications of this have the following sequences:

Enniatin A: $\overline{\text{(LMeIle-DHyv)}_3}$

Enniatin B: $\overline{\text{(LMeVal-DHyv)}_3}$

Enniatin C: $\overline{\text{(LMeLeu-DHyv)}_3}$

All three have alternating L-peptide units and D-ester units, the former being always N-methylated but with different side chains, while the latter is throughout associated with D-hydroxyisovaleric acid, abbreviated Hyv. As an initial step in the study of the conformation of this antibiotic, the peptide and the ester groups were taken to have the same dimensions as far as the backbone chain C^{α}-C-X-C^{α} is concerned. It is then found that cyclization can be attained by having the dihedral angle ϕ anywhere between 60° and 130°. The corresponding value of ψ for which the ring is closed can be worked out. A set of values at intervals of 5° for ϕ are shown in Table 2A, and the structure is schematically represented in Fig. 9. The structure has the symmetry of a 6-fold rotation-reflection axis -- that is, each unit can be rotated by 60° about the axis through the centre and the next obtained by then reflecting it in the plane of the paper. The oxygen atoms of the carbonyl group point towards the inside of the ring formed by the units and they are alternately above and below (so that three are above and three below), the six of them being roughly at the corners of an octahedron. The distance from the centre to the oxygen atom, indicated by the symbol R(M-O), which are the lower limits for the binding of the various monovalent alkali ions, are given in Table 2B. The radius of the circle passing through either the top three, or the bottom three, oxygen atoms in the structure, as given in Fig. 9, is indicated by the symbol r(O) in Table 2A. The value of r(O) indicates whether the ion will be able to slip out of, or into, the space between the three oxygen atoms, while r(M-O) gives the distance between the centre of the ion to all the six oxygen atoms, when it is bound inside symmetrically. The data given in the last two columns of Table 2A, namely which ions are bound and which ions can be released by the antibiotic, are obvious from the data on r(O) and r(M-O), on comparison with Table 2B.

Table 2A also contains the energy of the peptide chain for enniatin B and enniatin C which contain NMeVal and NMeLeu respectively. This energy has been calculated using conventional conformational theory (1), but does not contain the contribution from the electrostatic attraction between the metal ion and the oxygen atoms. These data regarding the energy of the two peptides for different conformations, which will be specified by

Table 2A. Variation of Energy and Ion-Binding Properties of Enniatin with Dihedral Angle Phi.

ϕ	ψ	r(0)	r(M-0)	V(kcal/mole) NMeVal	V(kcal/mole) NMeLeu	Atom* Release#	Atom* Bind†
70°	305°	3.00Å	3.50Å	-3.9	-5.0	Na,K,Rb	Cs
75	308	2.90	3.40	-4.3	-5.1	Na,K	Rb,Cs
80	311	2.75	3.25	-3.9	-5.3	Na	K,Rb,Cs
85	314	2.65	3.15	-2.9	-5.2	Na	K,Rb
90	317	2.55	3.05	-1.2	-5.0	Na	K.Rb
95	320	2.45	2.90	1.0	-4.6	--	Na,K
100	322	2.35	2.80	4.0	-4.2	--	Na
105	325	2.25	2.65	8.4	-3.5	--	Na
110	328	2.15	2.55	12.4	-2.8	--	Na
115	331	2.05	2.45	15.2	-2.5	--	--
120	334	2.00	2.35	18.6	-1.2	--	--

* Lithium is not listed. It is not bound even at $\phi = 120°$.
\# r(0) > R(M-0). † r(M-0) > R(M-0).

Table 2B. Radii of Atoms Involved in Ion-Binding of Enniatin.

Atom	Radius R	R(M-0)#
Li*	0.60Å	2.1Å
Na*	0.95	2.5
K*	1.33	2.85
Rb*	1.48	3.0
Cs*	1.69	3.2
0†	1.52	--

* R = Ionic radius
† R = Van der Waals radius
\# R(M-0) = R(M) + R(0), rounded off to 0.05Å

the angle ϕ, suggest a possible mechanism of action of the peptide in transporting ions, and also, at the same time, the reason why enniatin B containing NMeVal transports K^+ ions preferentially, while enniatin C with NMeLeu behaves differently, in transporting Na^+ and K^+ with equal ease (10). We shall briefly mention the theory, but without details, which are reserved for a fuller publication.

The electrostatic energy depends both on the distance between the central positive ion and the negatively charged oxygens, and on the charges held by these atoms. Assuming the charge of the metal positive ion to be one electron unit, the charge on the negative oxygen atom is approximately 0.5 unit, but this may vary according to the ionization of the medium, and other circumstances. The electrostatic attraction between the two may also be affected by the medium. Thus, in the absence of the metal ion, enniatin B will have a conformation corresponding to a small value of $\phi \approx 75°$ for which it has minimum energy. This permits both sodium and potassium ions to enter into the space inside the cage formed by the oxygen atoms. As soon as the ion enters inside, there is an appreciable attraction between the opposite charges of the ion and the oxygens, which makes the oxygen atoms close around the metal ion, decreasing r(M-0) and increasing ϕ. Although this leads to an increase in the energy of the peptide chain as such (see

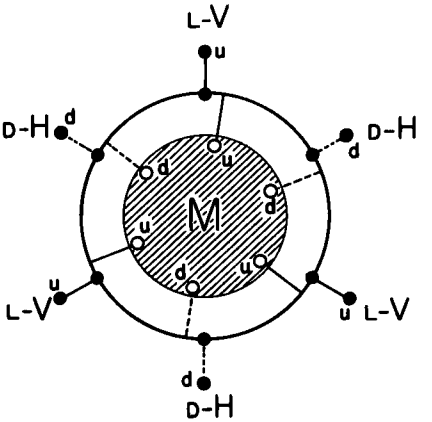


Fig. 9. Schematic representation of the structure of the enniatin backbone. (●= α- and β-carbon atoms; o= carbonyl oxygens; M=metal ion) The α-carbon atoms are all nearly in the plane of the paper, while the oxygens are alternately above and below.

column 5 of Table 2A), this is compensated by the increase in electrostatic attraction, so that we may expect bound structures with $\phi \approx 95^{\circ}$ for K^+ and $\phi \approx 110^{\circ}$ for Na^+.

However, the situation is quite different for enniatin B and C, when it comes to the release of the ion. A rough estimate of the energy of attraction between the metal and oxygen ions is of the order of 10 to 15 kcal/mole for the interaction between Na^+ and K^+ and one O^- atom. If column 5 of Table 2A is examined, it will be seen that there is a difference in energy of about 5 kcal/mole between the fully binding and releasing states of the peptide for K^+ ions, while this difference in energy is more than 13 kcal/mole for Na^+ ions, for enniatin B. It is suggested that the release of the ion takes place by some change in the system which reduces the ionization of the carbonyl oxygens and appreciably reduces the electrostatic attraction between the metal ion and the carbonyl oxygens. Then K^+ would be expected to be much more readily released than Na^+. It appears that enniatin B does transport K^+ ions more readily than Na^+ (10). On the other hand, on examining the data in column 6 of Table 2A referring to enniatin C, the difference in energy between the binding and the releasing states of the peptide are found to be quite small (<3 kcal/mole) for both Na^+ and K^+. Hence, the difference in the transport of these two ions is not expected to be as striking as for enniatin B, which is what is observed. (See p. 190 and Table 3 of Ref. 10).

It might be mentioned that the difference in the variation of the peptide chain energy with ϕ between enniatin B and C arises essentially because of the branching of the side chain at the β-carbon atom in B and the absence of this in C. This is further confirmed by the fact that enniatin A, which has NMeIle, behaves in a manner similar to enniatin B with NMeVal (10), since both Ile and Val have branched β-carbon atoms.

The structure described above from stereochemical considerations for enniatin is in good agreement with the preliminary data obtained from X-ray diffraction studies by Dunitz and coworkers (11). The energy values listed in Table 2A are not very accurate, particularly for enniatin B in the region of

$\phi > 90^{\circ}$. We shall not consider the theory in any more detail, except to state that conformational energy calculations can be expected to lead to a reasonable explanation of the ion-transporting behavior of the enniatins and their analogues. Many of the results in Table 2 of Ref. 10 can be explained, but the details are reserved for separate communication. For instance, the fact that Li^+ is not bound by any of the enniatins is clear, since ϕ must be greater than 120° for the bound state, for which the peptide energy will be highly positive and unfavorable.

The question whether the ester unit Hyv may also be replaced by an N-methyl peptide unit has not been fully answered by our studies. However, it appears that the ester unit has a greater flexibility than the N-methyl peptide unit, so that three of the oxygens may attach themselves more readily to the metal ion first, and the three others may then fold over them and close up, like the lid of a box. For this reason, $\overline{\text{[(DMeVal-LMeVal)}_3\text{]}}$ would very likely not exhibit ion-transport properties.

(b) Valinomycin. Unlike enniatin, valinomycin contains twelve residues and does not contain any N-methyl amino acid residues, although it is a depsipeptide and contains alternating peptide and ester units. The sequence of the residues is also of the type LDDLLDDL..., unlike the sequence LDLD... for enniatin. In fact, the structure may be denoted by the sequence $\overline{\text{[(LDDL)}_3\text{]}}$, in which the residues, in sequence, are LVal, DHyv, DVal and LLac.

Disregarding for the case of the backbone structure the difference between a peptide and an ester unit, the nature of the conformation of the cyclic dodecapeptide $\overline{\text{[(LDDL)}_3\text{]}}$ is as shown in Fig. 7 of Section 4. As discussed therein, it has the shape of a narrow cylinder formed by the ribbon. The units are folded up and down in the ribbon, but each pair is twisted in the same sense about the axis of the cylinder for every bend, whether of the LD or the DL type. Since four residues form the repeating unit of this chain, four pairs of dihedral angles $(\phi_1,\psi_1),\ldots,(\phi_4,\psi_4)$ are necessary for defining its conformation. However, it is reasonable to assume that the structure has an approximate 6-fold rotation-reflection axis, since the DL and LD bends are related by a reflection symmetry, so that we may take $(\phi_3,\psi_3) = (-\phi_1,-\psi_1)$ and $(\phi_4,\psi_4) = (-\phi_2,-\psi_2)$. With this restriction, there are only four dihedral angles, corresponding to two of the residues, that have to be specified. Even so, the variations possible in the values of these dihedral angles, which would still make the resultant structure close up and have the required symmetry, are very large. Therefore, only a representative set of values of these angles are given here in Table 3, which would correspond to an M-O distance for a bound alkali ion of approximately 2.9 Å, corresponding to the K^+ ion which is bound by valinomycin. As will be seen from Fig. 7, this binding is very similar to what exists in enniatin (Fig. 9), three oxygens being above, and three below, the metal ion. However, the radius of the oxygen atoms is only about 1.9 Å, which is appreciably smaller than the corresponding value in enniatin of the order of 2.4 Å, when binding potassium, so that the oxygen cage is narrower, but taller than in enniatin. Since the peptide units are not N-methylated, NH···O hydrogen bonds occur systematically along the length of the ribbon and they serve to keep the ribbon rigid in the form of a cylindrical ring. The

Table 3. Typical Data for the Conformation of the Valinomycin Ring Structure.

ϕ_1	ψ_1	ϕ_2	ψ_2	N...O	NH∧NO	r(O)	r(M-O)
108°	280°	271°	183°	3.04Å	16°	1.95Å	2.8Å
112	276	273	185	2.89	14	1.9	2.9
112	282	271	187	2.88	13	1.9	2.85

hydrogen bond length of the structure reported in Table 3 is satisfactory for peptide NH···O bonds.

During the binding and release of the potassium ion, conformational changes should obviously occur in the peptide units. Such changes are readily possible in the valinomycin structure, although the details are difficult to assess with a simple theory of the type presented here. However, it may be mentioned that the values of (ϕ_1,ψ_1), (ϕ_2,ψ_2) reported in Table 3 agree reasonably well with the preliminary X-ray crystallographic data of Steinrauf and coworkers, which was kindly made available to the authors for reference by Dr. Steinrauf (Indianapolis). Preliminary calculations and model-building examinations indicate results of the following types for the valinomycin analogues:

(i) It is not possible to have a closed ribbon-like structure with $\overline{\text{(LDDL)}_2}$ cyclized and having a good binding of ions.

(ii) Also the structure of $\overline{\text{(LDDL)}_3}$ for which the distance r(M-O) is smaller than 2.9 Å has unfavorable energy, so that Na^+ ions are not likely to be firmly bound by valinomycin, as observed (see e.g. Ref. 10).

(iii) $\overline{\text{(LDDL)}_4}$ can have the shape of a cylindrical ribbon, but the oxygen cage would be a little too large for K^+, so that its binding would be rather small, as observed (10). The eight oxygen atoms would be disposed four above, and four below, the centre, the two squares formed by the two sets of oxygen atoms being twisted with respect to each other by 45° about the common cylindrical axis.

It would be worthwhile trying to calculate the chain energy of valinomycin by theoretical methods for different values of the dihedral angles and thus try to obtain the minimum energy conformation for this peptide. It is likely to be not easy, since there are several uncertain factors involved such as the metal-oxygen binding energy and so on. However, a few of the facts observed by Merrifield et al. (12) in connection with synthetic analogues of valinomycin seem to be theoretically understandable. Thus, denoting the sequence DVal-LLac by A and LVal-DHyv by B, Merrifield found that the peptide B-A-B-A-B-A showed appreciable ion permeability, comparable with the cyclic peptide valinomycin, namely $\overline{\text{B-A-B-A-B-A}}$. This is obviously due to the fact that the open peptide has a twist in the same direction for both the combinations A and B as shown in Fig. 7, so that the resultant sequence would be hydrogen-bonded as a ribbon curved into a form very similar to the cyclic structure. On the other hand, if we take the sequence -B-B-B-A-A-A-, whether cyclized or open, this does not have all the oxygen atoms pointing in toward the axis of the cylinder formed by the ribbon.

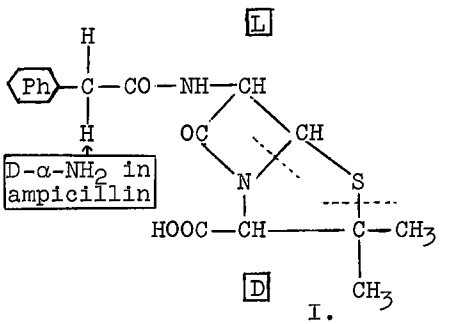
Only four of the six oxygen atoms are pointing inward, while two will be pointing outward (figure omitted). As a consequence, the capacity of this oxygen cage to hold an ion like K^+ is more limited than for valinomycin, although the size of the cage could be expected to be suitable for this purpose. So the data reported in Table 1 (p. 218) of Ref. 12 becomes understandable on the basis of conformational theory.

Thus conformational theory, properly applied, is seen to lead the way for an understanding of ion transport by peptides and depsipeptides.

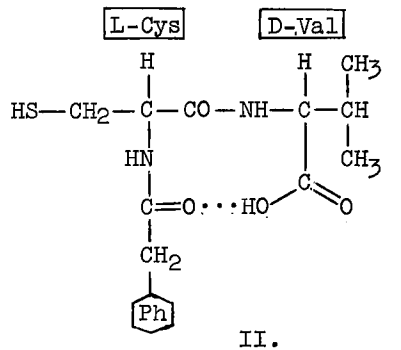
6. MECHANISM OF ANTIBIOTIC ACTIVITY.

(a) D residues and LD bends. It is an interesting fact that almost all antibiotics contain D amino acid residues, and also unusual side chains in which the racemization has led to an interchange of the positions of the α-hydrogen and the side chain group at the D α-carbon atoms, but have not otherwise changed the absolute configuration of the side chains (8). It would be interesting to speculate on how the occurrence of the LD sequence lends these peptides with special antibiotic activity. We shall consider this with special reference to the occurrence of the LD or DL bend. It is well known that several antibiotics, including penicillin, interfere with some mechanism involving short peptide chains having a sequence of alternating L and D residues, which cross-link the polysaccharide chains in the cell wall (13). It is very likely that the process involved requires the recognition of the LD (or DL) bend in the peptide by the enzyme, in which case, it could be competitively inhibited by the LD bend of the antibiotic, by its attaching itself to the same site in the enzyme. That this mechanism is reasonable is shown by the fact that, although penicillin itself (I), in its natural state, does not contain this bend, on reduction it has the structure II, which has the typical LD bend stabilized by a hydrogen bond across it. The absolute configurations of L-Cys and D-Val are readily deduced from the crystal structure determination of D-α-aminophenylacetamidopenicillanic acid (ampicillin) reported by Hodgkin and coworkers (14).

It is not suggested that this particular mechanism is necessarily valid for the action of penicillin, but the idea is to indicate such possible types of mechanisms that may be considered in connection with the activity of antibiotics which have LD sequences, or structures that could lead to such sequences. One



I.



II.

should look for the occurrence of similar LD sequences in some portion of the biological system on which a suitable enzyme acts, and which is necessary for the biological activity of the system concerned, which could then be inhibited by the antibiotic. Thus one sees in the action of these materials a much more general significance for the term "antibiotic", meaning "leading to action against the normal biotic activity", rather than the restrictive sense in which the term "antibiotic" is used in chemotherapy, namely to denote the destruction of disease-producing organisms. If the study of antibiotics is generalized in this manner, it is very likely that the studies can lead to more significant results, which would be of value even in such fields as anti-carcinogenic action and so on. (We are grateful to Dr. M. Bodanszky for discussions on some of the aspects discussed in this paragraph).

(b) Repeating sequence of L and D residues. We have seen that a chain having alternating L and D residues can have two highly stable conformations -- namely the LD or DL ribbon and the right- or left-handed LD helices. The latter can bind ions, and presumably gramicidin A does have this conformation when it binds ions. Whether the ion-binding property itself may lead to antibiotic effects is a question which is not quite clear. If so, it can explain the antibiotic behavior of examples like enniatin, valinomycin and gramicidin A.

On the other hand, the LD or DL ribbon has a number of hydrogen-bonding CO and NH groups exposed at regular intervals along its length. As mentioned in Section 4, this structure can bind to some sequences of base pairs in the DNA chain and also attach itself by H-bonds to phosphate groups on either side of the base binding site. This possibility of the repeated LD sequence having a strong tendency to bind itself to nucleic acid chains may be an operative factor which inhibits biological processes such as protein synthesis or DNA replication, and thus lead to antibiotic activity. A few examples of antibiotics binding to specific sequences of the DNA chain are known. For instance, actinomycin complexes specifically with guanine in DNA (15). Therefore the stereochemistry of such associations between peptide chains and nucleic acid chains is worthy of further detailed study.

(c) Synthesis of artificial antibiotics. Since we now have a reasonable understanding of the mechanism of action of peptide antibiotics, we may briefly speculate on the types of synthetic analogues which may be expected to be active and the method of synthesizing them.

(i) We have seen that a single LD bend found in several antibiotics is quite likely to be the site responsible for their activity. Therefore various cyclic peptides having one or more LD (or DL) sequences are suitable materials for the study of antibiotic activity. In these examples, the use of the special sequence of LPro attached to a hydrophobic D residue would be particularly interesting as the bend would then be very stable. The sequence DPhe-LPro is found in gramicidin S and in tyrocidine.

(ii) Synthetic analogues of gramicidin A would also be interesting since they could take up the LD ribbon structure, which apparently has the property of binding to nucleic acids. From what has been stated in Section 4 about attempts to synthesize

peptides by the Merrifield method, it is obvious that long chains of this type cannot be readily synthesized by adding alternately L and D residues, as cyclization is likely to occur at about six residues. Therefore, it would be necessary to imitate the process by which the D residues are synthesized in nature, which is by the racemization of L residues (8). For instance, if a polypeptide is made composed of a repeating sequence of a dipeptide containing amino acids A and B, say having the sequence ABAB..., in which all the residues are of the L configuration, and then if the B residues alone were racemized to take up the D configuration, then it would be possible to obtain the sequence LA-DB-LA-DB..., which would be an excellent synthetic analogue of gramicidin A. Many of the conformational features of repeating LD sequences discussed in this paper could conceivably be tested by making use of such analogues. The enzymology of racemizing enzymes and their specificity would therefore deserve special study with the view of synthesizing new antibiotics composed of L and D peptide units which may be tailor-made to have required properties.

In conclusion, there seems to be every hope that it will be possible to imitate nature and produce artificial compounds containing peptides which can be used in a variety of ways such as for destroying bacteria and other pathogens, in regulating biological processes and even in counteracting uncontrolled proliferation of cells in tumors.

Acknowledgements

We would like to thank several colleagues in Chicago, and also various persons with whom discussions were carried out during the Cleveland symposium, for their encouragement and for their critical comments on the various ideas proposed here. In particular, we wish to thank Dr. E.R. Blout for discussions on biologically active cyclic peptides, which stimulated our studies on the enniatin- and valinomycin-like compounds. We are very grateful to the USPHS for Grant No. AM 11493 and the Jane Coffin Childs Memorial Fund for a special grant to G.N.R. which made these studies possible.

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STUDIES OF SELF-ASSOCIATION AND CONFORMATION OF POLYPEPTIDES

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Ever since the tetrahedral nature of the carbon atom was proposed stereochemistry has played a major role in the development of our structural concepts in organic chemistry. For the study of most of the smaller molecules the relatively rigid directional bonds permit the possible spatial arrangements to be assigned with certainty by classical methods except for the cases where there is free rotation about the carbon bonds. Even for this problem the newer spectroscopic techniques such as nmr now permit accurate assessment of the probability of the different rotamers.

Modern organic chemistry, however, is no longer restricted to the precise study of small molecules. Highly effective separation techniques make available well characterized pure preparations of natural products such as polypeptides, proteins, nucleic acids, etc. Precise assignments of the covalent structures of many such substances are to be found in the literature, even though their molecular size and complexity is 10, 100 or 1000 times the size of molecules usually considered to be small. For these substances the stereochemistry becomes very complicated because of the almost limitless possibilities and because the forces acting on each segment of the molecule vary so widely. For such substances the term stereochemistry has been largely replaced by the term "conformation." The study of conformation has rapidly grown in importance in biochemistry because it is realized that the selective catalytic action and "recognition" sites of biologically active substances depends on, or is related to the conformation or spacing of certain structural groups as well as on the nature of the groups.

Not only are these substances available in high purity from natural sources but synthetic organic chemistry has recently been improved to the point where they are becoming available by synthetic means. There is thus great need for more precise methods for studying the stereochemistry of large molecules, most of which are built up as covalently bound chains containing ordered sequences of a relatively few small structural components such as the amino acids or nucleotides.

The shape of these chain-like large molecules can be determined primarily by covalent cross links but also by the noncovalent intrachain interaction of groups that "recognize" each other. The latter can be influenced by the solvent environment, as is well known. Another possibility concerns

a specific interaction with a site or sites on a neighboring molecule of the same species. This then results in self-association. Obviously once the covalent structure of a large molecule becomes assigned, the next step in a complete understanding of the structure in solution requires an understanding of both the conformation and association behavior.

Many different methods for studying conformation and association behavior have been under investigation. The following incomplete list contains methods well known and a few less well known for the purpose:

1. X-ray diffraction
2. The electron microscope
3. Sedimentation in the ultracentrifuge and diffusion
4. Birefringence of flow
5. "Melting out curves"
6. Countercurrent distribution
7. Thin film dialysis
8. Rotary dispersion and circular dichroism
9. Proton exchange
10. Fluorescence
11. Nuclear magnetic resonance
12. Energy minimization calculations

The first member of this list gives information only on substances that crystallize in forms suitable for study but for these substances the results seem conclusive and cannot be challenged by the other methods. Although the major more rigid features of the conformation shown in the crystal seem to hold when the solute is placed in solution there is still room for conformational change under the influence of the solvent. Considerable speculation on this point can be found in the literature with evidence both for and against the thesis (1, 2, 3) depending on one's viewpoint. The most certain view to take is that the conformation shown to be present in a crystal may be essentially the same as in solution but not necessarily so. There is now ample evidence that the conformations of many large solutes are highly sensitive to a change in the solvent environment.

In this paper no attempt will be made to discuss all the major methods for studying conformation and association behavior. Rather than attempt to do so a number of observations made and approaches developed during the past twenty-five years in the Authors' laboratory will be discussed. These deal chiefly with the cyclic antibiotic polypeptides which happen to be excellent models for testing many of the concepts and methods used for studying conformation and association behavior. The covalent structural formulae of four representative types are given in figures 1 - 4. These are all compounds intermediate in size between simple compounds and the more complex proteins. The cyclic structures greatly restrict the numbers of conformational forms possible in linear peptides.

The simplest of these is gramicidin S-A. When it was first isolated and shown to have five different amino acids in a unique sequence (4) the true molecular size was not certain (5). It was shown unequivocally to be a decapeptide by the partial substitution method (6) and countercurrent distribution (CCD). The uncertainty was much greater in the case of the tyrocidines, figure 2, because in contrast to the behavior of gramicidin S-A (which really is a tyrocidine) they show a strong tendency for self-association. Again the method of partial substitution (7)

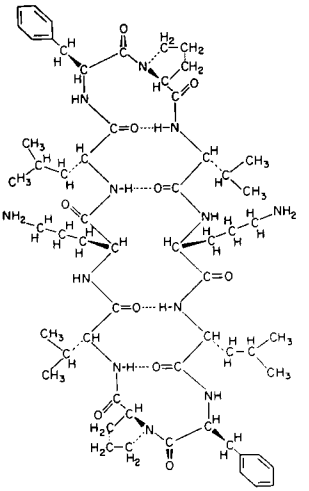
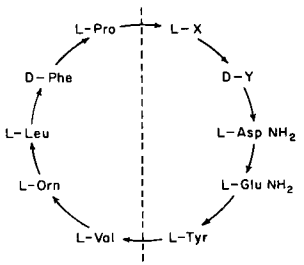


Fig. 1. Structural formula of gramicidin S-A



Tyrocidine	Residue X	Residue Y
A	Phe	Phe
B	Try	Phe
C	Try	Try

Fig. 2. Covalent structural formulae of the tyrocidines

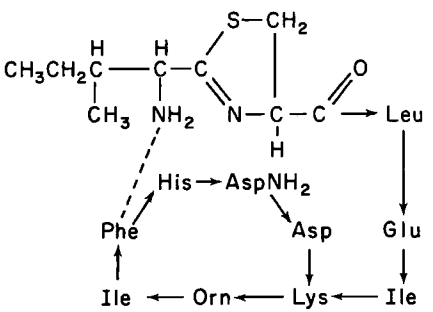


Fig. 3. Covalent structural formula of bacitracin A

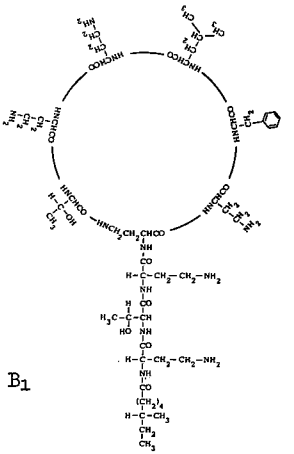


Fig. 4. Covalent structural formula of polymyxin B_1

$$A = A_0 (1 - r/R)^2$$

Fig. 5. Drawing illustrating the probability of different sized particles entering a pore. A = free cross-sectional areas, A_0 = cross-sectional area of pore, r = particle radius, R = pore radius

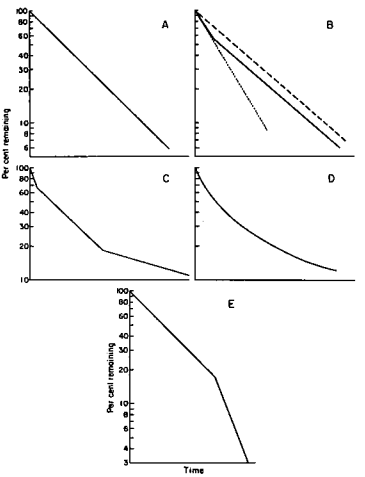


Fig. 6. Typical thin film dialysis escape plots. A = a single solute behaving ideally, B = a mixture of two diffusional sizes ..... and - - - whose sum gives ——, C = a mixture of three diffusional sizes, D = a mixture of several diffusional sizes or a solute behaving nonideally, E = a solute showing self-association

and CCD unequivocally showed the basic unit to be a decapeptide. Later the self-association behavior was thoroughly studied by CCD (8) and by the ultracentrifuge (9).

With the larger molecules problems of overall shape and association-dissociation have been classically studied by free diffusion methods. In the study of intermediate sized solutes a possible way of enhancing the differences revealed by free diffusion came to our attention (10, 11, 12). This approach employed the principle of restricted diffusion through cellophane membranes by a method called "thin film dialysis." It requires that the "porosity" of the membrane be adjusted to a critical size for the range of solute size to be studied. Experimental conditions are then adjusted so that relative rates of diffusion through the membrane caused solely by a concentration gradient depend only on the probability that the particle enter the pores. It can be seen from the hypothetical situation of figure 5 that as the size of particle approaches the limit of pore size the probability of it entering becomes much less and the size discrimination increases. Since these conditions imply very slow diffusion per cm^2 of membrane the overall rate must be increased by a large membrane area/retentate volume. Hence the name "thin film dialysis."

Through the study of rigid models well characterized by x-ray diffraction such as the homologous cycloamyloses (12) the limit of discrimination for the thin film dialysis technique has been shown to be about 2 - 3% of the effective diffusional diameter of the molecule.

Since we now know from nmr studies that molecules of this size are tumbling at the rate of 10^{-9} to 10^{-11} times per second the longest cross section of the molecule would be expected to be the controlling dimension and a spherical molecule would have a considerably smaller diffusional diameter than an asymmetrical one of the same volume. The thin film method therefore should be very sensitive to conformational changes caused by changes in temperature or solvent environment. Since the method was first proposed over ten years ago much data have been collected all of which support such reasoning. By comparative studies with solutes of known size and conformation reliable estimates of diffusional size can be made. This of course does not give information as to degree of asymmetry versus tightness of packing. Thus when the conformation of a molecule is perturbed by a change of solvent conditions it may retain its overall shape but become larger or smaller in size. This could not be distinguished from a change in assymmetry by the thin film method.

In studying changes of conformation there is always the troublesome problem of possible changes in hydration or solvation. Any firmly bound solvate naturally contributes to the diffusional size of a molecule. Similarly self-association causes a slower diffusion than would be expected from an ideal monomer. However, in the case of self-association strong concentration dependence is usually noted. The thin-film method is especially useful in this case for the reason that the results of the thin film method are interpreted by a plot, figure 6, showing the rate of escape. An ideal pure solute gives a straight line but a mixture of solutes of different diffusional size shows deviations. A characteristic deviation for self-association is that of reverse curvature. Ways of confirming the suggestions implied by the different types of curves have been well described in the literature (13). A combination of results from the ultracentrifuge and the dialysis technique are particularly informative. Gramicidin S-A, figure 1, has behaved as an ideal solute

throughout extensive investigations. On the other hand the tyrocidines are all non-ideal both in the ultracentrifuge and in thin film dialysis. The rate is anomalously influenced by a change in concentration, temperature and the addition of hydrophobic bond breaking additives to the solvent (14). In figure 7 the effect of alcohol is shown. The dissociating effect of alcohol gives a faster diffusion rate. It is interesting that in the absence of alcohol a reasonably straight line is obtained which is about the same rate found with this membrane for the diffusion of insulin. Insulin strongly dimerizes under these conditions and diffuses as a solute of molecular weight approaching 12,000. This indicates that the preferred n-mer size of the tyrocidine micelle contains about 8 monomers, a conclusion recently supported by an extensive ultracentrifuge study (15). The effect of changes in concentration and temperature for three tyrocidines is shown in figure 8.

With a basic knowledge of the overall shape of the molecules and the effect of changing solvent at hand a better basis for inquiry into the conformational details of individual parts of the molecule can be made. Spectroscopic methods are the most promising for this purpose, particularly high resolution nmr.

Some of the possibilities are shown by the nmr study of gramicidin S-A (16) which seems to make certain the conformation shown in figure 1. Even the most probable side chain conformations have been revealed (17). The existence of the four cross-linking hydrogen bonds has been confirmed by an independent tritium-proton exchange study (18). The details of these studies are too lengthy to be presented here.

The details of a similar study with tyrocidine B will soon be published (19). It is a more complicated molecule than gramicidin S-A and shows an interesting concentration dependence in aqueous solution. As shown in figure 9 the well known phenomenon of dipolar line broadening eliminates the signals almost entirely except at very low concentration (20). Addition of methanol as shown in figure 10 makes a spectrum possible. Similarly the line broadening is reduced by raising the temperature, figure 11. These results are interpretable as being caused by the association-dissociation phenomenon so well documented by thin film dialysis and the ultracentrifuge.

It is interesting to ask if association causes a change in the conformation. The evidence we have been able to accumulate strongly suggests hydrophobic interaction (21) as the cause of the aggregation. nmr relaxation studies (19) indicated that water is bound to tyrocidine B in a way that is not influenced by the degree of association. On the other hand nmr relaxation studies with gramicidin S-A indicated the absence of bound water. With tyrocidine B there appears to be a certain time dependency in the association-dissociation as shown by both the ultracentrifuge (9) and thin film dialysis (14). This could be the cause of the straight line in the top curve of figure 7. Otherwise the result is not consistent with the results of figure 8. The time dependency could be caused by the existence of more than one metastable conformational form of the monomer, only one of which would have the conformation favorable to association.

In any case it appears that an n-mer micelle of about 8 monomers is first favored (15) and that the monomers are held together by the hydrophobic regions of the molecule but in such a way that a hydrophyllic region of each monomer remains exposed to the solvent and carries the single molecule

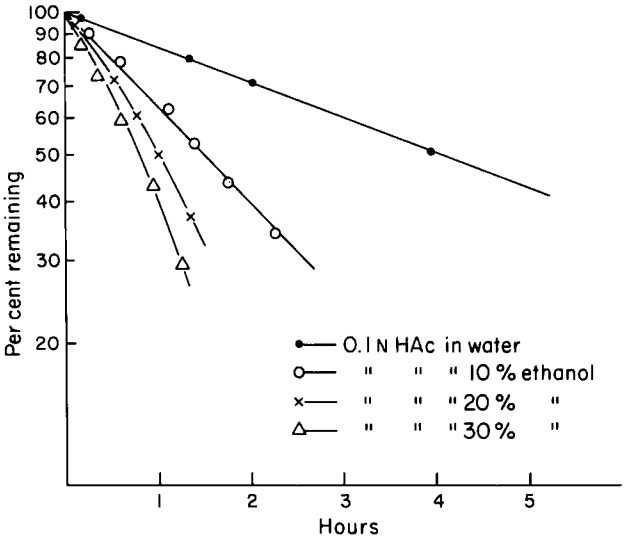


Fig. 7. Effect of ethanol on thin film dialysis escape plots of tyrocidine B

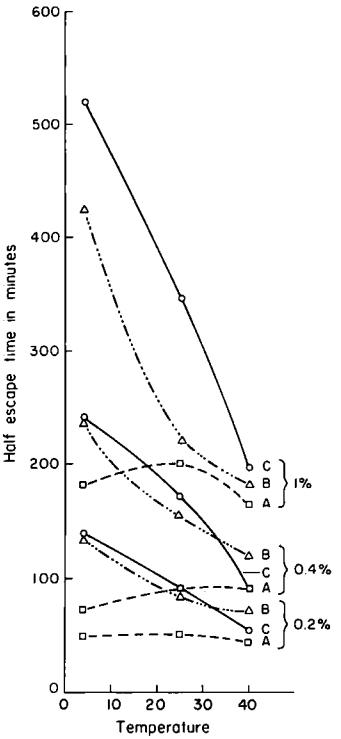


Fig. 8. Comparative effects of changes in concentration and temperature on the half escape times of tyrocidines A, B and C

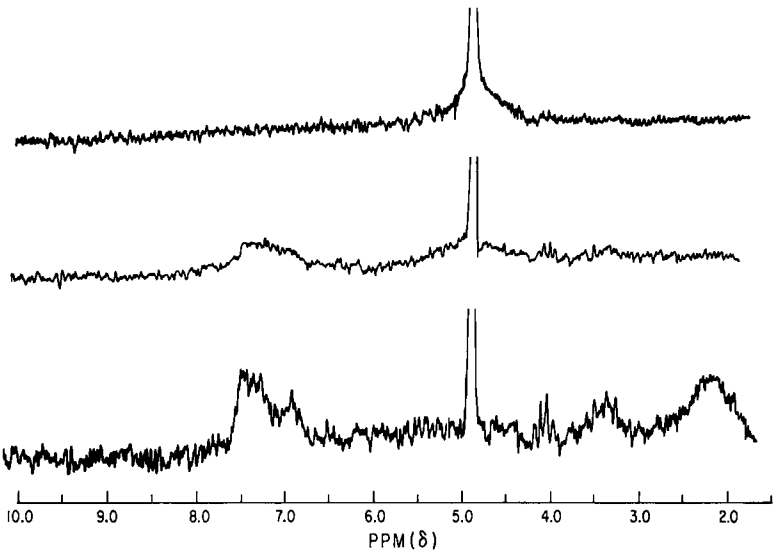


Fig. 9. Effect of concentration on 100-MHz spectrum of tyrocidine B in D_2O at 60°. Top pattern = 6%, middle pattern = 2%, bottom pattern = 1%.

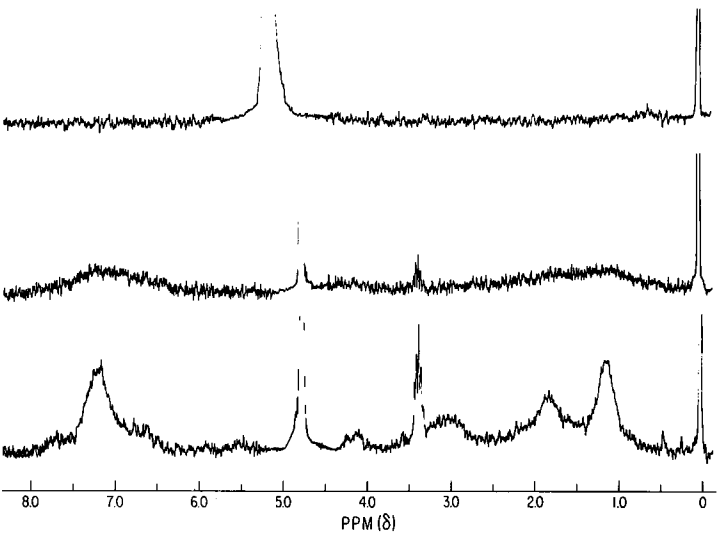


Fig. 10. Effect of methanol on 60 MHz nmr pattern of 8% tyrocidine B at 25°. Top = 100% D_2O, middle = 80% -20% CD_3OD, bottom = 50% D_2O - 50% CD_3OD.

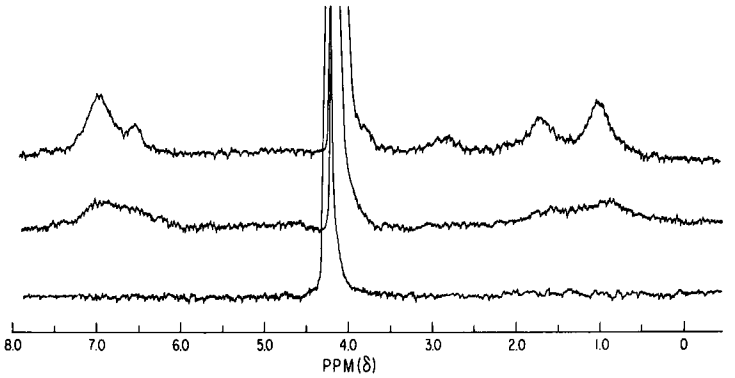


Fig. 11. Effect of a change in temperature on the 60 - MHz nmr spectrum of 8% tyrocidine B in D_2O. Top = 100°, middle = 80°, bottom = 45°

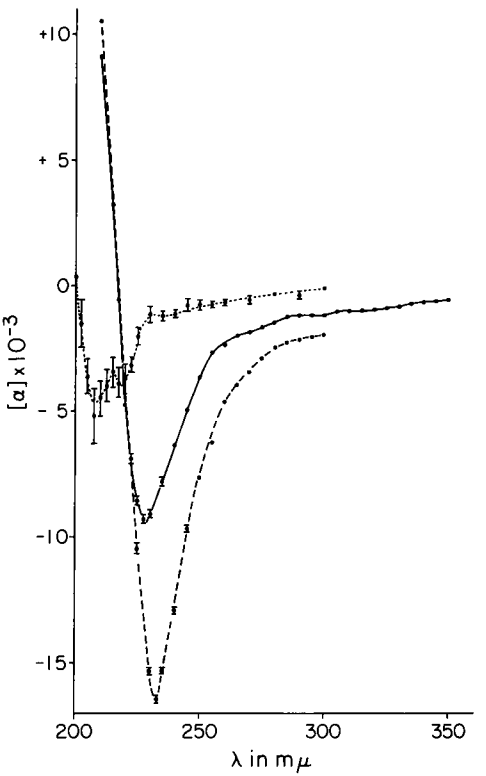


Fig. 12. Rotatory dispersion curves in H_2O. - - - = gramicidin S-A —— = tyrocidine B, ····· = linear tyrocidin B

of bound water. The nmr and ir spectra are sufficiently like those of gramicidin S-A to be quite certain that an antiparallel pleated sheet conformation is present.

Under certain conditions in which a higher concentration in aqueous solution is forced by dilution of a more concentrated alcohol solution with water a gel will be formed. This gel seems to have an interlocking system of fibrils when examined under the electron microscope (15).

With the above behavior of gramicidin S-A and the tyrocidines as a foundation it is interesting to consider their rotatory dispersion and circular dichroism spectra. The ord spectra shown in figure 12 was published from this laboratory in 1965 (22) and the cd spectra (23) later was shown to be entirely consistent with the ord spectrum. These results are highly significant because following a suggestion of Simmons and Blout (24) this type of spectra have become widely accepted as due to a helical conformation.

Numerous papers have been published with proteins, polypeptides and nucleotides in which the degree of helicity of the molecule has been estimated from such data.

The results shown in figure 12 and published more than five years ago should have served as a warning that this type of ord and cd spectra could be caused by structures other than a helix but little attention was paid to it and indeed strenuous attempts to were made in other laboratories to show that gramicidin S-A really was helical. This possibility is now ruled out and the flat structure originally proposed by Schwyzer (25) has been shown to be correct. One must now take the view (26) that the type of ord spectra shown in figure 12 can indicate helicity but not necessarily so unless other evidence such as x-ray diffraction studies confirm its presence. Quantitative deductions are also hazardous because the spectra may be caused by a very small region of the molecule showing an intense deviation.

By using the Corey-Pauling-Koltun models it is now possible to arrive at the dimensions of gramicidin S-A. These approximate 19 x 11 x 6A, values consistent with the x-ray data of Hodgkin and Oughton (27) at the 6 Å level of resolution. Using these dimensions as fixed points of reference it is now possible to estimate with considerable accuracy from thin film dialysis data the diffusional sizes of bacitracin A, figure 3, and polymyxin, figure 4. Here the 19Å dimension is the effective one for reasons given above. The data in table 1 were published in 1964 (28) and subsequent experiments have only confirmed these relative rates of dialysis.

Thus polymyxin B_1 should have a diffusional diameter approximating 25 Å which is too small a diffusional diameter if the polypeptide side chain ending in the isopelargonic group should be fully extended from the ring. On the other hand the ring fragment alone even with the side chains of the diaminobutyric acid residues fully extended cannot give a diffusional diameter of 19 Å. The data on the ring alone are consistent with it being as fully extended as the covalent bonds will allow. Nmr studies reveal the lack of a rigid compact hydrogen bonded structure in both the ring fragment and in intact polymyxin B. From the dialysis data the side chain appears to be at least partly folded back on the ring to give a more globular molecule than would be offered by one with the peptide side chain fully extended.

Table 1

Solute	Molecular Weight	T/2 (hr)*
Carbowax 1000	950-1050	2.4
Angiotensin I	1282	0.95
Tyrocidine B (sing split)	1348	2.1
Cyclohexaamylose	972	2.1
Polymyxin B_1	1208	5.6
Ring fragment of polymyxin B_1	762	1.4
Gramicidin S-A	1142	2.4
Bacitracin A	1420	1.8

* T/2, 50-percent escape time.

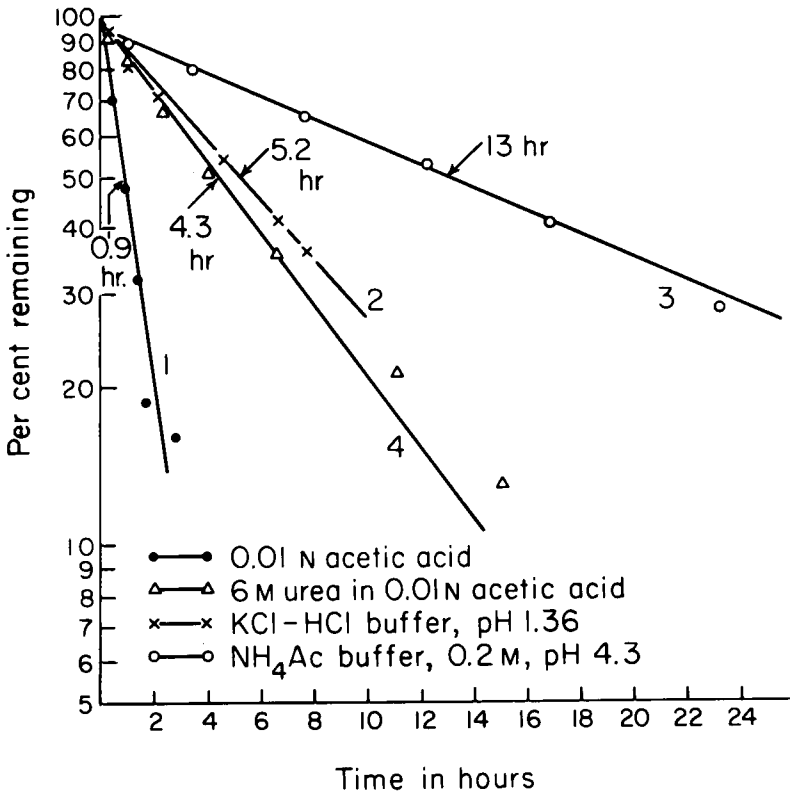


Fig. 13. Effect of the addition of salt or urea and change of pH on the diffusional size of β-ACTH

Bacitracin A offers interesting possibilities. Very early in the structural studies it was postulated from chemical behavior that the polypeptide side chain was folded back on the ring to bring the N-terminal isoleucine residue very close to the phenylalanine-histidine linkage. This would insure a rather globular structure. The thin film data of table 1 are consistent with this idea since its rate of dialysis is slightly higher than that of gramicidin S-A even though it is a dodecapeptide. Recently, data from a study of the binding of zinc to bacitracin A has confirmed this conformation (29). A single atom of zinc was shown to bind at two sites on the N-terminal region and at two sites on the histidine residue without significantly changing the dialyzability. A reasonably close packed globular molecule of slightly less than 19 Å diffusional diameter is indicated. Tritium-hydrogen exchange studies (30) and nmr have indicated at least one or more slowly exchanging protons depending on the solvent environment. Much nmr data on bacitracin A and polymyxin B_1 have been accumulated but the final interpretation is being reserved until still more data on these and model compounds become available.

A wide variety of linear polypeptides of different numbers of residues have been studied by the thin film method. These have been hormones or synthetic analogs from different sources and degradation products from protein. They have included the angiotensins, bradykinin, ACTH and parathyroid hormones. All these behave in 0.01 N acetic acid, salt free solution, at a pH approximating 3.3 and at 25° with the thin film dialysis method as if they were globular and of minimal diffusional size. Except for a few (the angiotensins and bradykinin) all show a marked increase in diffusional size when salt or urea is added to the solution as is shown by figure 13 with β-ACTH which contains 39 amino acid residues. They also give straight line dialysis patterns. Such substances have been spoken of as "random coils" since they do not show structure as detected by the ord and cd spectra ascribed to the helix or any of the denaturation phenomenom of proteins. Most of them are separable by CCD or other liquid-liquid partition systems. However, the thin film dialysis method seems to indicate that they do have a preferred conformation in a certain solvent environment. This conformation is easily perturbed by a relatively minor change in the solvent environment. It therefore seems doubtful if in a polypeptide there can be such a state as a true random coil.

In general the shorter polypeptides appear to be less perturbed than the longer ones by a change in solvent environment. This could be expected, reasoning from steric hindrance and the conformations allowed by energy minimization calculations. Angiotensin II and bradykinin definitely have a preferred conformation. Data have been presented to indicate that angiotensin has two interconvertible conformations (31, 32, 33).

With very short peptides the relative conformational contribution to the diffusional size becomes much greater. Thus in a study of dipeptides (14) striking differences have been found. In the data of table 2 which were collected with membranes of the same porosity it can be seen that the diffusibility of L-leucyl-L-tyrosine is not influenced by a change of solvent environment except by a pH that removes the charge on the α-amino group whose pK is 7.5. This apparently removes the constraint provided by the attraction of the charged amino group for the carboxyl group. The tyrosyl and leucyl side chains would be expected to be in close proximity because of their hydrophobic nature. This provides the most compact conformation possible.

Table 2

50% escape times in minutes for peptides in standard membranes at 25° and in different solvent environments

Solvent	L-Leu-L-Try	L-Tyr-L-Leu	L-Lys-L-Lys	L-His-L-His	L-Arg-L-Lys	L-Glu-L-Glu	L-Lys	L-Leu-L-Ser
	(40°)							
H_2O	50 22	48	100 *	53	124	52	20	
0.01 N HAc, pH 3.5	53 21	48	381	110	174	40		41
20% Ethanol, 0.01 N HAc	43 32	51	125	98	185	75		
20% Ethanol	44 42	44	93	44	107	105		
0.1 M NaCl	58 42		570	60	275	200		
0.01 N HAc, 0.1 M NaCl	58 40	48	590	150	170	110		
0.01 M PO_4, pH 8.5	100 53		206	68	120	430		104
0.01 M Borate pH 8.5	101 40		95	73 +	78 +	520		
0.01 M Borate pH 8.5 0.1 M NaCl	78 34		190	65	110	540		
0.01 M Tris, pH 8.5	115 94		109	138	76	420		
0.01 M PO_4, pH 8.5 20% Ethanol	95 93		115	110	148	220		

* Reverse curve

\+ Positive curve

In unpublished experiments it was found that L-tyrosyl-L-tyrosine behaved similarly. The diketopiperazine of L-tyrosyl-L-tyrosine, however, showed a slower dialysis rate by a factor of 1.5 than the dipeptide. The Corey-Pauling-Koltun models show that in this case the ring constriction prevents the two aromatic side chains from being closely associated. One must be extended away from the ketopiperazine ring and form a structure with a larger diffusional diameter. This interpretation is in accord with the nmr data of Kopple (34).

Examination of the data of table 2 will show that the dipeptides with charged side chains behave quite different than those with uncharged side chains. Thus L-lysyl-L-lysine shows a considerably slower dialysis rate than L-leucyl-L-tyrosine at the lower pH range. The conformational extremes possible with a dipeptide are shown in figures 14 and 15. L-leucyl-L-tyrosine apparently assumes the more globular compact one while L-lysyl-L-lysine assumes the more extended one. This is only to be expected because of the repulsive effect of the two positive charges on the ϵ-amino groups. The conformation shown still permits the α-amino and the carboxyl to be closely associated as in the case of the more compact form. Removal of the charge on the α-amino group by changing the pH to 8.5 markedly increases the dialysis rate of L-lysyl-L-lysine as contrasted to the behavior of L-leucyl-L-tyrosine. This indicates a partial collapse of the extended form.

With L-glutamyl-L-glutamic the data of table 2 show a similar behavior except that here a low pH promotes a more rapid dialysis and the higher pH with all the groups ionized promotes a slower dialysis.

The data with L-lysyl-L-lysine reveal another interesting feature. At the lower pH the dialysis plot showed a reverse curvature and concentration dependence. Salt slowed the dialysis but alcohol increased it. This behavior was shown by the tyrocidines and is characteristic of associating systems. On the other hand all the dipeptides without charged side chains showed ideal behavior. These results could suggest that the proper alignment and distribution of charges could play a role in intermolecular interaction and also in the intramolecular interactions which influence the conformation.

Some interesting comparisons can be drawn from thin film dialysis data obtained with a series of alanine and glycine peptides. The data are shown in table 3. These data were collected with the same membranes used for table 2. The longest and shortest cross sectional dimensions possible are given in table 3. These were measured from the scale molecular models when the conformation was in the fully extended form and when it was in the most compact form allowed by the models. Comparison of the half escape times for the di, tri and tetra peptides shows that the alanines follow the fully extended differences more closely. If the most compact form were present there should be little difference between the tri- and tetra-alanines. The dialysis data seem to indicate that the polyalanines of this length behave as rather rigid extended rods.

The glycines on the other hand should have much greater rotational freedom and therefore greater conformational mobility. The data in table 3 are completely consistent with this view. The behavior noted is also consistent with the theories of energy minimization (35, 36) and the allowed conformations so predicted.

In table 3 there is shown rather small differences in the half escape

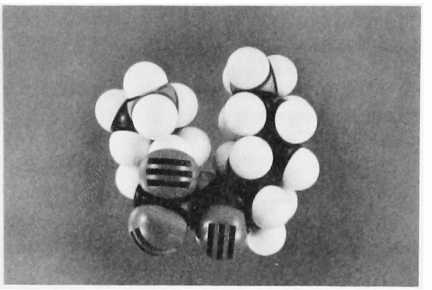


Fig. 14. Conformation most collapsed with Corey-Pauling-Koltun models for a dipeptide such as L-lysyl-L-lysine

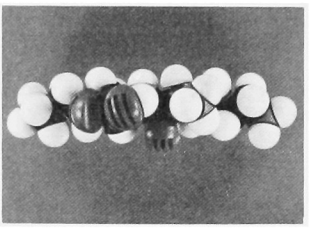


Fig. 15. Conformation most extended with Corey-Pauling-Koltun models for a dipeptide such as L-lysyl-L-lysine

Table 3

Comparative dialysis rates for a series of alanine and glycine polypeptides at 25°

Peptide	MW	50% escape times, min.	Longest axis in Å Extended	Collapsed
L-Ala	89	14	6.4	6.4
L-Ala-L-Ala	170	43	10.4	9.2
D-Ala-D-Ala	170	44	-	-
L-Ala-L-Ala-L-Ala	231	87	14	11.5
L-Ala-L-Ala-L-Ala-L-Ala	302	163	17	11.5
D-Ala-L-Ala-L-Ala-L-Ala	302	145	-	-
L-Ala-D-Ala-L-Ala-L-Ala	302	130	-	-
L-Ala-L-Ala-D-Ala-L-Ala	302	140	-	-
L-Ala-L-Ala-L-Ala-D-Ala	302	159	-	-
Gly	75	8	6.0	6.0
Gly-Gly	132	19	10.4	8.0
Gly-Gly-Gly	189	28	14	8.5
Gly-Gly-Gly-Gly	246	40	17	9.0

times of the diasterioisomeric tetra alanines. Little significance was given to this possibility until a study was discovered of the same isomers made in 1965 by Beacham et al (37). They estimated the degree of extension of the diasterioisomers of the tetraalanines by comparing dipole moments and found the L,L,L,L isomer to be the most extended while the D residue as a middle residue gave a molecule with the amino and carboxyl groups nearest together.

Acknowledgments

This work was supported in part by a National Institute of Health Grant No. AM-02493.

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SYNTHESIS OF THE 2-15 N-TERMINAL SEQUENCE OF HEN EGG-WHITE LYSOZYME AND RELATED STUDIES

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As the spatial arrangement assumed by a protein is strictly dependent on its primary structure, it is clearly important to study if the influence of the amino acid sequence on the protein conformation is primarily due to short-range interactions or whether important contributions come from long-range interactions between amino acid residues quite far apart in the sequence.

It has proposed (1) that the conformation assumed by a polypeptide chain, during the biosynthesis, is not a random process but proceeds sequentially from the amino terminus.

According to this mechanism a polypeptide segment derived from the amino terminus of a protein, will tend to assume a conformation similar to that found in the complete protein.

Studies on the properties of isolated small segments of protein molecules can provide a direct way to confirm this hypothesis. Conformational studies in solution on the isolated N-terminal eicosapeptide of bovine pancreatic ribonuclease (2) i.e. S-peptide, have shown that this fragment, which is 50% helical in both the crystalline ribonuclease A (3) and S (4), is not helical in water solution when separated from the other part of the molecule, i.e. S-protein.

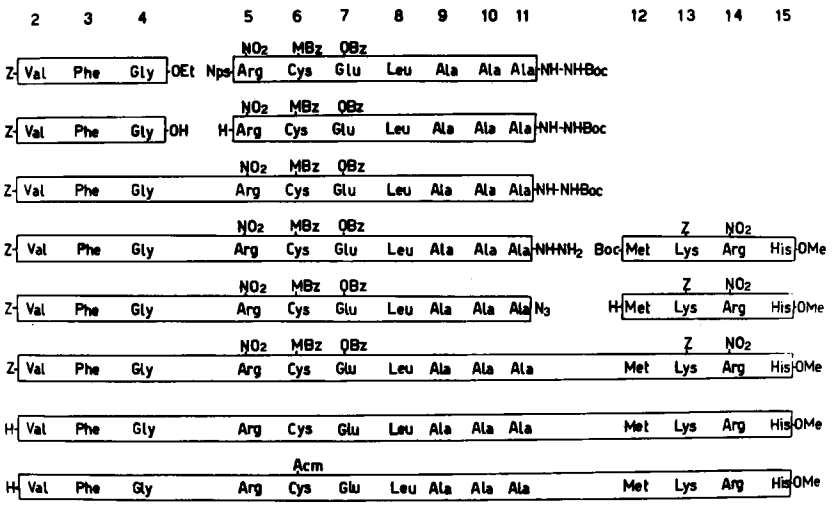
The obtained results indicate that the S-peptide undergoes a coil-to-helix conformational transition induced by the presence of S-protein and do not agree with the hypothetical sequential mechanism mentioned before. In order to ascertain if these findings can be considered quite general and to obtain evidence whether the incomplete peptides, formed in vivo, begin to fold from the N-terminus we decided to extend this kind of study to other proteins.

Hen egg-white lysozyme is one of the most extensively studied enzymes. Its primary structure, the disposition of its disulfide bonds, its spatial arrangement, its active center, specificity and mode of action have all been investigated in detail.

The tertiary structure of lysozyme, determined by X-ray analysis by Phillips and his group (5), shows that this molecule contains three runs of helix, each about ten residues long (5-15; 24-34 and 88-96), for which the axial translation per residue and the number of residues per turn fall close to the alfa-helix values given by Pauling and Corey.

Even in this case, as in the ribonuclease molecule, one helical portion includes residues in the N-terminal sequence. Therefore we decided to study the conformational features in solution of the synthetic N-terminal segment of hen egg-white lysozyme. As the lysine residue in position 1 is not included in the helical portion, which goes from 5 to 15, and is not essential for the catalytic function, as demonstrated by Jolles (6) we synthetized the 2-15 sequence.

The general scheme of the synthesis is shown in the figure.



Synthesis of the 2-15 sequence

In order to submit the labil methionine residue at the least possible number of steps, two principal subunits, corresponding to the sequences 2-11 and 12-15, were built up as shown in the scheme.

The thiol group of cysteine in position 6, which in the native molecule forms a disulfide bond with the residue in

position 127, was present as p-methoxybenzyl derivative (7) during the synthesis and as the S-acetamidomethyl derivative (8) in the final product.

In the step-wise elongation the activation of the carboxyl groups was achieved by the active ester method with the exception of the valyl residue which was introduced, as benzyloxycarbonyl derivative, by the mixed anhydride procedure. As often as racemization can occur, the condensation of the peptide subunits have been carried out by an azide coupling step.

The use of the t-butyloxycarbonyl hydrazide of the alanyl residue in position 11 allowed the construction of the heptapeptide 5-11 from the carboxyl and with retention of the hydrazide protected function which can be easily liberated once assembly of the desired subunit has been achieved.

The sequence 5-11 was then treated with thioacetamide (9) to remove selectively the α-NH_2 protecting group and coupled, "via" the dicyclohexylcarbodiimide-N-hydroxysuccinimide procedure (10) with the tripeptide 2-4.

The so obtained decapeptide was treated with trifluoro acetic acid, to remove the t-butyloxycarbonyl protecting group from the C-terminal hydrazide, and condensed, "via" an azide coupling step with the tetrapeptide methionyl-N^{ε}-benzyloxycarbonyllysyl-N^{ω}-nitroarginylhystidine methyl ester: the latter was prepared from the corresponding N^{α}-t-butyloxycarbonyl derivative by treatment with trifluoro acetic acid.

The resulting protected tetradecapeptide 2-15 was first treated with anhydrous hydrogen fluoride with the presence of anisole (11) and methionine (12) to remove all the protecting groups with the exception of the C-terminal methyl ester. Subsequent treatment of this solution with hydroxymethylacetamide (13) protected the thiol group of cysteine in position 6 as the acetamidomethyl derivative. After removal of the hydrogen fluoride the crude material was dissolved in water and purified by ion exchange chromatography on Amberlite IRC 50 column, using 0.2 M ammonium acetate, with pH gradient, as the eluent. The desired product was eluted from the column at pH 9.5 and desalted on a Bio-Rex column.

The peptide was then eluted from the resin with aqueous acetic acid and lyophilized. The homogeneity of the obtained material was assessed by paper electrophoresis at different pH values after treatment with aqueous thioglycolic acid to reduce any methionine solfoxide to methionine (14).

The amino acid analysis of the acid hydrolysate gave the expected amino acid ratio with the exception of the S-acetamido methyl cysteine which is partially decomposed during the hydrolysis. The synthetic tetradecapeptide was digested by aminopeptidase M, and gave amino acid ratios in good agreement with the expected values.

To determine whether the synthetic 2-15 N-terminal fragment of lysozyme assumes the helical conformation, present in the native molecule, we carried out its C.D. spectra in water solution and in presence of helicogenic solvent.

The shape and the features of the C.D. curve in water indicate the absence of ordered structure, but, in 50% trifluoroethanol the conformational features of the synthetic fragment are those typical of an helical structure.

The behaviour of the 2-15 sequence of lysozyme, which is not helical in water but is able to undergo a solvent-induced conformational transition, is strictly paralleled by the ribonuclease S-peptide (2).

The results obtained by studying isolated fragments of the ribonuclease and lysozyme molecules seem to point out that long range interactions are needed in a protein molecule as stabilizing points for the native conformation.

The authors wish to thank dr. Tamburro for carrying out the circular dichroism measurements.

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A PROTON MAGNETIC RESONANCE STUDY OF EVOLIDINE, A CYCLIC HEPTAPEPTIDE

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Detailed analysis of high resolution proton magnetic resonance spectra of oligopeptides promises to be a useful technique for gathering information about the conformation of peptides in solution, although the number of compounds investigated carefully has been limited. The spectrally most complex peptides for which conformational conclusions have been drawn from nmr data have been Gramicidin S, with five spectrally distinct residues, (1,2) and oxytocin, with nine (3,4). Evolidine, cyclo-Ser-Phe-Leu-Pro-Val-Asn-Leu (all L-),(5,6) is an intermediate case. It gives spectra with sharp lines and seemed worth investigating. This report proposes a conformation for the evolidine backbone based on 220 MHz proton magnetic resonance spectra of its dimethyl sulfoxide solutions.

Clues to oligopeptide backbone conformation from proton spectra come primarily from the resonances of the peptide (CONH) and α-protons. If a single conformation predominates, possible values for the conformational angle ϕ of each residue can be obtained from spin-spin coupling between these protons. This coupling is dependent on the dihedral angle of the H-C-N-H system, which for an L-residue equals $|240 - \phi|$. In addition, whether peptide protons are exposed to or shielded from hydrogen bond accepting solvents (e.g., dimethyl sulfoxide, methanol or water) may be inferred from the dependence of their chemical shifts on temperature (2,4, 7,8) or solvent (9); internal protons are assumed to show the smaller changes. Together with the peptide sequence, these indications may be used in model building to derive an approximation to the preferred backbone conformation or, at least, to limit the possibilities.

The necessary first step in the use of proton magnetic resonance spectra of a peptide is identification of individual proton resonances with individual amino acid residues. In many oligopeptides completing this task is impossible because of overlap of lines, even at the highest available magnetic fields, but for evolidine at 220 MHz a fairly complete analysis has been possible. The details of this analysis are described elsewhere (10). In brief, peptide protons were related to α-protons by double irradiation experiments. Then, using the deuterium-exchanged (N-D) form of the peptide, the α-proton lines, which are fewer and more clearly resolved in the absence of coupling to the peptide proton, were identified by their multiplicity and/or by demonstration of their coupling to specific β-protons at higher field.

In evolidine, the valine α-proton was identified as a triplet that becomes a doublet in the N-D peptide, and the proline α-proton resonances

were recognized by their immutability on deuterium exchange. The serine α-proton was shown to be coupled to a resonance pattern at 3.6 ppm (tetramethylsilane reference) corresponding to the β-protons of model serine derivatives, and the asparagine and phenylalanine α-protons were shown to be associated with protons with resonance patterns in the 2.5 - 3 ppm region. These last were sorted on the basis of the β-β proton coupling constants indicated by model phenylalanine and asparagine derivatives.

Chemical shift changes of the peptide protons were followed in dimethyl sulfoxide over the range 20 - 80° , and an estimate of the exchange lifetimes of the peptide protons in 50 per cent methanol - 50 per cent dimethyl sulfoxide was also made. Key results of the spectral analysis and these experiments are given in the Table.

TABLE

PEPTIDE AND α-PROTONS OF EVOLIDINE IN DIMETHYL SULFOXIDE, 20°

Residue	δ_{NH} ppm	$d\delta/dT$ ppm/degr	$\tau_{1/2}$ exch min	J_{HCNH} Hz	δ_{α} ppm	H-C-N-H angle, degrees
Ser	8.01	-0.0048	30	9.2	4.28	170
Phe	7.39	-0.0014	38	7.3	4.43	150
Leu*	8.94	-0.0079	52	4.5	3.86	30 or 120
Pro	-	-	-	-	4.26	-
Val	8.23	-0.0040	~500	7.8	3.70	150
Asn	7.79	-0.0020	~500	5.2	4.36	15 or 130
Leu*	8.31	-0.0057	2	2.5	4.22	55 or 105

(* The α- and peptide proton resonances of the two leucine residues were separately identifiable, but the above assignment to specific positions in the sequence is made because it affords a conformational model that fits the total of data better than the alternative. The spectra themselves give no obvious information on this point.)

The chemical shifts of peptide protons identified as belonging to the asparagine and phenylalanine residues of evolidine exhibit markedly lower temperature sensitivity than those of the remaining protons; the coefficients for the two residues approach those observed for non-hydrogen bonding protons such as, for example, those attached to the aromatic ring of the phenylalanine (ca.-0.001 ppm/degr). In contrast, the coefficients for the leucine peptide protons and the NH protons of the asparagine carboxamide side chain (-0.006-7 ppm/degr) are close to that observed for N-methylacetamide. It seems reasonable to suggest that the asparagine and phenylalanine peptide protons are not involved in intermolecular hydrogen bonds to solvent, bonds that would, on the average, be fewer at higher temperatures. Arguments for this interpretation of temperature insensitivity have already been given (7,8) and it is supported by the fact that it has led to results consistent with other evidence for Gramicidin S (2) and oxytocin (4), as well as for the potassium complex of the depsipeptide valinomycin (2).

H-C-N-H coupling constants for the six primary amino acid residues in evolidine can be extracted from the peptide N-H region of its spectra, and are also given in the Table above. Use on these observed values of a correlation between H-C-N-H coupling and the H-C-N-H dihedral angle leads to the set of dihedral angles given at the right of the Table above. The correlation employed was established from observations of a set of dipeptide models,

and is described in another paper of This Symposium (11).

The spectrum of the deuterium-exchanged form of evolidine in formamide-$\underline{d}_2$ is not significantly different from its spectrum in dimethyl sulfoxide: there are no major changes in chemical shift of the α-protons, and the α-β coupling constants remain almost unchanged, indicating no major conformational difference in the two solvents. Chemical shift changes induced by going to 75 per cent pyridine or 90 per cent benzene (remainder dimethyl sulfoxide) have also not given an indication of conformational change and have not proved useful in elucidating or confirming details of conformation in this case.

Conclusions drawn from the spectral data, $\underline{i}.\underline{e}.$, possible values of ϕ for six of the seven amino acid residues plus the indication that the asparagine and phenylalanine peptide protons are directed inward, or otherwise shielded from solvent, by the peptide ring, were used as input for manipulation of CPK and Dreiding molecular models. A solution of the conformational problem consistent with the observations is a conformation of the kind indicated in Figure I, with the conformational angles shown in the ϕ-ψ plane of Figure II. This model contains a cis Leu-Pro peptide bond. Models with a trans Leu-Pro bond do not match all of the data; in particular, they cannot be constructed with the required H-C-N-H dihedral angles for the asparagine residue.

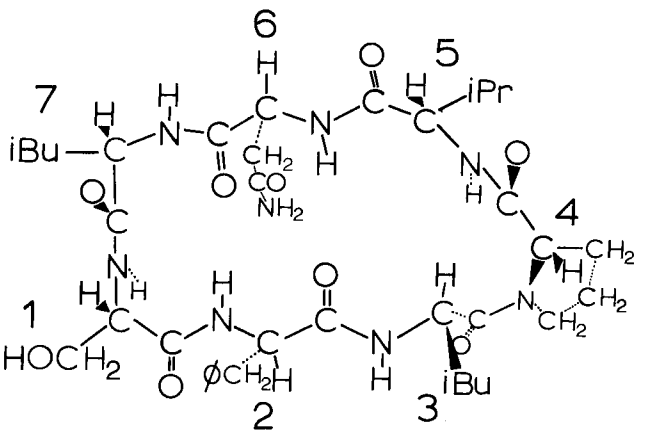

Figure I. Proposed backbone conformation for evolidine.

Spin-spin coupling between α and β-protons of amino acid residues are related to rotation about the C_α-C_β bond (12). Data from the evolidine spectra indicate major predominance of one α-β rotamer only for the asparagine residue ($J_{\alpha\beta}$ = 3 and 4.5 Hz). That rotamer has both β-hydrogens gauche to the α-proton. If this feature is added to the model advanced above, there appears a possible hydrogen bond between a side chain carboxamido proton of asparagine and the carbonyl oxygen of the Leu-Pro bond. There is independent evidence for this interaction, in that the carboxamido protons come into resonance 0.3 - 0.4 ppm to lower field in evolidine than corresponding protons in dimethyl sulfoxide solutions of oxytocin or simpler model compounds. (7.3 and 7.8 ppm vs 7.0 and 7.4 ppm).

In the evolidine model, the Leu-Ser turn has the conformation proposed by Venkatachalam (13) for two L-residues that form a bend to allow an

intramolecular hydrogen bond between residues 1,4 to each other. The resonance of the phenylalanine peptide proton that forms this hydrogen bond is at the high-field end of the peptide proton region. This agrees with its being close to and above the plane of the Leu-Ser peptide link. A high-field position for the resonance of protons similarly situated has also been found in cyclic hexapeptides, oxytocin and Gramicidin S. There is a possible inconsistency of this conformation with the limited deuterium exchange data, in that the asparagine and valine peptide protons are the most slowly exchanging, not that of phenylalanine. However, it is not likely that evolidine is completely rigid, and the exchange rates may reflect the ease with which small conformational changes can occur to permit exchange, while the nmr data indicate a time average in which the preferred conformation is heavily weighted.

This work was supported by a grant from the National Institute of General Medical Sciences, U.S. Public Health Service (GM 14069).

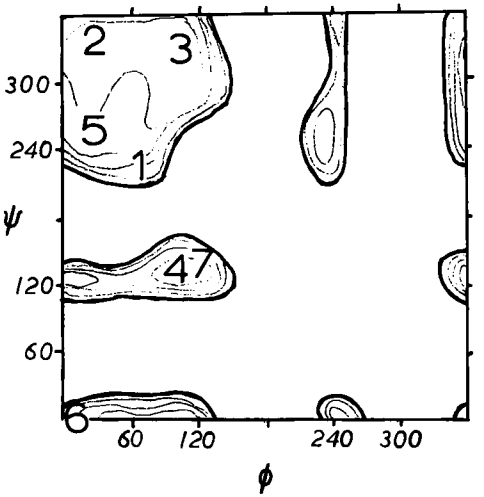

Figure II. Conformational parameters of the proposed evolidine conformation. Residues numbered as in Figure I.

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PROTON NMR STUDIES OF BIOLOGICAL MACROMOLECULES IN H_2O

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High resolution proton NMR measurements are usually run in D_2O solution to simplify the spectra. NMR investigations in H_2O permit one to observe resonances of exchangeable protons. Glickson, McDonald and Phillips[1] showed that the indole NH proton of tryptophan can be observed between -10 to -11 ppm in this way. It is well known that hydrogen bonds can shift proton nmr peaks several ppm to lower fields[2-4] and also can reduce their rates of exchange with solvent. Therefore, peaks from hydrogen bonded protons of amino acids and nucleic acid bases in macromolecules in H_2O might be shifted below -10 ppm and be narrow enough to be observed. This study reports the proton nmr studies of the heme proteins myoglobin[5a] and hemoglobin[6] and the nucleic acid, t-RNA^{yeast}_{phe}[7] in H_2O.

Myoglobin. The nmr spectra of diamagnetic proteins in D_2O give resonances between 0 and -9 ppm. Myoglobin has 950 protons and the spectral envelope in this region prohibits a detailed analysis. The nmr spectra of diamagnetic oxy Mb (sperm whale) in H_2O at pH 7 and 9 in the regions -9 to -15 ppm and +2 to +4 ppm are presented in Figure 1. Comparison with spectra run in D_2O indicate that the resonances shown between -10 to -15 ppm arise from exchangeable protons while non-exchangeable protons account for the peaks at -9.7 and +2.84 ppm. From an investigation[5a] of the effects of ligand, spin, pH, temperature, species variation and computer simulation of spectra,[8] assignments for the resonances in Figure 1 are outlined in Table 1. The -11.9 ppm guanidinium NH resonance of ARG G19 involved in a hydrogen bond with the CO_2^- groups of Asp B8 or Glu A16 is shifted 5 ppm downfield from its position in the absence of a hydrogen bond. At pH 7.4, the full width at half height of this resonance is 110 Hz giving a lifetime of $\tau = \frac{1}{\pi\Delta\nu} = 3$ msec in the hydrogen bond.

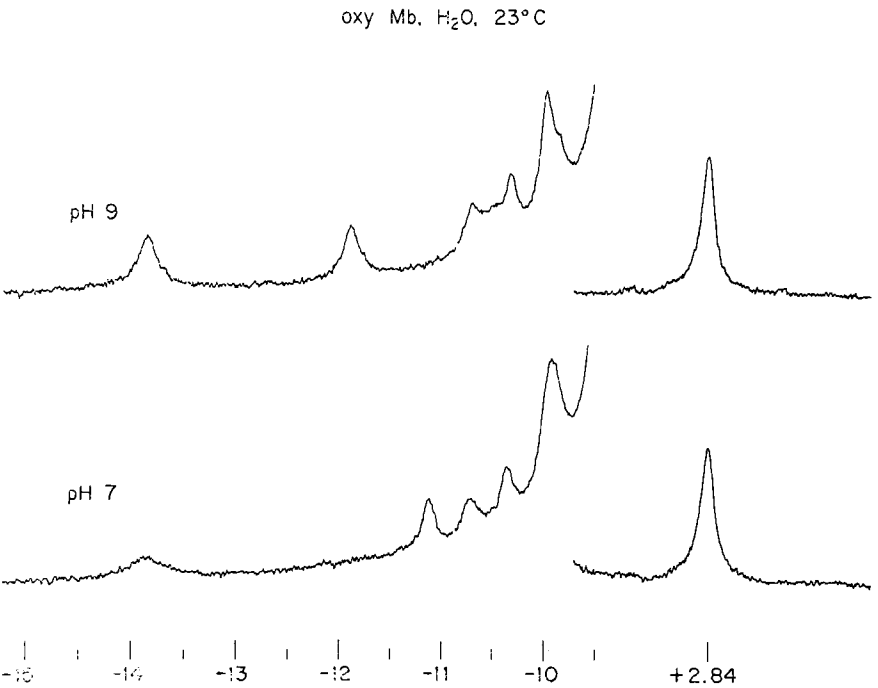

Figure 1 Proton 220 MHz nmr spectra of oxy Mb (sperm whale) in 0.1 M phosphate buffer in H_2O, 23°C at pH 7 and pH 9

TABLE 1

Amino Acid Assignments to Resonances in Figure 1

Resonance ppm	area at pH		Assignment
+ 2.85	2.8	7,9	CH_3, Val E11
- 9.7	~2	7,9	Porphyrin CH
-10.3	0.7	7	Indole NH, Trp
-10.7	0.7	7	
-11.15	0.7	7	Indole NH, Trp
-11.9	1.2	9	NH, Arg G19
-13.8	1.4	9	NH, His

Hemoglobin. Well resolved proton nmr resonances have been observed in hemoglobin derivatives between -10 and -15 ppm (Fig. 2).[6] They appear in H_2O but not in D_2O solution and therefore come from exchangeable protons. Table 2 lists their areas and chemical shifts. The resonances between -10 and -11.5 ppm arise from tryptophans at A12α, A12β and C3β in human Hb. The absence of shifts upon deoxygenation in the β subunit and non-cooperative $(\beta)_4$ tetramer suggest that these tryptophan resonances are sensitive probes to structural differences between oxygenated and deoxygenated forms of cooperative hemoglobin tetramer. From studies at lower pH it was observed that the -12.18 ppm HbO_2 resonance is shifted 2 ppm downfield to -14.14 ppm in Hb, while the peak near -13 ppm remains unchanged in the two forms. The low-field shifts and slow exchange rates in water of these resonances suggest their participation in hydrogen bonds and/or hydrophobic environments. Identification of these resonances with specific amino acids must await further experiments. The absence of these resonances in the subunits, their distance from the heme and the large shift upon deoxygenation suggest that the one proton /αβ dimer resonance which moves from -12.18 ppm in HbO_2 to -14.14 ppm in Hb is an indication either of quartenary structure, or of a tertiary structural change which is related to quartenary structure.[9]

$t\text{-}RNA^{yeast}_{phe}$. NMR studies of complementary Watson-Crick pairs of nucleosides in non-aqueous polar solvents have shown that the exchangeable hydrogen bonded ring NH protons of uridine and guanosine give sharp resonances at lower fields than the non-exchangeable protons.[4] This suggested that it might be possible to observe and resolve the corresponding lowfield resonances from the relatively few hydrogen bonded protons of t-RNA in aqueous solution.

Figure 3 outlines the lowfield proton nmr region of $t\text{-}RNA^{yeast}_{phe}$ in H_2O between -10 to -15 ppm.[7] The resonances in this region are absent in D_2O and arise from exchangeable protons. The integrated intensity of the peaks between -10 to -15 ppm were shown to correspond to 23 ± 3 exchangeable protons. The narrowest resonances have line widths of 50 Hz. On raising the temperature, certain of these lowfield resonances are differentially broadened and disappear above 61.5°C. Since hydrogen bonding does not shift[4] amino protons nearly as far downfield as the ring NH protons,[4] we assume that in t-RNA only NH ring protons will be observed in the -12 to -15 ppm region.

All the data discussed above were carried out at 220 MHz and represent time averaged spectra to improve signal to noise.

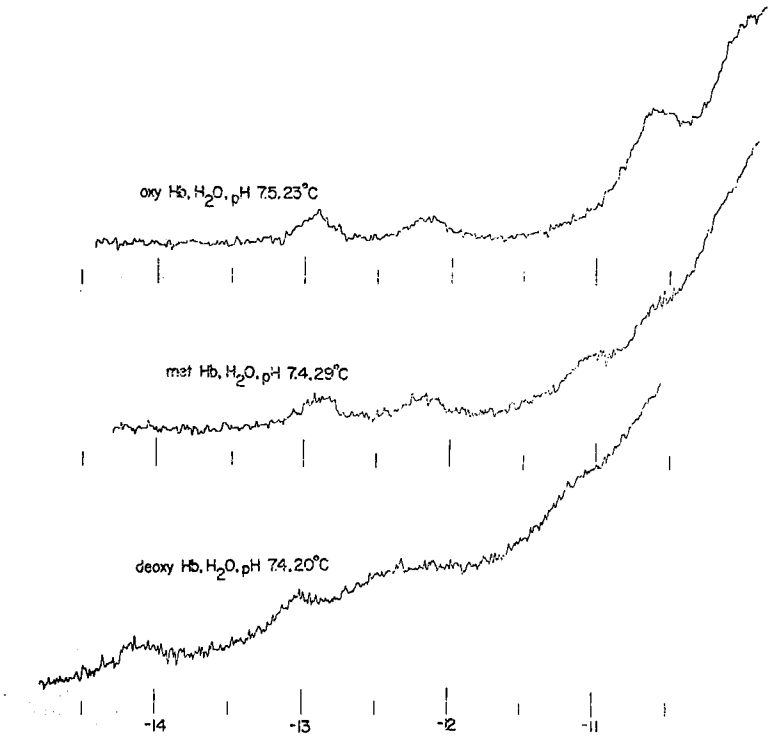

Figure 2 Proton 220 MHz nmr spectra of human HbO_2, met Hb and Hb in 0.1 M phosphate buffer in H_2O at pH 7.4. In the Hb spectrum, the broad resonance at -12.4 ppm is nonexchangeable.

TABLE 2

Chemical Shifts (ppm) Assignments to Resonances in Figure 2

Areas/αβ dimer indicated in brackets.

oxy		-10.58 (2-3)	
deoxy	-11.17 (1)		-10.21 (1)
oxy	-12.20 (1)	-12.90 (1)	
deoxy		-13.07 (1)	-14.14 (1)

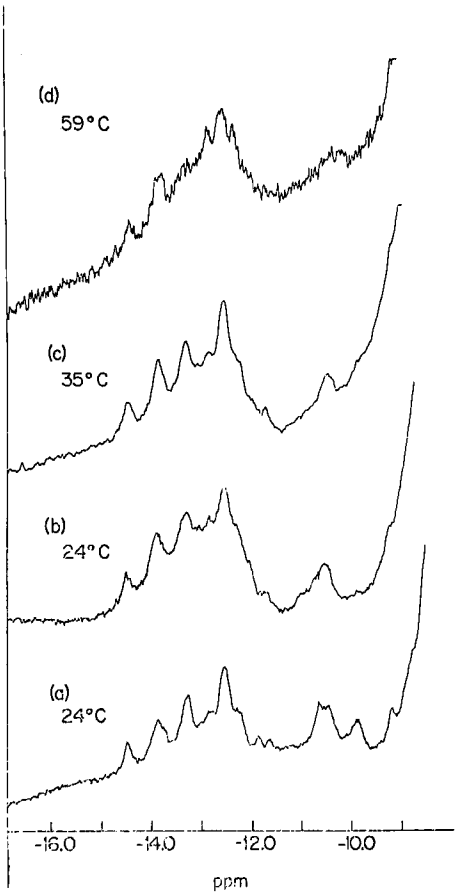

Figure 3 Proton 220 MHz nmr spectra of t-RNA$^{phe}_{yeast}$ 80 mg/ml in H_2O. (a) dialysed sample, 24°C, (b)-(d) sample in aqueous solution before dialysis containing high salt

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ANGIOTENSIN-CONVERTING ENZYME INHIBITORS FROM THE VENOM OF BOTHROPS JARARACA

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Introduction. After the original observation by Ferreira (1) that an alcoholic extract of the venom of Bothrops jararaca potentiated some of the biological activities of bradykinin, Bakhle (2) reported in 1968 that a similar extract inhibited the conversion in vitro of angiotensin I to angiotensin II by the angiotensin-converting enzyme from dog lung.

Ferreira et al. (4) and Greene et al. (3) recently described a procedure for the fractionation of this crude extract that led to the isolation of nine peptide fractions possessing bradykinin-potentiating activity. Ferreira et al. (5) showed that these peptide fractions also inhibit angiotensin-converting enzyme. Kato and Suzuki (6) have isolated peptides with bradykinin potentiating activity from the venom of Agkistrodon halys Blomhoffii.

Concurrently with and independently of the studies described in the previous paragraph, our interest in the inhibitory activity reported by Bakhle (2), with respect to the angiotensin-converting enzyme, led us to further fractionate this activity in an alcoholic extract of venom of B. jararaca prepared according to the method of Ferreira (1). Fractions were tested for inhibitory activity in an in vitro-assay for angiotensin-converting enzyme developed by Cushman and Cheung (7).

Isolation Studies. A schematic description of the fractionation is given in Figure 1. All chromatographic separations were run on columns at room temperature in the following manner: Sephadex G-25, equilibrated and developed with 0.2M acetic acid; CM-Cellulose, equilibrated with 0.005M ammonium acetate and developed stepwise with the same buffer and with 0.2M acetic acid; DEAE-Sephadex, equilibrated with 0.005M

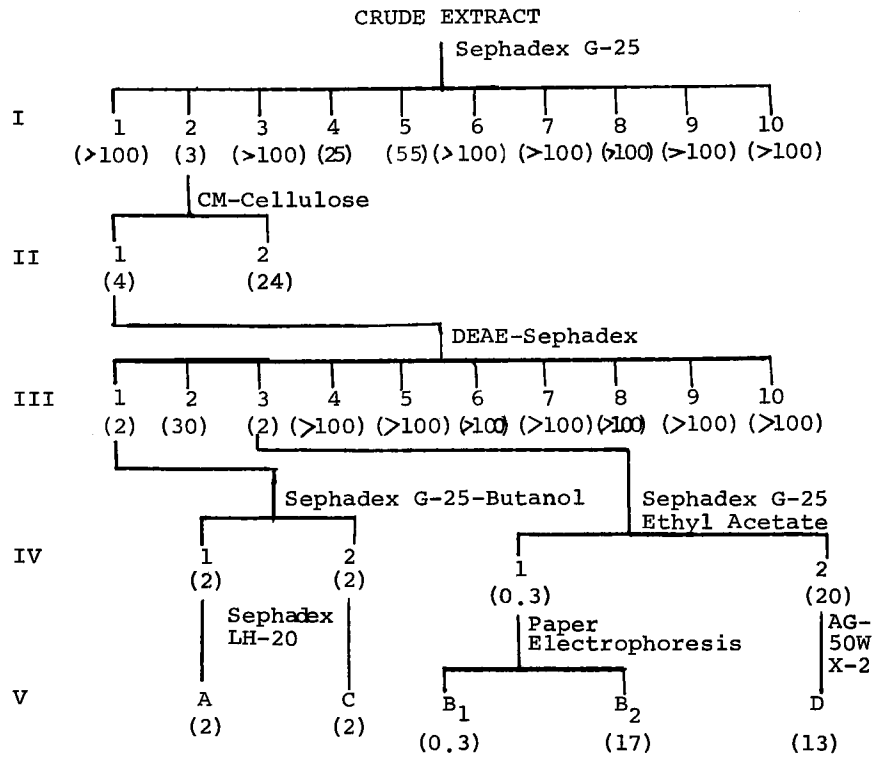


Figure 1 Fractionation of the alcoholic extract of venom of B.jararaca. The number in parenthesis under each fraction indicated the concentration, in mcg/ml, needed to inhibit 50% of the converting enzyme activity (7).

ammonium bicarbonate and developed with a linear gradient of the same buffer 0.005 to 1.0M; Sephadex G-25-Butanol, equilibrated and developed with n-butanol, pyridine, acetic acid, water (30:20:6:24); Sephadex G-25-Ethyl acetate, equilibrated and developed with ethyl acetate, pyridine, water (20:10:11); Sephadex LH-20; equilibrated and developed with methanol; AG-50W-X2, equilibrated with pH 3.1 pyridine-acetate buffer and developed with a linear gradient of pyridine acetate buffer pH 3.1 to 5.0 (8). The progress of the fractionation was followed by paper and thin-layer chromatography, electrophoresis, and amino acid analyses. The purification of some fractions was not pursued further because of the comparatively low activity (e.g., I-4, II-2).

Fractions V-A, V-B_1, V-B_2, V-C and V-D were the only components obtained in amount sufficient for structural studies; their amino acid composition is shown in Table 1.

Fraction	Asp	Thr	Ser	Glu	Pro	Gly	Ile	His	Arg	Trp
V-A		0.77		2.24	5.58	0.16	0.86		1.0	0.84
V-B_1	0.66			2.01	4.50		1.0	0.90		0.80
V-B_2	0.99		0.89	1.05	4.10	1.0	0.91			0.71
V-C	0.29			2.02	4.27		0.92		1.0	0.82
V-D				2.00	5.00	2.76	0.94		1.0	0.91

TABLE 1

Amino Acid Composition of Peptides Isolated from B.Jararaca Venom.

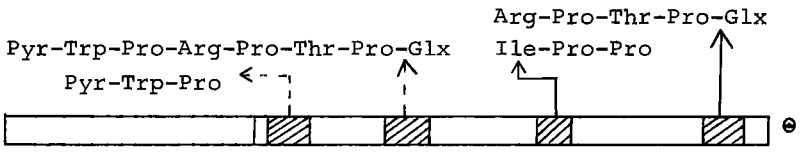
Fraction V-A is contaminated with a glycine-containing peptide that can be separated only partially by Sephadex LH-20 chromatography. Fraction V-C has an impurity that contains either aspartic acid or asparagine, but the small amount available did not allow further purification.

Structural Studies. The lack of free N-terminal amino groups (ninhydrin reaction negative) precluded direct Edman degradation of the peptides. Mass spectrometry of fraction V-A after methylation with diazomethane showed a strong peak at M/e 112, suggestive of an N-terminal pyroglutamyl residue. Greene et al. (3,4) have postulated the same structural feature for the fractions they isolated from venom of B.jararaca, and in one of those fractions (pentapeptide V-3-A) this structural assignment has been demonstrated by degradation and synthesis.

Digestion of several of these peptides with chymotrypsin under different conditions produced no significant cleavage, probably because of the large number of proline residues. Similar observations were made with trypsin (10), except for V-D, partial digestion of which was obtained under the conditions described for the cleavage of Arg-Pro bonds (11). Papain (12), on the contrary, was found to be the enzyme of choice for the complete digestion of these peptides. As will be shown below, complete hydrolysis was observed for Gln-Ile and Ser-Trp bonds, and partial cleavage in the case of Asn-Ile, Asn-Trp and Pro-Arg bonds.

The presence of tryptophan in all the peptides isolated suggested chemical degradation with N-bromosuccinimide (NBS) (13) as a substitute for enzymatic digestion. This technique was successfully applied to V-A and V-D.

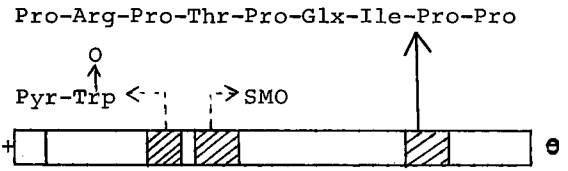
The mixtures obtained after digestion with papain or oxidation with NBS were fractionated by preparative high-voltage electrophoresis on paper, with formic acid-acetic acid buffer (pH 1.9; 65 volt/cm). Figures 2 to 8 illustrate the separations



Thr		0.9		1
Glu	1	2		1
Pro	1	3	2	2
Gly		0.1		
Ile			1	
Arg		1		1
Ninhydrin	-	-	+	+
Sakaguchi	-	+	-	+
Ehrlich	+	+	-	-
Rydon	+	+	+	+

PAPAIN DIGESTION OF V-A

Figure 2



Thr		0.7	1
Glu	+	2	1
Pro		5	5
Gly		0.3	
Ile		1	1
Arg		1	1
Ninhydrin	-	-	+
Ehrlich	-	-	-
Sakaguchi	-	+	+
Rydon	+	+	+

NBS OXIDATION OF V-A

Figure 3

SMO: Starting material oxidized

O
Trp: Oxidized Trp residue

obtained with each of the fractions studied. Ninhydrin-positive bands were eluted and aliquots analyzed for N-terminal amino acid by dansylation (14), and for their content of amino acids. The remainder was degraded by the Edman-dansylation (15) procedure. The solid arrows indicate the sequences identified by those procedures. Ninhydrin negative bands were eluted and the amino acid composition of each was determined. Broken arrows indicate the sequence postulated

for these fragments, based on the amino acid composition of the whole peptide and the Edman sequence of the other fragments. A few minor bands were observed besides those illustrated, but the small amounts available precluded obtaining useful structural information from them.

Assignment of the side chain amides was based mainly on the electrophoretic mobility of the peptides themselves or of the dansyl intermediates of the Edman degradation. In the case of V-A, both dansyl intermediates—DNS-Pro-Thr-Pro-Glx-Ile-Pro-Pro— and DNS-Ile-Pro-Pro—showed a charge of -1, indicating a glutamine residue. On the contrary, in the case of V-D the mobility of DNS-Pro-Gly-Pro-Glx-Ile-Pro-Pro showed a charge of -2, pointing to a glutamic acid. The electrophoretic mobility of V-B_1 and V-B_2 at 9.3 indicates a net charge of -1 for both peptides and, since the papain digestion releases a C-terminal Ile-Pro-Pro, as in the case of V-A, it was concluded that the Asx and Glx were asparagine and glutamine, respectively. On the basis of the data summarized in Figures 2 to 7 and the amide assignments described above, the following structures are proposed:

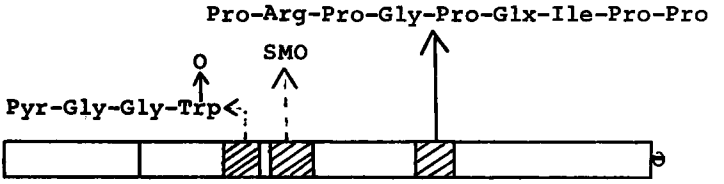
V-A Pyr-Trp-Pro-Arg-Pro-Thr-Pro-Gln-Ile-Pro-Pro
V-B_1 Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro
V-B_2 Pyr-Ser-Trp-Pro-Gly-Pro-Asn-Ile-Pro-Pro
V-D Pyr-Gly-Gly-Trp-Pro-Arg-Pro-Gly-Pro-Glu-Ile-Pro-Pro

Interpretation of the results of papain digestion of V-C (Fig. 8) was somewhat complicated, because this fraction is a mixture of two components. On the basis of the specificity of papain for this class of compounds, and assuming the presence of only one residue of tryptophan per mole of peptide, it was concluded that the two components of V-C have the following structures:

V-C_1 Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro
V-C_2 Pyr-Asn-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro

Assignment of the amide side chains was based on the electrophoretic mobility of V-C, which is identical to that of V-A at all pH values from 3 to 9.3.

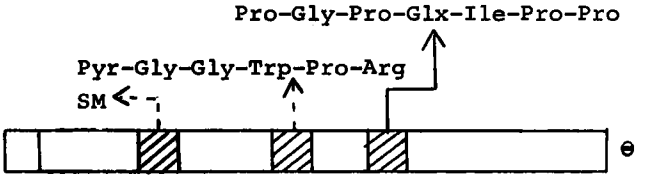
Synthetic Studies. The sequences proposed above for V-A, V-B_1, V-B_2, V-C_1, V-C_2 and V-D were synthesized using the solid-phase procedure developed by Merrifield (16). The purified synthetic materials showed the same chromatographic and electrophoretic mobilities as did their natural counterparts, and gave the same electrophoretic patterns upon papain digestion. The assays for inhibition of angiotensin-converting enzyme *in vitro* by natural and synthetic peptides were in similar agreement.



Glu	1	2	1
Pro		5	5
Gly	2	3	1
Ile		1	1
Arg		1	1
Ninhydrin	-	-	+
Ehrlich	-	-	-
Sakaguchi	-	+	+
Rydon	+	+	+

NBS OXIDATION OF V-D

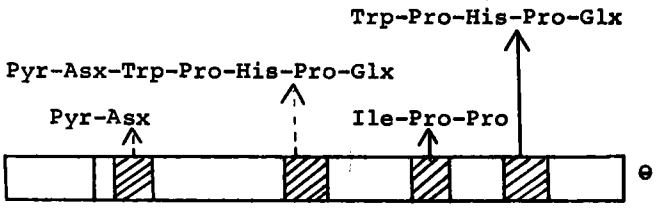
Figure 4



Glu	2	1	1
Pro	5	1	4
Gly	3	2	1
Ile	1		1
Arg	1	1	
Ninhydrin	-	-	+
Ehrlich	+	+	-
Sakaguchi	+	+	-
Rydon	+	+	+

TRYPSIN DIGESTION OF V-D

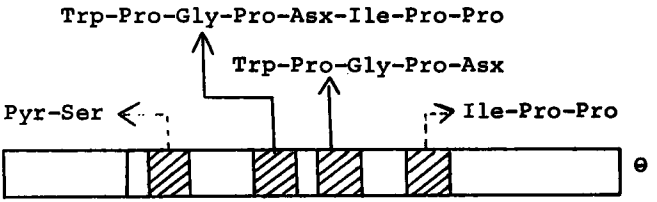
Figure 5



Asp	1	1		
Glu	1	2		1
Pro		2	2	2
Ile			1	1
His		1		
Ninhydrin-		-	+	+
Ehrlich	-	+	-	+
Pauly	-	+	-	+
Rydon	+	+	+	+

PAPAIN DIGESTION OF V-B_1

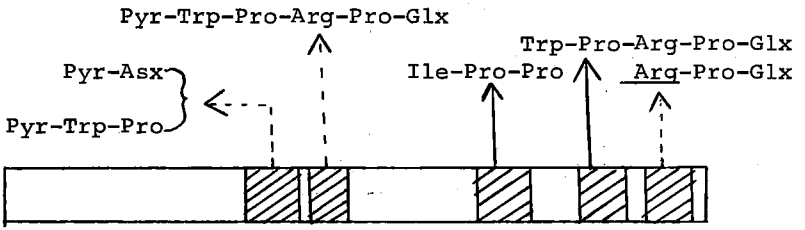
Figure 6



Asp		1	1	
Glu	1			
Pro		4.5	2	2.5
Gly		1	1	
Ile		1		1
Ser	1			
Ninhydrin	-	+	+	+
Ehrlich	-	+	+	-
Rydon	+	+	+	+

PAPAIN DIGESTION OF V-B_2

Figure 7



Asp	0.4				
Glu	1	2		1	1
Pro	0.6	2	2.5	2	1
Ile			1		
Arg		1		1	1
Ninhydrin	-	-	+	+	+
Ehrlich	+	+	-	+	-
Sakaguchi	-	+	-	+	+
Rydon	+	+	+	+	+

PAPAIN DIGESTION OF V-C

Figure 8

Achnowledgment. The authors wish to express their gratitude to Dr. D. W. Cushman and Mr. H. S. Cheung for the assays of angiotensin-converting enzyme inhibition.

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SYNTHESIS OF BRADYKININ-POTENTIATING PEPTIDES ISOLATED FROM THE VENOM OF AGKISTRODON HALYS BLOMHOFFII

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Introduction. In 1965, Ferreira found the presence of peptide-like substances in the venom of Bothrops jararaca, which potentiates bradykinin action on isolated smooth muscles (1). Recently, Ferreira et al. isolated one component of these potentiating factors, and the amino acid sequence was determined to be Pyr-Lys-Trp-Ala-Pro (2). This compound was then synthesized using the solid-phase method, and its biochemical and pharmacological properties were studied in detail (3). Of special interest was the finding that this compound is a potent inhibitor of the angiotensin converting enzyme located in dog lung (4). Two of the present authors (H.K. and T.S.) had also isolated five different bradykinin-potentiating peptides A, B, C, D, E from the venom of the Japanese snake, Agkistrodon halys blomhoffii (5); subsequently, they elucidated the amino acid sequences of peptides B, C, and E, as follows: B. Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro (6). C. Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro (7). E. Pyr-Lys-Trp-Asp-Pro-Pro-Pro-Val-Ser-Pro-Pro (8).

The present communication deals with synthesis of potentiators B and C to confirm their structures, and with biological activities of their fragment-peptides in order to elucidate the structure-activity relationship of these potentiators.

Synthesis of Peptides. Potentiator B was synthesized as illustrated in Fig. 1. Mainly, t-amyloxycarbonyl (Aoc) group (9) was used for the protection of α-amino groups except in the case of an arginyl residue, on which the p-methoxybenzyloxycarbonyl [Z(OMe)] group (10) was used for the N^{α}-protection. These N^{α}-protective groups were removed with trifluoroacetic acid before elongation of the peptide-bonds. Protection of the functional groups of arginyl and lysyl residues were carried out with nitro and p-chlorobenzyloxycarbonyl [Z(Cl)] groups, respectively. The Z(Cl)-group is more resistant to trifluoroacetic acid than the ordinary benzyloxycarbonyl group, but is removed easily with liquid hydrogen fluoride (HF) in a presence of anisole (11). The protected C-terminal tri-, tetra- and penta-peptides were obtained as syrupy or amorphous materials, and were purified by silica-gel column chromatography. Attachment of Z(OMe)-nitroarginyl residue to the C-terminal penta-peptide benzyl ester gave finally a crystalline material.

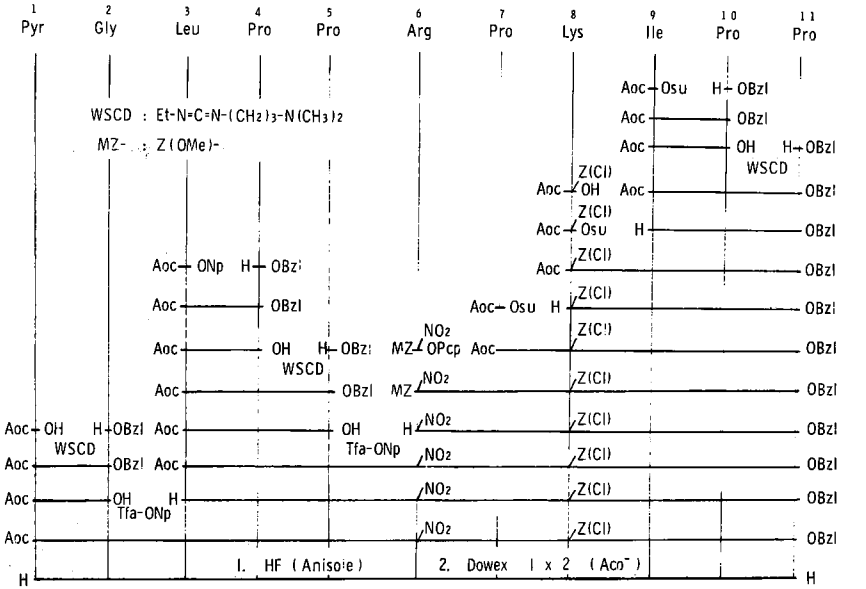

Fig. 1. Synthesis of Potentiator B

Then, a fragment, Aoc-Leu-Pro-Pro-OH, and N-terminal Aoc-Pyr-Gly-OH were coupled successively by the trifluoroacetate procedure (12) to obtain protected potentiator B. All the protective groups were removed simultaneously by the HF-procedure at 0°C (11), and the final product was obtained as the mono acetate by passing through a column of Dowex 1 x 2 (acetate form) to remove excess HF followed by lyophilization; $[\alpha]_D^{18}$ -204° (c 0.3, H_2O).

Anal. Found: C, 51.8; H, 7.7; N, 15.9%. Calcd. for $C_{58}H_{95}O_{15}N_{15}\cdot 5\ 1/2\ H_2O$: C, 51.9; H, 8.0; N, 15.7%.

Amino acid anal. after HCl hydrolysis. (105°C, 48hrs.)

Glu	Gly	Leu	Pro	Arg	Lys	Ile
1.00	0.96	1.00	4.93	0.97	0.97	0.82

The potentiator C was synthesized as shown in Fig. 2. Since this structure has repeating three tripeptide-units, -Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro, this part was assembled by coupling respective three tripeptide fragments, successively, by the trifluoroacetate procedure (12). Finally, the same Aoc-Pyr-Gly-OH was coupled to the nonapeptide, and then the resulting protected undecapeptide was treated with HF to obtain free peptide C; $[\alpha]_D^{25}$ -253° (c 0.3, H_2O).

Anal. Found: C, 55.4; H, 7.6; N, 13.9%. Calcd. for $C_{51}H_{77}O_{13}N_{11}\cdot 3H_2O$: C, 55.4; H, 7.6; N, 13.9%.

Amino acid anal. after HCl hydrolysis. (105°C 48hrs.)

Glu	Gly	Leu	Pro	Ile
1.08	1.98	1.00	5.90	1.04

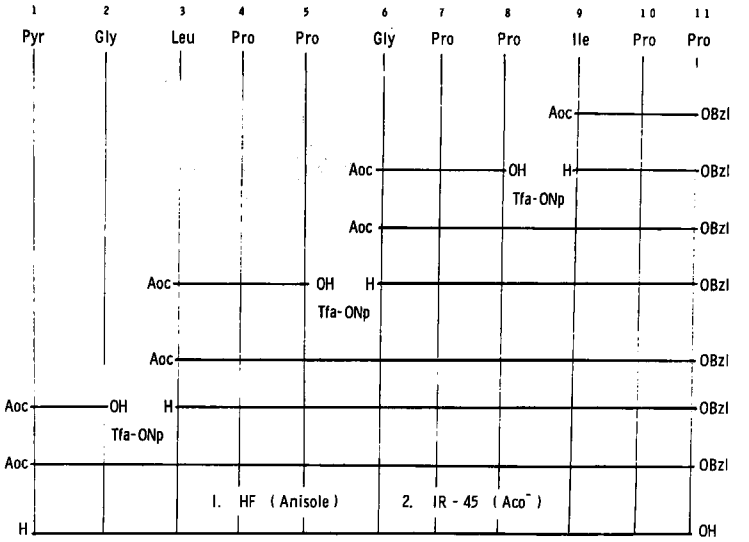

Fig. 2. Synthesis of Potentiator C

These synthetic materials were confirmed to have the same Rf-values and mobilities as the natural products by paper chromatography and by paper electrophoresis.

Biological Properties. Generally, bradykinin activity was determined by measuring the contraction of the isolated guinea pig ileum at 30°C in aerated Tyrode's solution containing 1mg of atropine per liter. The activity was also measured on the isolated rat uterus in oxygenated de Jalon solution for comparison. The bradykinin-potentiating activity of peptides was compared by the concentration of each peptide in an organ bath, which is required to increase the contractile response of the tissue induced by a dose of bradykinin to the same level of response induced by the double dose of bradykinin. Typical dose-response curves of ileum induced by the interaction of bradykinin and potentiators are illustrated in Fig. 3. Thus, biological activities of natural and synthetic potentiators B, C, and natural potentiator E were measured, respectively; the data are summarized in Table 1. As shown in the table, both natural and synthetic potentiators B and C showed practically the same activities. Although potentiator E was much less potent than B or C on guinea pig ileum, the potentiating activity was found to increase 50-fold on rat uterus. On the other hand, potentiators B and C showed only faint activities on rat uterus.

These peptides B and C were also found to inhibit angiotensin converting enzyme (dog lung) as has been observed by Ferreira et al. with their penta-peptide, Pyr-Lys-Trp-Ala-Pro (4). As can be seen in Table 2, the inhibitory activity of potentiator B was much stronger than that of C.

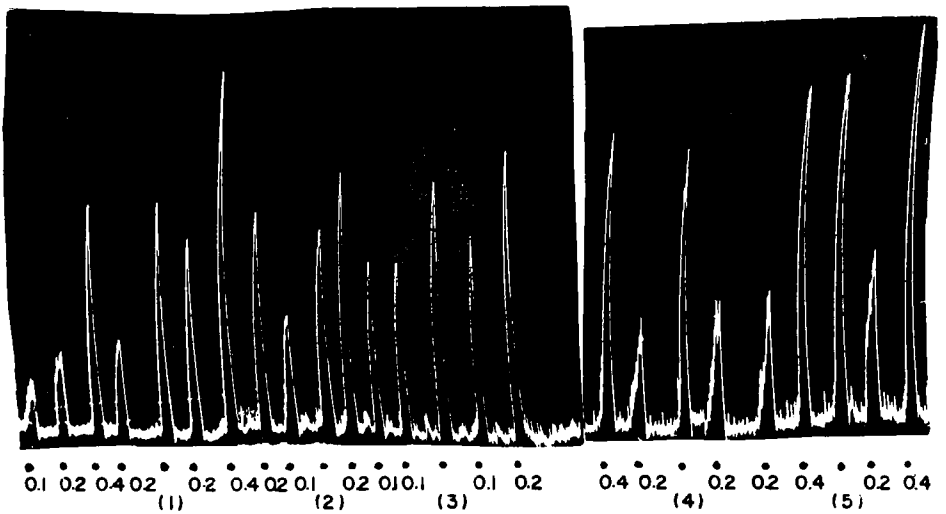

Fig. 3. Contraction of Isolated Guinea Pig Ileum.
Each number indicates the amount of bradykinin added (μg); (1) 5.2 mμmoles of Natural B + 0.2 μg of BK; (2) 5.2 mμmoles of Natural B + 0.1 μg of BK; (3) 6 mμmoles of Synthetic B + 0.1 μg of BK; (4) 13 mμmoles of Natural C + 0.2 μg of BK; (5) 10 mμmoles of Synthetic C + 0.2 μg of BK. (Volume of the organ bath: 16 ml)

Table 1

Biological Activities of Potentiators B, C and E

Compound	Concentration[a] mμmol/ml	
	Guinea pig ileum	Rat uterus
Natural B	0.33	>100
Synthetic B	0.38	
Natural C	0.63	>100
Synthetic C	0.62	
Natural E	46.8	0.93

(Bradykinin dose level : 0.01-0.5γ)
a. To potentiate 2-fold the bradykinin action.

Table 2

Inhibition of Angiotensin Converting Enzyme (Dog Lung) by Potentiators B and C*

Potentiator	Final Conc. 10^{-12} mol/ml	Inhibition %	
		Natural	Synthetic
B	10	11	15
	10^2	44	47
	10^3	83	78
	10^4	91	93
	130-150[a]	50	
C	10^2	19	7
	10^3	34	33
	10^4	67	70
	3000[b]	50	

(Angiotensin I substrate level : 5μg/ml)
a. Correspond to 0.19μg/ml. b. Correspond to 3.3μg/ml.

Structure-Activity Relationship. Fragments of potentiators B and C, which are obtained during syntheses of these potentiators, were subjected to the same assay system on guinea pig ileum for the bradykinin-potentiating activity. As shown in Table 3, minimum size of the biologically active fragment was Ile-Pro-Pro, which is a common C-terminal fragment of B and C; on the other hand, N-terminal fragments had no biological activity. Removal of the N-terminal dipeptide, Pyr-Gly, from B resulted in a lowering of the activity, but the same treatment with C gave a more potent nonapeptide fragment. Thus, the nonapeptide, Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro, was found to be the most potent peptide fragment so far tested on the isolated ileum.

Further modification of the biologically active tripeptide, Ile-Pro-Pro, was carried out to elucidate which structure is essential for the activity; the data are summarized in Table 4. Attachment of an acyl group to the N-terminus or of an amide group to the C-terminus resulted in less or no activity. Replacement of Ile with Leu, Val, Ala, Gly, or Pro also decreased the activity. Replacement of either proline residue in the

* The experiments on the inhibition of angiotensin converting enzyme with potentiators B and C were kindly undertaken by Dr. Y. S. Bakhle of Royal College of Surgeons of England.

Table 3

Bradykinin-Potentiating Activity of Fragments of Potentiators B and C

	Compound	Activity Ratio[a]
B	Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro	100
	Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro	50
	Arg-Pro-Lys-Ile-Pro-Pro	12
	Ile-Pro-Pro	2.5
	Pro-Pro	none
	Ile-Pro	none
	Pyr-Gly	none
	Pyr-Gly-Leu-Pro-Pro	none
C	Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	61
	Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	140
	Gly-Pro-Pro-Ile-Pro-Pro	8.5

a. Ratio = $\frac{0.38 \times 100}{\text{Conc. of test peptide (m}\mu\text{mol/ml) to potentiate 2-fold the bradykinin action}}$

original tripeptide with a glycine was also found to destroy the activity. Bradykinin-potentiating activity of oligomers of a tripeptide, $(Pro\text{-}Pro\text{-}Gly)_n$, which had been available from our stock, were also measured to check the relationship between biological activity and molecular weight; a weak but definite biological activity was observed only with the trimer. Thus, the size of a nonapeptide may be important to be favorable to exhibit the maximum potentiating activity. Further, it was found that although the unit-tripeptide, Ile-Pro-Pro, was the most potent of its analogs so far tested, the trimer, $(Ile\text{-}Pro\text{-}Pro)_3$, was much less potent than the natural fragment, Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro.

From the above results, it may be concluded that a regular molecule, X-Pro-Pro-Y-Pro-Pro-Z-Pro-Pro, would be potent in terms of bradykinin-potentiating activity when positions X and Z are both occupied with hydrophobic amino acid residues, and Y is a hydrophylic amino acid residue.

Further modification and testing of these peptides is being continued in our laboratory.

Table 4

Bradykinin-Potentiating Activity of Analogs of Ile-Pro-Pro

Compound	Activity Ratio[a]
Potentiator B	100
Ile-Pro-Pro	2.5
Gly-Ile-Pro-Pro	1.0
Aoc-Ile-Pro-Pro	0.1
Ile-Pro-Pro-NH_2	none
Leu-Pro-Pro	1.8
Val-Pro-Pro	0.6
Ala-Pro-Pro	0.2
Gly-Pro-Pro	0.1
Pro-Pro-Pro	0.2
Ile-Gly-Pro	0.5
Ile-Pro-Gly	none
$(Pro\text{-}Pro\text{-}Gly)_2$	none
$(Pro\text{-}Pro\text{-}Gly)_3$	1.0
$(Pro\text{-}Pro\text{-}Gly)_4$	none
$(Ile\text{-}Pro\text{-}Pro)_3$	6.0

a. See Table 3.

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SPECTRUM OF BIOLOGICAL ACTIVITIES IN CAERULEIN-LIKE PEPTIDES

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The structure of caerulein, a biologically active peptide, has been reported by Anastasi, Erspamer, and Endean (1).

Caerulein has been the object in our laboratories of an investigation, based on the synthesis of analogues, (2, 3, 4) aimed at finding: i) which amino acid and/or sequence is essential for the biological activity, ii) which kind of dissociation of the biological activities is achieved by chemical modifications of the molecule. We hoped also to find an analogue still retaining the activity of caerulein but more stable toward oxygen and acids and, possibly, an analogue which could act as an antagonist of caerulein itself. Not all the goals of this investigation have been so far achieved, but we have now gained sufficient knowledge of the structure -activity relationship to justify this note that summarizes all our findings.

$$\text{Pyr-Gln-Asp-}\underset{\displaystyle SO_3H}{\text{Tyr}}\text{-Thr-Try-Met-Asp-Phe-}NH_2 \quad \text{(caerulein)}$$

The elimination of amino acid residues from the N-terminal side of the molecule does not modify the spectrum of activity up to the heptapeptide.

$$\underset{\displaystyle SO_3H}{\text{Tyr}}\text{-Thr-Gly-Trp-Met-Asp-Phe-}NH_2$$

The loss of the sulphate residue reduces 1000 fold the activity on the gall bladder and on the gut, which are two of the more typical tests for a cholecystokinin-like activity. The same reductions are observed when the threonyl residue is suppressed or when the tyrosyl sulphate residue is moved further to the left by insertion of an amino acid residue. The presence of a tyrosyl sulphate residue in the right position seems therefore essential for a cholecystokinin-like kind of activity. However the fair activity of the D-tyrosyl sulphate analogue shows that the chirality of the tyrosine residue is of limited importance and it is therefore not surprising to find that the desamino analogue still has all the activity of the heptapeptide. We can further add that the tyrosine sulphate residue can be replaced without too great a loss of activity (and in order of decreasing activity) by a 3-chlorotyrosine sulphate, p.sulphonylphenylalanine, tyrosine phosphate, m.tyrosine sulphate, and 3,5-dibromotyrosine sul-

phate residue. On the contrary replacement of the tyrosine sulphate residue with a neutral residue such as tyrosine itself or p.sulphonamidophenylalanine yields compounds practically devoid of any activity on the gall bladder. We can therefore conclude that a cholecystokinin-like activity is to be found in peptides having a negatively charged residue in the appropriate position, but the steric requirements of such residue are not very strict and within these limits it can be changed considerably.

Since the C-terminal pentapeptide is common to gastrin, cholecystokinin and caerulein, it was conceivable that the different biological activities (e.g. gastrin has an extremely low activity on the gall bladder and on the gut) could eventually be related to the threonine residue that was accordingly replaced by Ala, Met, Abu (α.aminobutyric acid), Tyr, Val, Phe, Trp. All these analogues however had the same kind and order of activity of the heptapeptide, whereas suppression of the threonine residue yielded a peptide very similar to gastrin and with 1/1000 of the activity of caerulein in the gall bladder: in caerulein therefore, threonine plays a simple, but very important, spacing role.

The structure-activity relationship in the C-terminal pentapeptide, which is common to gastrin, has already been the object of an extensive study by Morley (5): in our case, as in the case of gastrin, replacement of the easily oxydized Met with Nle (norleucine), yielded a more stable and highly active compound. Finally, by combining the acid-stability of p.sulphonylphenylalanine with the stability toward oxygen of Nle, the analogue

$$\overset{SO_3H}{\underset{\downarrow}{\text{Phe}}}\text{-Thr-Gly-Trp-Nle-Asp-Phe-NH}_2$$

was prepared and found to be very stable but only moderately active. Most of the newly synthesized peptides were used to study the relationship between pancreatic exocrine secretion and pancreatic blood flow according to the procedures previously described (6, 7). In addition, a comparison was made with vasoactive peptides such as eledoisin, angiotensin II, and glucagon, to test specificity of the cardiovascular responses to caerulein and caerulein-like peptides.

All peptides which increased water secretion from the cannulated pancreatic duct, increased pancreatic blood flow too, while peptides inactive on exocrine secretion had no vascular action. The ratio between the relative potencies on these two tests remained fairly constant throughout the whole series of peptides examined.

Glucagon, contrary to caerulein, had no stimulant action on resting pancreatic secretion when infused intravenously from 1 to 10 μg/kg/min. It increased, however, pancreatic blood flow as well as total mesenteric blood flow whereas caerulein and caerulein-like peptides were active only on pancreatic vessels. In addition, glucagon was found to be a fairly potent antagonist to the pancreatic stimulating activity of caerulein. During an intravenous infusion of glucagon (10 μg/kg/min) four times higher doses of caerulein had to be injected to obtain a pancreatic secretory rate comparable to that present prior to the administration of glucagon. The dose-response curves for caerulein and caerulein-like peptides were shifted to the right, although a complete parallelism was not always observed.

Eledoisin and physalaemin, on the contrary, had a direct vasodilating

activity on nearly all vascular beds being, however, more active on intra-abdominal than on peripheral beds. Angiotensin, diverted visceral blood flow to the periphery at doses not affecting markedly arterial blood pressure.

From the above reported facts the following observations can be drawn:

1. The cholecystokinin-pancreozymin-like stimulation of the pancreas is accompanied by a functional vasodilatation of pancreatic blood vessels. Most probably a similar relationship between functional vasodilatation and pancreatic exocrine flow is operating also during the physiological process of digestion when cholecystokinin in released from the gut.
2. The responses of the cardiovascular system seem to be fearly specific for each type of peptides examined. Therefore we suggest that cardiovascular techniques can be profitably used to differentiate newly isolated peptides from known ones.
3. Van Dyck et al. (8) postulated a reduction in visceral blood flow to explain the antagonistic action of glucagon on exocrine pancreas stimulated either by secretin or by a combination of secretin and cholecystokinin. Since we have shown that glucagon increases pancreatic blood flow while inhibiting caerulein, we believe that a competition of glucagon for the receptors in the pancreas is a better explanation for ours and Van Dyck's results. This, however requires that two widely different structures such as those of secretin and cholecystokinin act on the same receptor, contrary to the belief that only chemically related agonists may have a common tissue receptor. Studies are in progress to elucidate this particular aspect of our results.

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NEW SHORT-CHAIN SYNTHETIC CORTICOTROPHIN ANALOGUES WITH HIGH CORTICOTROPHIC ACTIVITY

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During the last years many attempts have been made to modify the ACTH molecule, especially to reduce its chain length.

Various observations led to the conclusion that the sequence 6-9 and 6-10, respectively, play an important role in the biological action of this pituitary hormone.

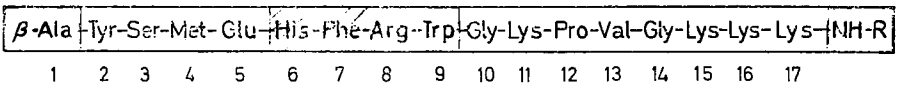
Limited modifications are still allowed within that area, but the consequence of these modifications is a marked loss of ACTH activity, except histidine is replaced by its very similar pyrazole analogue (1). Therefore these observations are not contradictory to the assumption that the topographic situation within this segment is responsible for the release of the actual effect of ACTH, provided that the segment is fixed to a specific receptor by appropriate binding sites.

In figure 1 these correlations are demonstrated by a heptadecapeptide with a slightly varied ACTH sequence. The hatched part is assumed to represent the smallest sequence exhibiting ACTH activity, the white and grey parts are the binding sites of the molecule.

It is known that protection of the amino and the carboxylic ends against enzymatic degradation leads to compounds with increased and prolonged activity. In figure 1 the protection is indicated by the grey parts (2,3,4).

Using this principle we studied the biological activity of ACTH peptides with respect to the contribution of the binding sequences, adjacent to the active sequence.

The amino end was blocked by ß-alanine and, under simultaneous reduction of the chain length, by carboxylic acids which can be considered as deamino amino acids (figure 2), furthermore by acids which are oxa-analogues of such deamino



R= $-(CH_2)_4-NH_2$

Fig. 1 [ß-Ala^1,Lys^{17}]Corticotrophin-(1-17)-heptadecapeptide-(4-amino-n-butyl)-amide

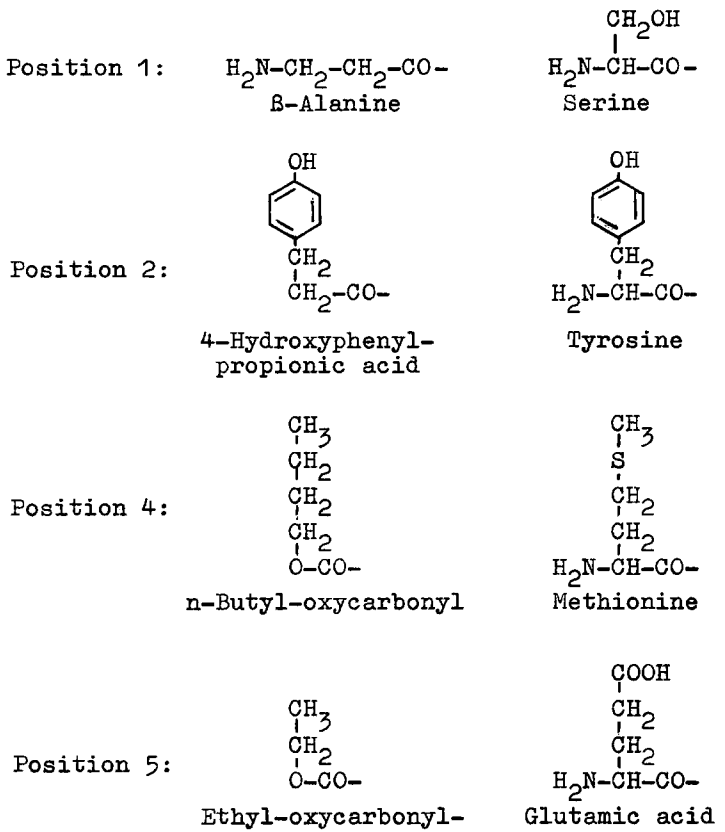


Fig. 2. Exchange of amino acids in ACTH analogues

amino acids.

To protect the carboxylic end we used basic amides which can be considered as derivatives of decarboxylated basic amino acids (figure 3). In these amides the positive charge of the protonated nitrogen of the corresponding amino acid, which seems to be essential for a strong binding of the peptide to the ACTH receptor, is still present.

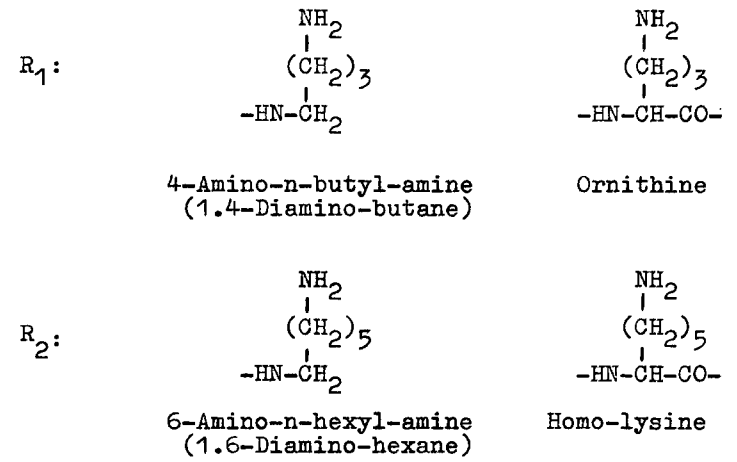


Fig. 3. Exchange of amino acids in ACTH analogues

In contrast to the unsubstituted amides the basic amides offer the advantage of increased stability against enzymatic degradation. Unsubstituted amino acid amides can be enzymatically cleaved from the carboxylic end of a peptide as described by Glass, Schwartz,and Walter (5).

Figure 4 shows the synthesis of the heptadecapeptide mentioned above. In the last step of the synthesis the decapeptide 1-10 is condensed with the C-terminal sequence. This is the known strategy of Schwyzer et al.(6), Hofmann et al.(7), and our group in former syntheses of peptides with ACTH sequence.

In the beginning, when we condensed with DCC, the yields were unsatisfactory, especially when the ε-amino group of lysine in position 11 was protected by Boc which gives rise to steric hindrance of the free α-amino group.

As the DCC-activated decapeptide reacts rather slowly, side reaction can occur with its own unprotected functional groups. Another side reaction is the rearrangement of the primary formed O-acyl-isourea to N-acyl-urea.

The first increase of the yield was achieved by the addition of 4-nitro-phenol. Compounds such as 4-nitro-phenol react with the DCC-activated decapeptide faster than the above mentioned

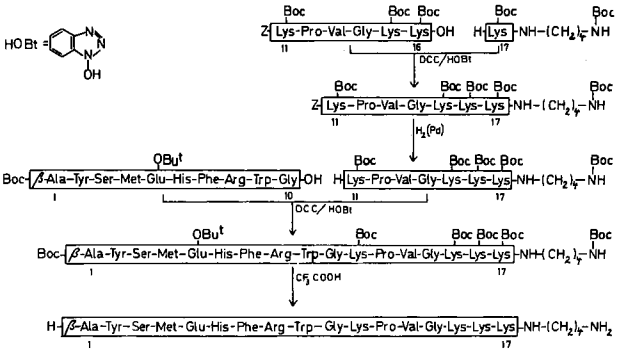


Fig. 4. Synthesis of [ß-Ala^{1},Lys^{17}] Corticotrophin-(1-17)-heptadecapeptide-(4-amino-n-butyl)-amide

rearrangement occurs. On the other hand they form decapeptide derivatives which are active enough to react with the amino component(8).

With pentachlorophenol, alredy used by Bajusz et al.(9,10), we obtained slightly better results. To our surprise the yield was not higher using hydroxysuccinimide instead of pentachlorophenol(3,10). Only when we had learned the outstanding properties of 1-hydroxy-benzotriazole in the DCC-reaction, we could achieve a further increase of the yield.(11). 1-Hydroxy-benzotriazole turned out to be an excellent reagent in the earlier condensation steps too.

In figure 5 some typical compounds are shown which were synthetized by following this scheme.

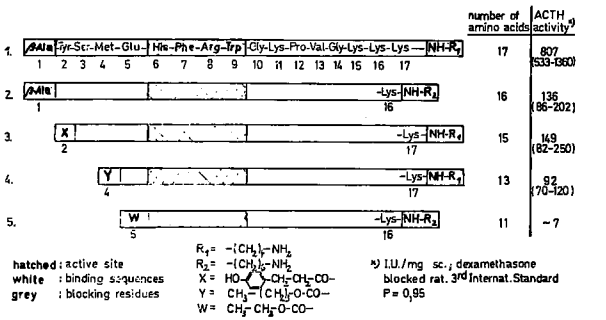


Fig. 5. Constitution and ACTH activity of synthetic corticotrophin analogues

The biological activities of the compounds were estimated by the ascorbic acid depletion test in Dexamethasone blocked

rats. According to our experience this modified Sayers test yields slightly higher values than the original test in hypophysectomized rats, when short-chain synthetic analogues are compared with the 3rd International Standard. But as it is very sensitive and convenient we used this modified test in all these experiments. In some cases, however, we found differences between subcutaneous and intravenious administration, and the steroidogenesis did not always correspond exactly to the ascorbic acid depletion.

The above mentioned compound 1 exhibits 800 I.U./mg and is the most active peptide of the series. When we reduce the chain length from the carboxylic end, we observe a decrease of the ACTH activity to 136 I.U./mg in compound 2 which however is still a remarkably high value for a hexadecapeptide. A similar effect is achieved by reducing the chain length from the amino end to a peptide with only 15 amino acids. This compound 3 exhibits 149 I.U./mg.

Compared to these peptides, the peptide amide with the normal sequence 1-16 exhibits an activity of only 1.4 I.U./mg and the pentadecapeptide amide 1-15 not more than 0.2 I.U./mg(10).

By further elimination of amino acids from the amino end of the chain, we prepared ACTH analogues like the trideca-peptide 4 with still 92 I.U./mg, and the undecapeptide 5. The latter peptide, however, with an activity of only 7 I.U./mg clearly shows the limitations of this procedure. This is no surprise because the degradation has reached now the active sequence of the molecule.

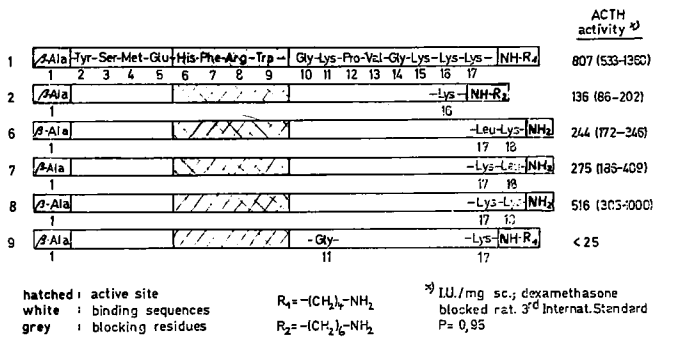


Fig. 6. Constitution and ACTH activity of synthetic corticotrophin analogues

In the compounds 6-9 (figure 6) we studied the contribution of the positive charges in the positions 11, 17, and 18 to the ACTH activity.

When we reduce the number of charges near the carboxylic end from 4 to 3, we find a decrease of the ACTH activity to about one half of the activity of compound 8, in which all charges are still present.

The comparison of compounds 7 and 2 shows, however, that

complete elimination of position 18 leads to a further loss of activity, although the number of charges is still the same. This demonstrates a marked influence of the side chain itself in position 18, whereas the amide group apparently does not play an important role (compare 1 and 8).

Lysine[11] contributes to a surprisingly high degree to the ACTH activity, presumably as a binding site; elimination of the side chain, which means elimination of the positive charge, results in an almost complete loss of activity in compound 9.

These studies show that the two binding sequences 1-5 and 11-17 contribute to a different extent to the binding to a specific ACTH receptor, and therefore to the biological activity of the compounds, the latter being more important than the first. This fact is further demonstrated by compound 9, in which the basic lysine in position 11 is replaced by glycine: the activity is drastically lowered.

That the sequence 6-10 has to be considered as the active sequence in ACTH, is proved by an experiment in which the decapeptide 1-10 is provided with the positive charge, necessary for the binding to the receptor (figure 7).

10 ß-Ala-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-NH-$(CH_2)_4$-NH_2

Fig. 7. [ß-Ala^1]Corticotrophin-(1-10)-decapeptide-(4-amino-n-butyl)-amide

Compound 10 exhibits a small but distinct ACTH activity (0.4 I.U./mg). Since we know that the N-terminal sequence 1-5 contributes to the ACTH activity only as a binding site, we have to look for the actual hormonal information within the sequence 6-10. The role of glycine^{10} is not cleared up by this experiment.

For clinical use compound 1 seems to be the most suitable one, since with respect to the intensity and duration of its effect, this peptide is superior to all other compounds presented here.

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SYNTHESIS OF SCOTOPHOBIN. A MEMORY CODE WORD

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Protein and peptide changes have been implicated in routine neural activity for some time and probably involve conformation structure shifts, as well as specific synthesis and break-down mechanisms. Further, various proteins have been associated with learning and information storage, transmission of data between the hypothalamus region and the pituitary, brain antibodies, nerve growth factors, and other miscellaneous neural compounds.

Specific chemical changes induced in the brain by learning are difficult to demonstrate by ordinary chemical methods. Under these circumstances, a bioassay method is employed in which doner animals are subjected to a training procedure aimed at inducing a specific acquired behavioral pattern. When training is complete, the brains of the donors are removed, extracted, and purified by one or more procedures. These products are administered to recipient animals, which are then tested to learn whether their behavior is influenced by this treatment. The results of the testing are then compared with those obtained with control recipients who have been given brain extracts from untrained donors.

The chemical transfer of learned behavior has been attempted by many groups, and, although the results remain controversial and their interpretation difficult, there appears to be no doubt of their validity. One such technique employs a passive avoidance task, which assumes the ability to obtain a high degree of reproducibility with a reliable dose-response curve. The paradigm is based on the innate preference of rodents for dark over lighted enclosures. The donors are trained to reverse this preference by receiving shocks when they run into a dark chamber. The recipients are tested without shocks and the degree of avoidance is measured by recording the time spent in the dark box both before, and after, injection of the extracts(1). The outstanding features of the experiment are that it allows one to detect a chemical correlate of learned information in the brain of the donors, provides a technique for assaying the material,

and assists in its isolation and characterization. Any potential application to the transfer of learning or induction of specific behavior is of lesser importance, since such a practical use is subordinated to the possibility of producing the substance by chemical synthesis rather than by extraction from the brain.

The procedure for the isolation of the transfer factor was as follows -- brain homogenates were centrifuged and the activity was found in the supernatant. Dialysis of this fraction, followed by concentration of the dialyzate, and precipitation with acetone gave an active material, which was stable to ribonuclease, but was destroyed by trypsin(2,3). Gel filtration and fractionation by thin-layer chromatography yielded an active product. Acid hydrolysis, in combination with a microdansylation procedure allowed the determination of the individual amino acids. A quantitative analysis indicated 15 residues were present in the peptide. After incubation with trypsin, two fragments were found and were subjected to a mass spectral study(4). The final sequence of the pentadecapeptide, which has been given the name "scotophobin" (from the Greek skotos = dark, and phobos = fear) is as follows:

Ac-Ser-Asp-Asn-Asn-Glu-Gln-Gly-Lys-Ser-Ala-Glu-Gln-Gly-Gly-Try-ONH_2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Assuming a synthetic peptide possesses the behavioral effect of the natural material, then the isolation will have been effected for the first member of a very important family of substances that serve for the coding of acquired information in the central nervous system. Whether these substances act by labeling the neural pathways or achieving the registration of experience in some other fashion is not known now, but their existence will considerable enhance our understanding of the processing of information in the brain. From a narrow medicinal chemistry standpoint, the presence in the brain of substances such as the one described points to the possibility of obtaining drugs with highly specific behavioral effects(5).

With these thoughts in mind, a synthesis of scotophobin was designed along the following lines -- first, the presence of glycyl residues at positions 7, 13, and 14 permits one to join smaller fragments here without fear of racemization, while the replication of the tripeptide glutamyl-glutaminyl-glycine at 5-7 and 11-13 allows an economy in the construction of these two units(6).

The actual synthesis proceeded in the following manner, and began with sequence 14-15. N-Benzyloxycarbonyl-O-t-butyl-tyrosine(I) was converted into a mixed anhydride (4-methylmorpholine and isobutyl chloroformate), which on treatment with ammonia gave the corresponding amide (II). In turn, hydrogenation formed the free amine (III), which was coupled with N-benzyloxycarbonyl-glycine (IV) to furnish N-benzyloxycarbonyl-glycyl-O-t-butyl-tyrosinamide (V). This dipeptide was hydrogenated to afford the corresponding amine(VI).

The sequence 10-13 was prepared by joining methyl glycinate (VII) and N-benzyloxycarbonyl-glutamine (VIII), via the mixed anhydride procedure, to yield methyl N-benzyloxycarbonyl-glutaminyl-glycinate (IX). Hydrogenation gave the amine (X), which was combined with N-benzyloxycarbonyl-γ-t-butyl-glutamic acid (XI) through the aid of a mixed anhydride to form methyl N-benzyloxycarbonyl-γ-t-butyl-glutamyl-glutaminyl-glycinate (XII). This tripeptide was deprotected as usual to give the amine (XIII) and reacted with N-benzyloxycarbonyl-alanine (XIV) by a mixed anhydride to yield the desired tetrapeptide (XV). Hydrolysis of XV by dilute alkali in a mixed solvent system produced methyl N-benzyloxycarbonyl-alanyl-γ-t-butyl-glutamyl-glutaminyl-glycinate (XVI).

Sequence 8-15 was obtained as follows: XVI was combined with VI through a water-soluble carbodiimide reagent to give N-benzyloxycarbonyl-alanyl-γ-t-butyl-glutamyl-glutaminyl-glycyl-glycyl-O-t-butyl-tyrosinamide (XVII). Hydrogenation afforded the amine (XVIII). A mixed anhydride coupling of N-benzyloxycarbonyl-ε-t-butyloxycarbonyl-lysine (XIX) with methyl O-t-butyl-serinate (XX) formed the dipeptide (XXI). Hydrolysis produced N-benzyloxycarbonyl-ε-t-butyloxycarbonyl-lysyl-O-t-butyl-serine (XXII), which was joined to the hexapeptide amine XVIII by a water-soluble carbodiimide to yield the octapeptide N-benzyloxycarbonyl-ε-t-butyloxycarbonyl-lysyl-O-t-butyl-seryl-alanyl-γ-t-butyl-glutamyl-glutaminyl-glycyl-glycyl-O-t-butyl-tyrosinamide (XXIII). Removal of the benzyloxycarbonyl group gave the amine (XXIV).

Sequence 3-7 was synthesized in the following manner: tripeptide amine XIII was added to N-benzyloxycarbonyl-asparagine (XXV) by reagent "K" to give methyl N-benzyloxycarbonyl-asparagyl-γ-t-butyl-glutamyl-glutaminyl-glycinate (XXVI). Hydrogenation produced the amine (XXVII), which was joined to XXV by "K" to form the pentapeptide methyl N-benzyloxycarbonyl-asparagyl-asparagyl-γ-t-butyl-glutamyl-glutaminyl-glycinate (XXVII). The hydrolysis of XXVII was unsuccessful, probably due to the low solubility of the starting product. Some cleavage of the asparagyl-asparagyl bond was noted, too, possibly as a result of a transpeptidation rearrangement.

This forced a change in the synthetic scheme, so the tripeptide XII was hydrolyzed to the corresponding acid (XXVIII). In turn, XXVIII was added to the octapeptide amine XXIV to produce N-benzyloxycarbonyl-γ-t-butyl-glutamyl-glutaminyl-glycyl-ε-t-butyloxycarbonyl-lysyl-O-t-butyl-seryl-alanyl-γ-t-butyl-glutamyl-glutaminyl-glycyl-glycyl-O-t-butyl-tyrosinamide (XXIX). Hydrogenation gave the amine (XXX), which was joined to N-benzyloxycarbonyl-asparagine N-hydroxysuccinimide ester (XXXI) to form the dodecapeptide (XXXII). Hydrogenation gave the amine (XXXIII), followed by coupling to XXX, to form N-benzyloxycarbonyl-asparaginyl-asparaginyl-γ-t-butyl-glutamyl-glutaminyl-glycyl-ε-t-butyloxycarbonyl-lysyl-O-t-butyl-seryl-alanyl-γ-t-butyl-glutamyl-glutaminyl-glycyl-glycyl-O-t-butyl-tyrosinamide

(XXXVI). This material, called scotophobin-13, was tested and found to be biologically inactive.

The dipeptide 1-2 was prepared as follows: N-benzyloxycarbonyl-O-t-butyl-serine (XXXVII) was reacted with methyl β-t-butyl-aspartate (XXXVIII), via the mixed anhydride method, to yield the dipeptide (XXXIX). Hydrogenation produced the amine (XL), which on treatment with acetic anhydride yielded methyl N-acetyl-O-t-butyl-seryl-β-t-butyl-aspartate (XLI). Hydrolysis of XLI formed the acid (XLII), purified as the dicyclohexyl ammonium salt (XLIII). A water-soluble carbodiimide coupling in the presence of N-hydroxysuccinimide with XXXV gave N-acetyl-O-t-butyl-seryl-β-t-butyl-aspartyl-asparaginyl-asparaginyl-γ-t-butyl-glutamyl-glutaminyl-glycyl-ε-t-butyloxycarbonyl-lysyl-O-t-butyl-seryl-alanyl-γ-t-butyl-glutamyl-glutaminyl-glycyl-glycyl-O-t-butyl-tyrosinamide (XLIV). Removal of the blocking groups with trifluoroacetic acid, followed by chromatography, yielded the desired pentadecapeptide (XLV). Biological data on this product is, unfortunately, not available at this time.

In conclusion, a synthetic route to scotophobin, a peptide considered to possess memory properties, has been developed. Whether a new and novel field of peptide chemistry is now available for exploration remains to be seen in the near future.

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6. All amino acids are L; satisfactory microanalytical data was obtained from the intermediates; thin-layer chromatography employed silica gel H as the support, acetic acid -n-butanol-water (1:4:1) or chloroform-methanol (9:1) for development, and iodine or ninhydrin for detection.

NEW POLYPEPTIDE WITH MULTIPLE BIOLOGICAL ACTIONS*

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During an investigation of the comparative vasoactivity of peptide fractions from porcine lung and small intestine, a vasoactive intestinal polypeptide (VIP) was extracted (1) and more recently, purified (2). Some chemical characteristics and biological effects of this peptide are here summarized.

CHEMICAL FEATURES

VIP is a strongly basic polypeptide. It is even more basic than secretin, with which it is closely associated through most of the purification procedures. Lacking tryptophan, cysteine/cystine, glycine and proline, VIP has 28 amino acid residues: 2 alanine, 5 aspartate/asparagine, 2 arginine, 1 glutamate/glutamine, 1 histidine, 1 isoleucine, 3 leucine, 3 lysine, 1 methionine, 1 phenylalanine, 2 serine, 2 threonine, 2 tyrosine, and 2 valine.

It is thus distinct from the other biologically active peptides secretin (3), glucagon (4), "substance P" (5) and the kinins (6).

The vasoactivity of VIP is destroyed completely by -chymotrypsin, and almost completely by trypsin, but not by carboxypeptidase B.

On cleavage with cyanogen bromide, an N-terminal octadecapeptide and a C-terminal decapeptide are obtained, both of which are inactive.

BIOLOGICAL ACTIONS

These were determined for the most part in anesthetized dogs, though some effects were duplicated in rabbit and rat.

Cardiovascular System. This is the major site of action of VIP. Bioassay during purification was based on the vasodilator action of the peptide on the femoral arterial circulation of dogs. This action is particularly notable for its relatively long-lasting duration, several times longer than bradykinin.

Aside from peripheral systemic vasodilation (apparent in doses of a few ng per kg), VIP induces a comparable splanchnic vasodilation, a lowering of systemic arterial blood pressure, and an increase in cardiac output. Both heart rate and stroke volume are increased and preliminary evidence suggests that myocardial contractility is also enhanced.

*Supported by grants from Swedish Med. Res. Council, NIH (HE-04226), Am. Heart Assoc., NTRDA & a Career Development Award from NHLI (HE-K_3-18,432).

Respiratory System. VIP stimulates respiration in the same doses that augment total blood flow, and in smaller doses if infused directly into the carotid artery. The respiratory stimulation is due to a direct action of the peptide on the carotid chemoreceptors, and results in increased ventilation (+81 percent), with a drop in arterial blood P_{CO_2} (-3 mm Hg) and a rise in Po_2 (+6 mm Hg).

Glucose Metabolism. VIP has a hyperglycemic effect that is at least 30 percent as pronounced as that of glucagon. This effect is not attributable to B-adrenergic stimulation. In vitro, the peptide increases glycogenolysis in slices of rabbit liver.

Central Nervous System. Deeply anesthetized dogs (pentobarbital) show electroencephalographic evidence of a tendency to wake up after intravenous or intra-carotid infusions of 1-2 mg per kg of the peptide.

Direct Action on Smooth Muscle. As reported earlier (7), VIP has a direct relaxant effect on several isolated smooth muscle organs: rat stomach, chick rectum, chick rectal cecum, guinea-pig trachea and guinea-pig gall-bladder. These actions are not mediated by α- or B-adrenergic receptors, histamine, serotonin or acetyl choline.

Other Actions. VIP also has a moderate secretin-like action on pancreatic secretion, and a choleretic effect. Other possible metabolic actions, e.g., on lipolysis and adenyl cyclase system, have not been investigated.

POSSIBLE SIGNIFICANCE

1. In the normal state, VIP could serve to promote intestinal blood flow during digestion.

Because the peptide is inactivated in the liver, its action is probably limited to the splanchnic bed.

2. If the ability of the liver to take up VIP is impaired, such as in liver failure and after porto-caval shunt operations, the peptide could reach the systemic circulation at sufficiently high levels to produce changes similar to those described above in animals.

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vasoactive polypeptide, intestine, vasodilator, hypotension, splanchnic blood flow, hyperventilation, hyperglycemia, glycogenolysis, smooth muscle, liver failure.

ISOLATION AND STRUCTURAL ELUCIDATION OF OVINE HYPOTHALAMIC THYROTROPIN (TSH) RELEASING FACTOR (TRF)

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In the 1930's, the concept arose that the secretion of hormones from the pituitary gland was controlled by an area of the brain called the hypothalamus (see historical material in(1)). As shown in Figure 1, the location of the pituitary gland is at the base of the brain, just below the hypothalamus which has no precise boundaries but which is located near the third ventricle immediately behind the chiasma of optic nerves. In the sheep, this area weighs about 5 g. The pituitary gland itself is divided into two segments: 1) the neurohypophysis (posterior pituitary gland) which secretes vasopressin and oxytocin, and 2) the adenohypophysis (anterior pituitary gland) which secretes ACTH, or adrenocorticotropin; TSH, or thyroid stimulating hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; GH, growth hormone; and LTH, prolactin.

The concept has been that the hypothalamus produces substances which are transported through a vascular system to the adenohypophysis where they stimulate the release of the various pituitary hormones. Over the years, physiological and anatomical evidence has accumulated which supports the proposed existence of factors which control the secretion or, in the case of prolactin, the inhibition of secretion of each of the pituitary hormones (1). Until very recently, however, the necessary proof of the existence of these substances by their purification and chemical characterization had not been produced.

It is the purpose of this report to summarize the isolation, chemical characterization, and synthesis of one such factor, TRF, which stimulates the secretion of TSH or thyrotropin. The scheme of purification of our most recent batch of TRF from approximately 300,000 sheep hypothalami is summarized in Table I. After 11 stages of purification involving 6 different systems, we recovered from 300,000 brain fragments approximately 1 mg of material having a constant specific biological activity using an *in vivo* mouse assay (2,3,4,5,6) over the last stages of purification (5,6).

The material is extremely potent, releasing TSH into the plasma when injected into the jugular vein of a mouse at doses of 5 ng or less and in *in vitro* experiments, in which excised pituitary glands are incubated in a medium; a few picograms of TRF are sufficient to cause release of TSH into the medium (4); TRF is also effective orally (7,8). The factor shows

a high degree of specificity with regard to the hormone released from the pituitary gland, having no effect on the release of ACTH, LH, or MSH, nor has it shown any oxytocic, smooth muscle, or vasopressin activity (6). It shows little or no species specificity; for example, crude TRF of porcine origin (9) and synthetic TRF (10,11) produce rises of plasma TSH when given intravenously to humans.

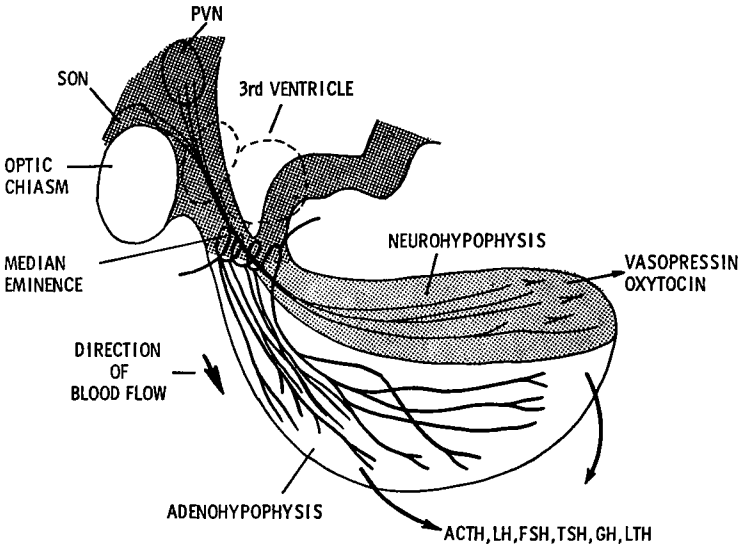

Fig. 1
The hypothalamic area corresponds to the dark stipled zone, around the third ventricle, at the base of the brain; the median eminence is the junction area between hypothalamus and pituitary stalk. SON, supra optic nucleus; PVN, paraventricular nucleus.

In work reported in January 1969 (5), we showed that analysis of 6 N HCl hydrolysates of purified TRF yielded the amino acids His,Glu and Pro in equimolar ratio and that these three amino acids accounted for at least 81% of the weight of the preparation. One can calculate that these amino acids would contribute 86% of the weight of the tripeptide monoacetate molecule. These results confirmed and extended earlier reports (12,13) that these amino acids were the principal ones found in TRF preparations at various stages of purification. Quantitative estimates of the amino acids in these earlier preparations, however, showed a maximum contribution of 30% of the weight by these three amino acids (13).

With our observation (5) that the contribution of these three amino acids to the major portion of the structure of TRF was now quite obvious, we tested for TRF biological activity a series of tripeptide isomers, synthesized by Studer et al.(14), composed of the amino acids Glu, His, Pro in equimolar ratio. We found, as shown in Table II, that the 6 tripeptide isomers in which glutamic acid was linked by the alpha-carboxyl group were devoid of biological activity (4,15), confirming the earlier results of Schally et al. (16).

Reasoning from the observation that TRF was ninhydrin-negative and that no end terminus could be detected by dansylation (17), we presumed that the end terminus was protected.

Table I. Sequence of Purification for Isolation of Ovine TRF

Stage	No. of fragments x 1,000[a]	Weight[b]	TRF units/mg[c]
1. Lyophilized sheep hypothalamus	294	25 kg	
2. Alcohol-chloroform extract	294	294 g	1
3. Ultrafiltration 'UM-3'	294	71 g	3
4-5. Gel filtration, 'Sephadex G-25', 0.5 M acetic acid, 2X	286	16 g	16
6-7. Partition chromatography, 0.01 percent acetic acid-n-butanol-pyridine(11:5:3),2X	280	246 mg	800
8-9. Adsorption chromatography, Norit/H_2O-ethanol-phenol,2X	275	4.2 mg	30,500(1,100-200,000)
10. Partition chromatography, n-butanol-acetic acid-H_2O (4:1:5, upper phase)	273	2.0 mg	58,500(12,500-150,000)
11. Repeat 10	270	1.0 mg	57,000(12,000-124,000)

a) Number of hypothalamic fragments available at each step in the purification sequence; the continuous decrease in number from step 4 to 11 results from the removal of aliquots for pilot studies, repeated bioassays, and so on.

b) From step 3 to 11, the weights correspond to those of the TRF-active fractions at each step.

c) Specific activity (TRF U/mg) with 95 percent confidence limits in multiple four-point assays.

Table II. TSH-releasing activity of reaction products of synthetic tripeptides with acetic anhydride.

Peptide	TRF Activity[a] Untreated	Acetylated
H-Glu-His-Pro-OH	-	**
H-Glu-Pro-His-OH	-	-
H-Pro-His-Glu-OH	-	-
H-Pro-Glu-His-OH	-	-
H-His-Pro-Glu-OH	-	-
H-His-Glu-Pro-OH	-	-

a) All untreated peptides were given at doses of 20 to 300 μg/animal and acetylated peptides at 20 to 150 μg/animal in the mouse *in vivo* assay; -, not significant; **, $p \leq 0.01$, in the multiple comparison test of Dunnett (see 3).

Therefore, as one approach to the preparation of an active synthetic material we acetylated the individual tripeptides using acetic anhydride and tested the crude product mixtures for TRF biological activity. One, and only one, of these derivatives, that derived from the sequence Glu-His-Pro, had biological activity (Table II). After purification by column chromatography, we found several active fractions. One of the major fractions was shown on the basis of its comparison with totally synthetic material provided by Studer *et al.* (14), to be the pyroglutamyl derivative, that is pyroglutamyl-histidyl-proline (PCA-His-Pro-OH).

Although the compound PCA-His-Pro-OH had some biological activity, it was only 1/5000th as potent as the natural product. We then reasoned that TRF might be amidated on the carboxyl end, as is the case with a number of peptide hormones. We prepared a small amount of this material by first making the methyl ester and then converting it by ammonolysis to PCA-His-Pro-NH_2. The results of tests of biological activity are shown in Table III; it can be seen that the formation of the methyl ester increases the potency considerably and that amidation produced material which is not significantly different in biological activity from the natural product. The activity of the amide was confirmed using a totally synthetic compound prepared by Gillessen et al.(14). N-α-Acetyl Glu-His-Pro-OH, the major product of the acetylation reaction, had no activity.

Table III. Biological activity of ovine TRF and synthetic analogs.

Compound	TRF Activity
Ovine TRF	55,000 ± 9,900 U/mg
PCA-His-Pro-NH_2 (synthetic)	44,000 ± 1,100
PCA-His-Pro-OMe	28,600 ± 6,700
PCA-His-Pro-OH	10
H-Glu-His-Pro-OH	0
Nα-Ac-Glu-His-Pro-OH	0

Biological activity of ovine and porcine TRF is not destroyed by incubation with such proteolytic enzymes as trypsin, pepsin, pronase, carboxypeptidase A and B, or leucine aminopeptidase (18,19) and as further shown by Schally et al., biological activity of porcine TRF is not destroyed by papain or subtilisin (19). Resistance to proteolytic enzymes, together with other properties of TRF, such as failure to show an N-terminus, low content amino acids in the cruder products, etc., had earlier led several of us working in this field to consider the concept that TRF might not be a simple homomeric polypeptide (13,18,19).

An enzyme isolated by Robert Fellows and associates, pyroglutamyl-peptidase (PCA-ase), which cleaves pyroglutamic acid from peptides, does however completely destroy the biological activity of TRF. Treatment of PCA-His-Pro-OH and PCA-His-Pro-NH_2 by PCA-ase, produces a ninhydrin positive product which moves with a different mobility than the starting material and which shows a histidine N-terminus by dansylation and hydrolysis by the method of Woods and Wang (20) as shown in Table IV. Ovine TRF behaved the same as synthetic PCA-His-Pro-NH_2 in this system, thereby giving us our first direct evidence of the structure of the natural material (6).

Table IV. Effect of Treatment with PCA-ase on Mobility of Pauly Positive Zone on TLC[a].

Compound	Buffer Control		After PCA-ase	
	R_f	Ninhydrin	R_f	Ninhydrin
H-Glu-His-Pro-OH	0.18	+	0.19	+
Nα-Ac-Glu-His-Pro-OH	0.55	-	0.56	-
PCA-His-Pro-OH	0.32	-	0.14	+
PCA-His-Pro-NH_2 (synthetic TRF)	0.29	-	0.45	+
Ovine TRF	0.24	-	0.45	+

a) Silica gel, 1:1:1:1 n-butanol: ethyl acetate: acetic acid:water

We were able to show the identity of the totally synthetic PCA-His-Pro-NH_2 with TRF by a number of physical and chemical criteria. The natural and synthetic compounds are essentially identical in five different thin layer chromatography systems and in their infrared (Fig.2) and NMR (Fig. 3) spectra. Support for the presence of an amide group is also given by quantitative analysis of the ammonia content (Table V).

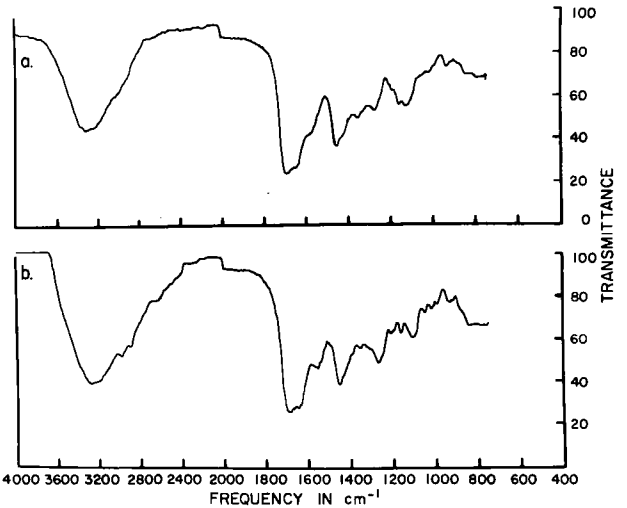

Fig. 2
Infrared spectra of (a) isolated ovine TRF and (b) synthetic PCA-His-Pro-NH_2, deposited as a film on Irtran-2; Beckman IR-9.

Table V. Ammonia and Histidine Contents in 6 M HCl Hydrolysates of Isolated Ovine TRF and Synthetic Peptides.

	No. of determinations	nmoles His	nmoles NH_3[a]	NH_3/His
Synthetic PCA-His-Pro-NH_2	6	38.4 ± 4.6[b]	40.3 ± 6.1	1.07
Synthetic PCA-His-Pro-OH	5	39.1 ± 3.3	0.4 ± 3.8	0.01
Ovine TRF	2	20.5 ± 2.1	22.1 ± 1.0	1.08
Ovine TRF unhydrolyzed	1	—	−2.0	—

a) Corrected for exogenous NH_3 in blank hydrolyzed with each determination; average blank=15.0 ± 5.7 nmoles NH_3 for seven determinations.

b) Standard error.

Dr. Desiderio has already quite extensively described in a paper at this meeting the role of low resolution and, even more significantly, of high resolution mass spectrometry, in the elucidation and indeed the proof of the structure of TRF. Figure 4, for example, shows a comparison of the methylated derivatives of natural and synthetic TRF as obtained by a low resolution mass spectrometer (6). We have, in addition, been able to

obtain a molecular ion of underivatized TRF in two different systems, one using chemical-ionization mass spectrometry in an MS-9 spectrometer in collaboration with Dr. Henry Fales at NIH, and recently in collaboration with Dr. Nicholas Ling, using our own Varian Mat CH-5 system at the Salk Institute. There is, therefore, conclusive evidence for the structural assignment PCA-His-Pro-NH_2 for ovine TRF (Fig. 5).

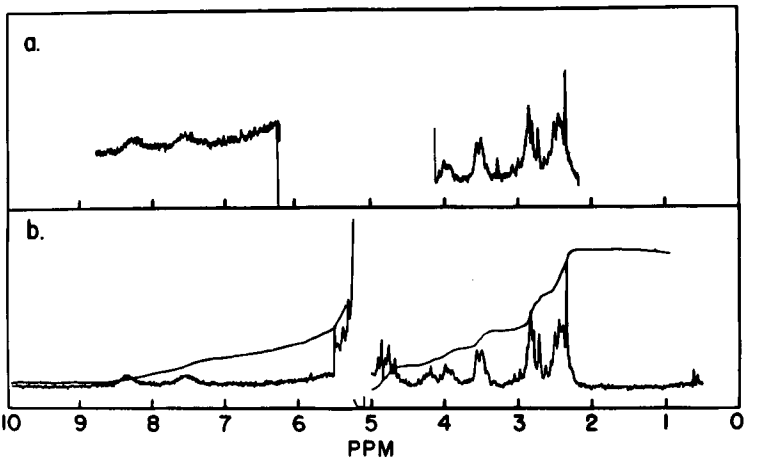

Fig. 3
Proton magnetic resonance spectra (100 MHz) of (a) ovine TRF acetate in D_2O, time-averaged for 276 scans, 500 s/scan, 500 Hz sweep width in regions shown, lock signal internal HDO (5.14 p.p.m. referenced to external capillary of tetramethylsilane in a separate experiment); (b) synthetic PCA-His-Pro-NH_2 (acetate) in D_2O, single scan 1,000 s/scan, sweep width 1,000 Hz; referenced to an external capillary of tetramethylsilane. Both spectra were obtained at 31°C.

While this work was being reported, Schally and co-workers were confirming our observations on the structure of ovine TRF with their closely related comparison of biological and chemical properties of porcine TRF with synthetic products (see 21), culminating in proof that the structure of porcine TRF is identical to that of ovine TRF (22).

Structural-functional studies on this molecule are still limited, but as shown in Table III, the total sequence PCA-His-Pro- is necessary for biological activity and the amide group is necessary for total biological activity. It would appear that a more nonpolar N-terminus favors biological activity over a charged N-terminus such as is the case with free acid. However, more extensive studies on the nature of the N-terminus have to be carried out in order to draw a conclusion on this point. The possibility of a glutaminyl N-terminus in the natural product prior to isolation has not been entirely ruled out (4,6); however, Folkers et al. (23) have reported that synthetic Gln-His-Pro-NH_2 has only a fraction of the biological activity of PCA-His-Pro-NH_2.

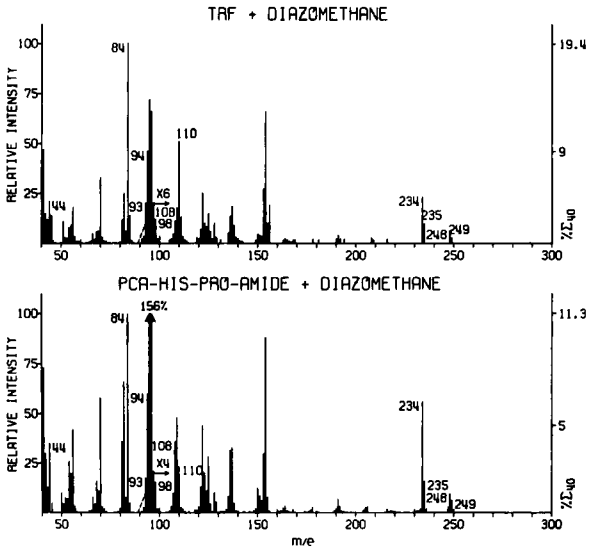

Fig. 4
Low resolution mass spectra of methylated ovine TRF and synthetic PCA-His-Pro-NH_2; LKB 9000; direct probe.

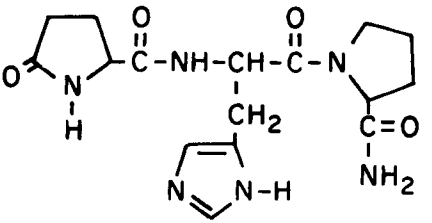

Fig. 5
Structure of ovine TRF.

In conclusion, one of the factors responsible for hypothalamic control of the pituitary gland, thyrotropin releasing factor, has been isolated, characterized, and prepared by total synthesis, thus opening the way to physiological and clinical studies not only on this releasing factor but, hopefully, for use as model studies on other hypothalamic factors controlling the secretion of the pituitary gland.

This work was supported in part by NIH Research Grants (AM 08290-01-06, HD 02577-01) and a Population Council grant (M68.0118).

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STRUCTURAL STUDIES ON NEOCARZINOSTATIN, AN ANTITUMOR POLYPEPTIDE ANTIBIOTIC*

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Neocarzinostatin (NCS), an antitumor polypeptide,was first isolated from the culture filtrate of *Streptomyces carzinostaticus* by Ishida *et al.* (1) during a screening program for antitumor antibiotics of low toxicity. To provide some background information, the known biological effects of NCS and several aspects of its mechanism of action are first briefly reviewed, followed by a description of the physical and chemical properties of NCS and by the account on our sequence analysis. The final section describes a convenient system, which has been developed in the course of this work, for the reduction of disulfide bonds in peptides and proteins and for S-alkylation.

Biological Effects of Neocarzinostatin. NCS has typical antibiotic characteristics. It is produced by a streptomyces strain and it possesses antimicrobial activity against some Gram-positive bacteria, *e.g. Sarcina lutea, Staphylococcus aureus,* or *Bacillus subtilis* (1).

NCS is highly effective against many experimental tumors in mice, including leukemia L1210 (2), ascitic leukemia SN36, ascitic sarcoma 180, or Ehrlich ascites tumor, at dose levels of 0.1 to 3.2 mg/kg/day x 6 (1). In these systems NCS has very favorable therapeutic indices ranging from 30 to 50. Tests carried out *in vitro* showed that it is selectively cytotoxic against mammalian tumor cell cultures and that normal mammalian cells are only affected at approximately 170 times higher doses (1). This striking selectivity appears to be due to differences in uptake of the polypeptide by the cells (3). Clinical tests began in Japan in 1967; the available preliminary results (4) are very encouraging. Table I lists some typical values for the biological activities of NCS.

The principal action of NCS on susceptible mammalian cells is arrest of mitosis at the preprophasic stage (G2 phase) of the cell cycle, *e.g.* in sarcoma 180 *in vivo* (5) and in HeLa cells *in vitro* (6,7). The primary effect is followed by gradual induction of chromosomal aberration, such as scattering, adhesion or aggregation, and by giant cell formation.

Studies on the molecular mechanism of action revealed that deoxyribonucleic acid (DNA) is the principal target of NCS action (7-9). Two

*This work is part of a collaborative project with Dr. Nakao Ishida, at the Dpt. of Microbiology, Tohoku University School of Medicine, Sendai, Japan.

Table I. Some Biological Activities of Neocarzinostatin (1,3)

In vitro[a]		
HeLa cells (ID_{50})	0.1-0.3	µg/ml
Normal primate cells: Monkey kidney, NS11 (Minimum degeneration dose)	50.0	µg/ml
Antibacterial activity (MIC)		
Sarcina lutea	0.2	µg/ml
Bacillus subtilis	32	µg/ml
Mycoplasma pulmonia	0.2	µg/ml
Minimum inducing concentration for *E. coli* phage λ (W1709)	0.025	µg/ml
In vivo[b]		
Toxicity (LD_{50}, mouse, i.p.)	2 - 5	mg/kg
Antitumor activity (MED, mouse)		
L1210	0.08	mg/kg/day x 13
Sarcoma 180 (ascites)	0.1-1.6	mg/kg/day x 6
Leukemia SN36 (ascites)	0.2-1.6	mg/kg/day x 6
Pyrogenicity (Max. nonpyrogenic dose, rabbit)	1	mg/kg/day
Therapeutic index	30-50	

[a] Purified neocarzinostatin, [b] Crude neocarzinostatin

independent effects have been observed: (a) selective inhibition of DNA synthesis without affecting ribonucleic acid (RNA) or protein biosynthesis, and (b) degradation of existing DNA.

The selective inhibition of DNA synthesis (a) is evident in Fig. 1 showing incorporation studies with radioactive precursors of macromolecular synthesis in a susceptible bacterium, *Sarcina lutea*. The incorporation of [^{14}C]thymidine into DNA was inhibited within a few minutes after the addition of the polypeptide, and the inhibition continued throughout the experimental period at concentrations above 0.5 µg/ml (Fig. 1A). The rates of RNA or protein synthesis, as measured by the incorporation of [^{14}C]uridine into RNA (Fig. 1B) or of [^{14}C]leucine into protein (Fig. 1C), were not inhibited for several hours (8). Similar results were obtained with HeLa cells (7).

Degradation of pre-existing DNA (b) occurs in *S. lutea* to an extent unknown for any other antibiotic (8). To measure this effect bacteria were grown in which the DNA was labeled by [^{14}C]thymidine. Fig. 2 shows the effects of various doses of NCS on the degradation of the prelabeled DNA, as measured by the radioactivity released into the acid-soluble fraction (Fig. 2A) and by that remaining in the DNA (Fig. 2B). For comparison the effects of mitomycin C, one of the few antibiotics known to cause DNA breakdown (10), on *S. lutea* DNA are shown. The potency of NCS in causing DNA degradation is several orders of magnitude higher than that of mitomycin C (approximately 100-fold on a weight basis or about 5000-fold on a molar basis). However, contrary to mitomycin C, which reacts with DNA (in *E. coli*) by forming covalent cross-links (11), NCS does not interact directly with DNA (8). DNA from NCS-treated cells does not differ from normal DNA in its physicochemical properties (8). When inhibitors of protein biosynthesis such as chloramphenicol or puromycin were administered simultaneously with NCS, the degradation of DNA was prevented (9). NCS thus seems to induce *de novo* protein synthesis as a requirement for effecting DNA breakdown. NCS itself exhibits no nuclease activity and does not degrade DNA *in vitro* (8).

The available experimental evidence suggests that the observed biological effects of NCS are caused by some indirect mode of action. This polypeptide antibiotic might function as an inducer or derepressor or some

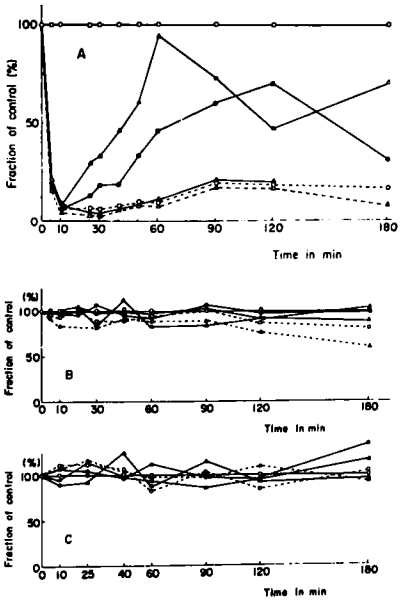


Fig. 1. Effect of neocarzinostatin on the incorporation of radioactive precursors into macromolecules by *Sarcina lutea*. Neocarzinostatin was added to the bacterial suspension at time 0. The radioactivity incorporated into macromolecules is plotted in percent of the untreated control. o——o, control; Δ——Δ, 0.1 μg/ml; •——•, 0.2 μg/ml; ▲——▲, 0.5 μg/ml; o---o, 2 μg/ml; Δ--Δ, 10 μg/ml. (A) [^{14}C]Thymidine incorporation into DNA, (B) [^{14}C]uridine incorporation into RNA, (C) [^{14}C]leucine incorporation into protein. [From (8)].

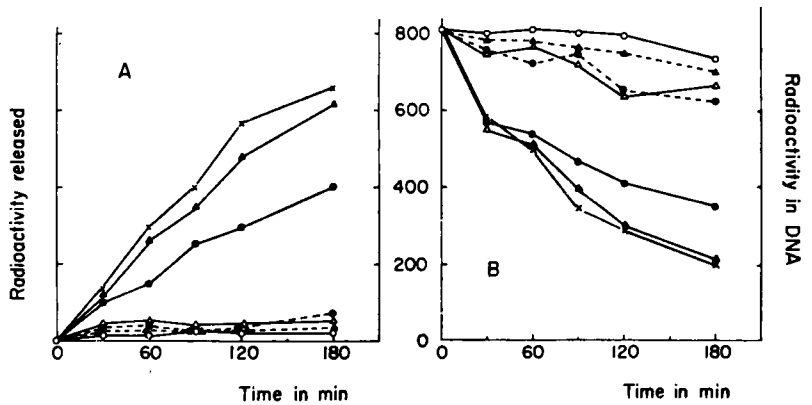


Fig. 2. Degradation of DNA by neocarzinostatin and by mitomycin C. Exponentially-growing bacteria were labeled with [^{14}C]thymidine and then exposed to neocarzinostatin and mitomycin C: o——o, untreated control; Δ——Δ, NCS, 0.1 μg/ml; •——•, NCS, 0.5 μg/ml; ▲——▲, NCS, 2 μg/ml; ×——×, NCS, 10 μg/ml; ▲---▲, mitomycin, 5 μg/ml; •---•, mitomycin, 10 μg/ml. (A) Release of radioactive DNA fragments into the acid-soluble fraction at the times indicated, (B) Decrease in radioactivity of DNA. [From (8)].

other type of regulatory agent for cellular events. Its action at a concentration range of 10^3 to 10^4 molecules per bacterial cell might indicate interaction with DNA-polymerase. Further experiments will be required to clarify the precise mechanism of action of neocarzinostatin.

Isolation and Characterization of Neocarzinostatin. NCS is produced by a microorganism, *Streptomyces carzinostaticus*, variant F-41* (1) and released into the culture beer. The isolation from the concentrated culture filtrate is carried out by ammonium sulfate precipitation, followed by dialysis and Sephadex G-50 chromatography to give crude NCS as a brownish powder. Material of this degree of purity has been used for clinical tests. Further purification for chemical studies is achieved by twice-repeated column chromatography on carboxymethyl cellulose by either gradient or stepwise elution using 0.1 *M* sodium acetate buffers pH 3.2 and pH 3.5 followed by desalting by dialysis and Sephadex G-50 gel filtration in 0.1 *M* acetic acid. The isolation and purification procedures are conveniently monitored by antibacterial assays using *S. lutea* agar plate cultures.

Purified NCS is obtained as an almost colorless powder which has been characterized (13) as homogeneous by (a) ultracentrifugation at pH 4.8 or 8 giving single symmetric peaks, (b) polyacrylamide gel electrophoresis at pH 4.6 and 8.2, (c) ion exchange chromatography (CM-cellulose), (d) Sephadex gel filtration, and (e) N-terminal end group analysis showing a single end group, alanine.

Physical and chemical properties of NCS are listed in Table II. These

Table II. Physical and Chemical Properties of Neocarzinostatin (13,14)

Nature	Acidic single-chain polypeptide
Source	*Streptomyces carzinostaticus*, var. F-41, culture filtrate
Molecular Weight	~9000 (from sedimentation and from amino acid composition)
Sedimentation Coefficient	$S_{20,W}$ = 1.44 S
Diffusion Constant	$D_{20,W}$ = 1.4 x 10^{-6} cm^2/sec
Partial Specific Volume	V_{ap} = 0.731 ml/g (from amino acid composition)
UV Spectrum	$E_{280\ nm}$ 12.8 (in 0.1 *N* NaOH), maximum at 277 nm (in 0.1 *N* HCl)
Chemical Reaction	Positive: Biuret, Folin, Ninhydrin, Sakaguchi, Pauly Negative: Molisch, Anthrone, Ellman (free SH)
End Groups	N-term., Alanine; C-term., Asparagine
Amino Acid Composition	Common amino acids, all of L configuration, no His, no Met
Conformation	Essentially no α-helix, fixed aromatic side-chains (from ORD, CD)

data show that NCS is an acidic polypeptide with a molecular weight of about 9000. Its isoelectric point lies at approximately pH 3.5. NCS gives a typical protein ultraviolet spectrum; the optical rotatory dispersion and circular dichroism spectra (14) show essentially no α-helix content. Negative reactions for carbohydrate prove that NCS is not a glycoprotein.

*When it became apparent that the original antitumor agent, carzinostatin (12), could not be prepared as a single homogeneous compound but remained consistently a two-component mixture, single spore isolation was carried out (1) giving the F-41 variant. The antitumor activity released into the culture beer by this variant was contained in a single entity, a polypeptide designated neocarzinostatin.

The amino acid composition given in Table III is remarkable for its

Table III. The Amino Acid Composition (13) of Neocarzinostatin

Lysine	1	Alanine	15 (14)
Histidine	0	Half-cystine	4
Arginine	2 (3)	Valine	9 (10)
Aspartic acid	10	Methionine	0
Threonine	10	Isoleucine	1
Serine	8	Leucine	5
Glutamic acid	4	Tyrosine	1
Proline	4	Phenylalanine	4
Glycine	13 (12)	Tryptophan	2
TOTAL 93	Amide 5 (6)	Calcd N, 15.9 Found N, 15.9	

high content of glycine, alanine, and the hydroxyamino acids serine and threonine. In a total of 93 constituent amino acid residues NCS contains no histidine nor methionine. The four half-cystine residues form two disulfide bonds since free sulfhydryl groups are absent according to negative results (13,16) with the Ellman reagent [5,5'-dithiobis(2-nitrobenzoic acid)] (17). Two tryptophan residues were determined (18) by Koshland's method using 2-hydroxy-5-nitrobenzyl bromide and by Scoffone's method using 2-nitrophenylsulfenyl chloride.

NCS was found to be resistant to enzymatic degradation by various proteolytic enzymes, such as trypsin, chymotrypsin, pepsin, papain, nagarse, pronase, leucine aminopeptidase or carboxypeptidase. Fig. 3 (lower curve)

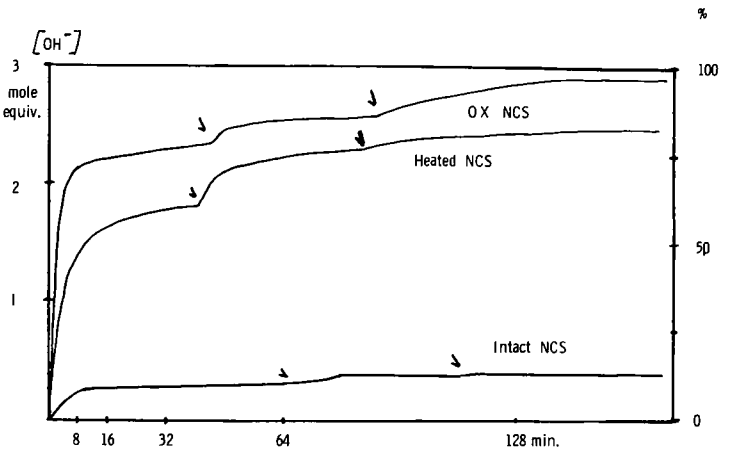


Fig. 3. The action of trypsin on native (intact), heat-denatured (heated), and performic acid-oxidized (OX) neocarzinostatin (NCS).

shows the resistance of NCS to treatment by trypsin for several hours. It could be established that NCS is not an inhibitor of proteolytic enzymes (13). D-Amino acid residues can also not account for the resistance of NCS to proteolytic digestion. The absence of D-amino acid residues from NCS was shown by negative results of assays with D-amino acid oxidase (13). Furthermore, NCS does not contain any unusual amino acid residues of N-methylated or unsaturated character. The resistance of NCS to degradation by proteolytic enzymes may be caused instead by some tightly packed

conformation. Support has been lent to this assumption by our observations of the strikingly unusual resistance of NCS to certain chemical modifications, such as disulfide bond reduction which will be described below.

NCS differs from most other peptide antibiotics by (a) the absence of D-amino acid residues, by (b) the absence of unusual amino acid residues and by (c) its relatively large size. It has been reported that several of those peptide antibiotics that contain unusual and D-amino acid residues are synthesized through unknown types of enzyme complexes, different and independent from messenger RNA-ribosomal systems (19). It is interesting therefore that NCS seems to contain only those amino acid residues that commonly occur in proteins and that these are of the L configuration.

Structural Studies on Neocarzinostatin. NCS contains one lysine and two arginine residues (Table III) and should be cleaved into four fragments by tryptic digestion. Since native NCS could not be cleaved by trypsin (Fig. 3), we attempted to reduce its two disulfide bonds completely and to alkylate the generated thiols. NCS exhibited a striking resistance to this chemical modification. Under standard conditions for complete protein disulfide bond reduction (20), *e.g.* in the presence of urea or guanidine hydrochloride at pH 8 to 9, essentially no or very little reduction was obtained in NCS by mercaptoethanol, dithiothreitol or sodium borohydride, see Table IV. Only under very drastic conditions, using

Table IV. Attempts at Disulfide Bond Reduction and S-Alkylation of NCS

No.	Solvent, pH	Reducing agent (moles per SS)	Reduction time (hr.)	Temp. (°C)	Alkylating agent	Alkylation time (hr.), temp (°C)	Reduction and Alkylation (%)
1	P, 8.4	DTT (10)	1.5	25°	ICH_2COOH	0.2, 25°	2.5
2	P-8MU, 9.1	$NaBH_4$ (27)	17	4°	DTNB	0.5, 25°	Undetected
3	P-5MG, 8.4	DTT (13)	1.5	25°	ICH_2CONH_2	0.25, 25°	8
4	P-5MG, 8.4	ME (320)	5	25°	ICH_2CONH_2	1, 25°	12
5[a]	P-8MU, 8.1	ME (90)	2	50°	ICH_2CONH_2	1, 25°	75
	Repeated	ME (400)	1	50°	ICH_2CONH_2	1, 25°	
6	liquid NH_3	Sodium (2)	0.25	-33°	Benzyl-Cl	1, -33°	95
7	liquid NH_3	DTT (20)	2	-33°	Benzyl-Cl	2, -33°	100

[a] Heat denatured NCS. P, 0.1 M Phosphate buffer; 8MU, 8 M urea; 5MG, 5 M guanidine x HCl; DTT, dithiothreitol; ME, mercaptoethanol.

heat-denatured NCS, elevated reaction temperature, very large excess of reducing agent, and twice-repeated treatment, could a 75% reduction and alkylation* be obtained (Table IV, No. 5) but the product was very impure and heterogeneous.

Most of our past studies were therefore done on performic acid-oxidized NCS or on heat-denatured NCS which were both easily digested by trypsin giving cleavage of three peptide bonds within two to six hours, see Fig. 3. Examples of patterns obtained by peptide mapping of these tryptic digests are shown in Fig. 4A and 4B. Many more spots were obtained than expected from four tryptic peptides. Nevertheless, the tryptic peptides T_2 and T_3 from the N-terminal part of the molecule could be isolated and purified by repeated ion exchange chromatography and Sephadex gel filtration. Tryptic

*Degrees of reduction and alkylation were mostly determined by measuring the amount of S-alkylcysteine in the amino acid analyzer after acid hydrolysis. Thiol determination by Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)] (17) agreed well with the results from amino acid analysis acc. to (15).

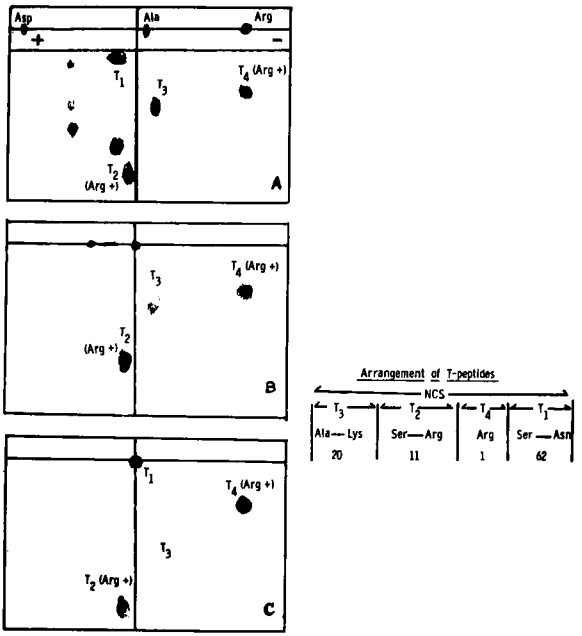


Fig. 4. Peptide maps of tryptic digests: (A) from heat-denatured NCS, (B) from performic acid-oxidized NCS, (C) from tetra-S-benzyl NCS.

fragment T_4 was found to be arginine. The partial sequences of the peptides T_2 and T_3 were established by standard procedures such as subfractionation by chymotryptic digestion, subtractive and dansyl-Edman degradation, and stepwise degradation by leucine amino peptidase and by carboxypeptidase. Peptide T_3 with 20 amino acid residues containing C-terminal lysine was identified as the N-terminal part of the molecule by determining that the N-terminal tetrapeptide sequence H_2N-Ala-Pro-Pro-Thr- was identical with that from native NCS. Peptide T_2 with 10 amino acid residues possessed a C-terminal arginine. Carboxypeptidase treatment of both heat-denatured NCS and of tetra-S-benzyl NCS (see below) gave the sequence of the C-terminal hexapeptide of NCS as being -Ser-Val-Ala-Ile-Phe-Asn-OH (21). The partial sequence which has been deduced from these studies is shown in Fig. 5.

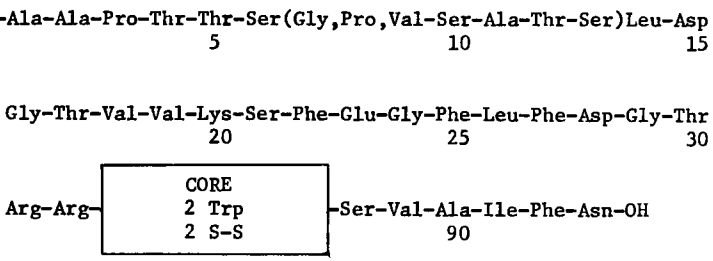


Fig. 5. Partial sequence of neocarzinostatin

The purification of the core region, tryptic peptide T_1 containing 61 amino acid residues, and of its chymotryptic subfragments, presented considerable difficulties. Each peptide consisted of a mixture containing many closely related peptides. Purification of each peptide required up to seven consecutive column chromatographic purifications resulting in heavy losses of material. We attribute the observed complexity obtained from oxidized NCS to the effect of performic acid (22) on the two tryptophan residues in T_1 leading to various different stages of oxidation. The tryptic core fragment obtained from heat-denatured NCS also gave rise to a multiplicity of peptides indicating additional side reactions of different nature.

At this stage it became evident that the conventional procedures of disulfide bond modification were not adequate for the sequence analysis of NCS. We decided to reexamine reduction and alkylation. The well-known sodium in liquid ammonia system, developed by du Vigneaud *et al.* for disulfide bond reduction (23), was applied. NCS dissolved readily in liquid ammonia; after treatment with sodium and addition of benzyl chloride a quantitative yield of tetra-S-benzyl NCS, giving a correct amino acid analysis, was obtained (see Table IV, No. 6). Thus, this procedure allowed complete reduction and alkylation under mild conditions. However, it has been reported that sodium in liquid ammonia treatment can give rise to peptide bond cleavage at proline residues (24) by Birch-type reduction. Examination of our S-benzylated product by amino end group analysis using dansyl chloride (25) revealed indeed that peptide bond fission had occurred at two or three different positions.

We therefore examined the reduction of disulfide bonds using mercaptoethanol, sodium borohydride, or dithiothreitol in liquid ammonia. Dithiothreitol [Cleland's reagent (26)] gave the best results. Essentially complete reduction was obtained with 20 molar excess of dithiothreitol in liquid ammonia at its boiling point temperature (about -33°) and quantitative benzylation was obtained by adding benzyl chloride (see Table IV, No. 7). A peptide map obtained from the tryptic digest of this material is shown in Fig. 4C. It contains essentially four spots as expected: T_3 being the N-terminal fragment which is followed by T_2. T_4 is a single arginine residue. T_1 contains the core fragment. It appears that the reduction of the disulfide bonds of NCS by dithiothreitol in liquid ammonia and the subsequent S-benzylation with benzylchloride proceeds essentially quantitatively without detectable side reactions. We hope that the tryptic core fragment obtained from tetra-S-benzyl NCS will provide uniform subfragments for completing the primary structure evaluation of NCS. For the final assignment of the positions of the disulfide bonds we have recently developed a new procedure based on the detection of cystine-containing peptides on peptide maps by 5,5'-dithiobis(2-nitrobenzoic acid) after reduction of the disulfide bonds by sodium borohydride (16,27).

A Convenient System for the Reduction of Disulfide Bonds in Peptides and Proteins and for S-Alkylation. The ease and convenience of the reduction of the disulfide bonds of NCS by dithiothreitol and the ready alkylation compared to the extreme difficulties which we encountered in aqueous phase led us to investigate whether this system can be used for other proteins as well. Furthermore, various other reagents for thiol alkylation were examined, in particular those leading to such useful derivatives as S-carboxymethyl, S-carbamidomethyl and S-aminoethyl derivatives. Some results are listed in Table V. Besides NCS, insulin and lysozyme were easily dissolved in liquid ammonia. Complete reduction was obtained by treatment with 20 equivalents of dithiothreitol per disulfide bond for periods of 2 to 6 hours in boiling liquid ammonia under reflux. So far, best alkylation results have been obtained with alkyl chlorides. Solid

Table V. Protein Disulfide Bond Reduction by Dithiothreitol in Liquid Ammonia and S-Alkylation.

Protein	Solubility in liq NH_3	SS Bonds	Reaction Time hr*	Alkylating Agent	S-Alkyl Cysteine	Conversion %	Unreacted Cystine	Solubility 0.1 M NH_4OH	S-Alkyl Protein aqueous
Neocarzinostatin	+++	2	2-4	$Cl-CH_2C_6H_5$	3.8	95	---	++	++ at pH 7
			3	$Cl-CH_2COOH$	4.2	100	---	++	++ at pH 7
Insulin	+++	3	2	$Cl-CH_3$	6.0	100	---	-	70% Formic Acid
			2	$Cl-CH_2C_6H_5$				+	
Lysozyme	+++	4	2-3	$I-CH_2CONH_2$	7.8	98	---	+	++ 0.1 M HOAc
				$I-CH_2COOH$	7.5	94	---	+	+ 50% HOAc
				$Br-C_2H_4-NH_2$	7.6	95	---	+	+ 0.1 M HOAc
Human Growth Hormone	--	2	24	$Cl-CH_2C_6H_5$	trace		not calc		
			4**	$Cl-CH_2C_6H_5$	3.7	93	---	-	--
Ribonuclease	- +	4	4-5	$Cl-CH_2C_6H_5$	3.8	48	2.0	-	--

*20 Equivalents of dithiothreitol per SS bond. **1000 Equivalents of dithiothreitol.

chloroacetic acid or chloroacetamide* (in 10-fold molar excess over the amounts of reducing agent used) were added to the liquid ammonia solution to give the S-carboxymethyl or the S-carbamidomethyl derivatives. To obtain S-benzyl substitution, liquid benzyl chloride is added through a pipette, and, even more conveniently, quantitative S-methylation can be achieved by bubbling a gas stream of chloromethane from a cylinder through the liquid ammonia solution for 1 to 2 minutes. Chemical cleavage of peptides at S-methylcysteine residues has been described by Gross *et al.* (28); however, a convenient mild procedure for the quantitative S-methylation of proteins had not been available. To isolate the reduced and alkylated peptide or protein derivatives, the liquid ammonia is allowed to evaporate to a small volume. The final 20 to 60 ml are shell-frozen in a liquid nitrogen bath and removed by freeze-drying under water pump vacuum with a KOH-drying tube between the pump and the flask. Fluffy powders are obtained consisting of the reduced and alkylated peptide or protein derivatives, of bis-S-alkyldithiothreitol, of 4,5-dihydroxy-*o*-dithione and, depending on its volatility, of excess alkylating agent. Trituration of the mixture with methanol or ethanol removes all reagents and by-products** leaving crude protein derivatives which often give correct amino acid analysis at this early stage of purification.

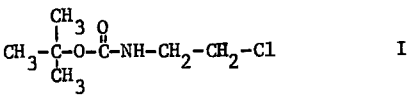
Human growth hormone (HGH) and bovine pancreatic ribonuclease A are insoluble in liquid ammonia. However, HGH also could be completely reduced in suspension using a 1000-fold excess of dithiothreitol. Subsequent benzylation gave tetra-S-benzyl HGH with a correct amino acid analysis. Alternatively, HGH was dissolved in a very small volume of water (*e.g.* 10 mg HGH in 0.5 ml H_2O) which was then added to liquid ammonia (*e.g.* 100 ml). The protein remained in solution and was readily reduced and alkylated

*Iodoacetic acid or iodoacetamide give considerable amounts of glycine or glycinamide. These side-products are only formed in minute amounts from chloroacetic acid and chloroacetamide.

**S-Carboxymethylated or S-carbamidomethylated products are contaminated with small amounts of glycine or glycinamide, which have to be removed by Sephadex gel filtration.

without requiring an extraordinarily high excess of dithiothreitol. Ribonuclease dissolved slowly in the presence of 20 equivalents of dithiothreitol within 4-5 hours. Addition of alkylating agents at this point gave 4 S-alkylcysteines. We still have to investigate whether this partial reaction is a consequence of a specific reduction of two susceptible disulfide bonds [as has been observed in aqueous solution (29)], and whether ribonuclease could be completely reduced by using larger amounts of reducing agents and longer reaction periods.

We have prepared a new reagent, *tert*-butyloxycarbonyl-aminoethyl chloride (Boc-aminoethyl chloride, I) which is a colorless liquid. Reduc-



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tion of cystine by dithiothreitol in liquid ammonia and reaction with I gave good yields of S-(N-*tert*-butyloxycarbonylaminoethyl)-cysteine. Treatment with trifluoracetic acid (30) afforded S-aminoethylcysteine. S-Aminoethylation of cysteine residues (thialysine formation) has been used by several investigators to introduce new sites for tryptic cleavage in peptide chains (31). Our reagent, I, might be useful for sequence analysis. It offers the advantage of selective tryptic digestion in two different stages, as schematically shown in Fig. 6. Reduction of disulfide

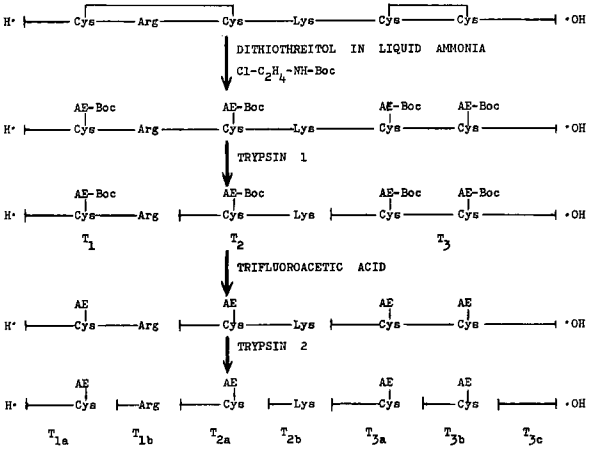

Fig. 6. Proposed strategy for selective tryptic fragmentation of proteins in two stages through the use of *tert*-butyloxycarbonylaminoethyl chloride (Boc-AE-Cl) for S-alkylation in liquid ammonia.

bonds by dithiothreitol in liquid ammonia followed by treatment with I will afford a S-(Boc-aminoethylated) protein derivative. Tryptic digestion will cleave the peptide chain at arginine and lysine residues but leave the Boc-aminoethylcysteine residues intact. Isolated tryptic peptides containing such residues then can be briefly treated with trifluoracetic acid which removes the *tert*-butyloxycarbonyl protecting group and creates aminoethylcysteine residues that will allow further subfragmentation by renewed tryptic digestion.

Acknowledgment. We wish to thank Dr. C. H. Li for helpful discussions and Mrs. H. Trzeciak, Mr. G. Johnson, Mr. J. Shaw and Mr. J. Daniel for technical assistance. This work was supported by Public Health Service Research Grants C-6516, National Cancer Institute, and FR-05526 from the Division of Research Facilities and Resources, National Institutes of Health.

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PROGRESS ON THE CLASSICAL SYNTHESIS OF "FRAGMENT P2" (RESIDUES 6-47) OF STAPHYLOCOCCAL NUCLEASE-T

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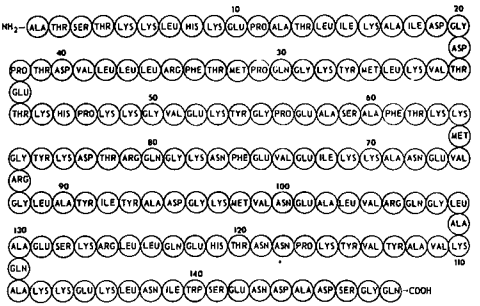
One approach in our laboratory to the problem of how individual amino acid side chains contribute to the determination of a unique biologically active, three-dimensional structure has involved synthetic studies of portions of staphylococcal nuclease. Figure 1 shows the primary amino acid sequence (1). It is a single polypeptide chain of 149 amino acids, devoid of cysteine. There is no evidence for intermolecular association and no covalent bridges are present to complicate the kinetics of structure formation. Furthermore, there is a rapid renaturation of nuclease to its native configuration after heat or acid denaturation (2).

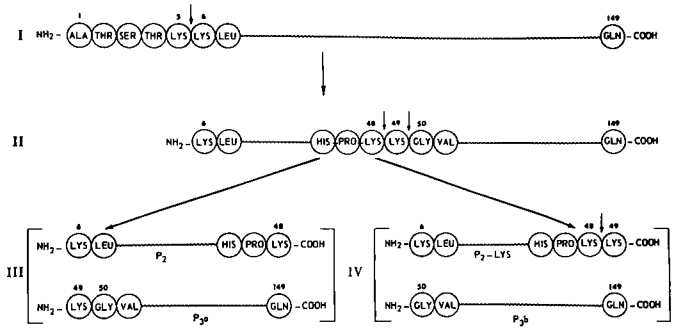
Figure 1
The amino acid sequence of staphylococcal nuclease (Foggi strain).



Nuclease contains 23 lysines and 5 arginines, all of which are cleavable by trypsin. In the presence of the inhibitor, deoxythymidine-diphosphate, and the metal ligand, calcium, digestion by trypsin yields only three fragments (3) (Figure 2). The first cleavage between lysine residues 5 and 6 results in a completely active peptide containing 144 amino acids. In the present discussion these will be called P1, which encompasses residues one through five, P2 (residues 6-48 or 6-49) and P3 (residues 49-149 or 50-149). None of these fragments has activity by itself. However, when the P2 and P3 fragments are combined, approximately

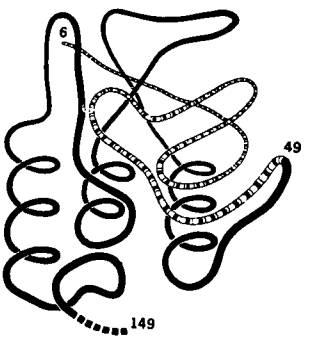
8% of the native nuclease activity is recovered. The P2-P3 complex is called nuclease-T (3,4).

Figure 2
Formation of nuclease-T by limited trypsin digestion of nuclease in the presence of deoxythymidinediphosphate and calcium ions.



The three-dimensional structure of nuclease has been elucidated by Cotton and co-workers at M.I.T. (5) (Figure 3). The P2 part contributes the portion containing the antiparallel pleated sheet. The P3 part contains the three helices. Both parts contain amino acids which are in contact with the inhibitor, which is bound in a centrally located, hydrophobic pocket. There is evidence that the conformation of nuclease-T is similar to that of native nuclease (6).

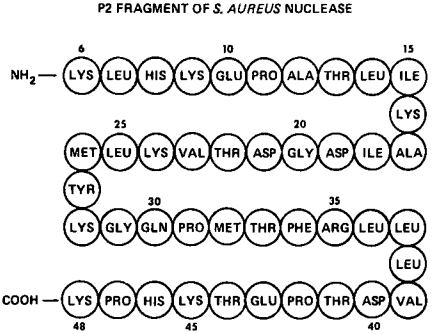
Figure 3
Schematic drawing of the three dimensional structure of nuclease-T. Fragments P2 and P3 are indicated by the striped and solid portions, respectively.



The sequence of P2 is shown in Figure 4. A study of the role of structural analogues on conformation and function has been underway for the past two to three years (7-10) and several analogues have been synthesized and tested by Drs. Ontjes and Chaiken using the solid phase method of Merrifield (11). Activities of the crude synthetic P2 is generally on the order of 2-5% of that elicited by "native" P2. Extensive purification

has recently resulted in small amounts of material with activities of 80% or better (12).

Figure 4
The sequence of fragment P2; the subject of the present synthetic study.



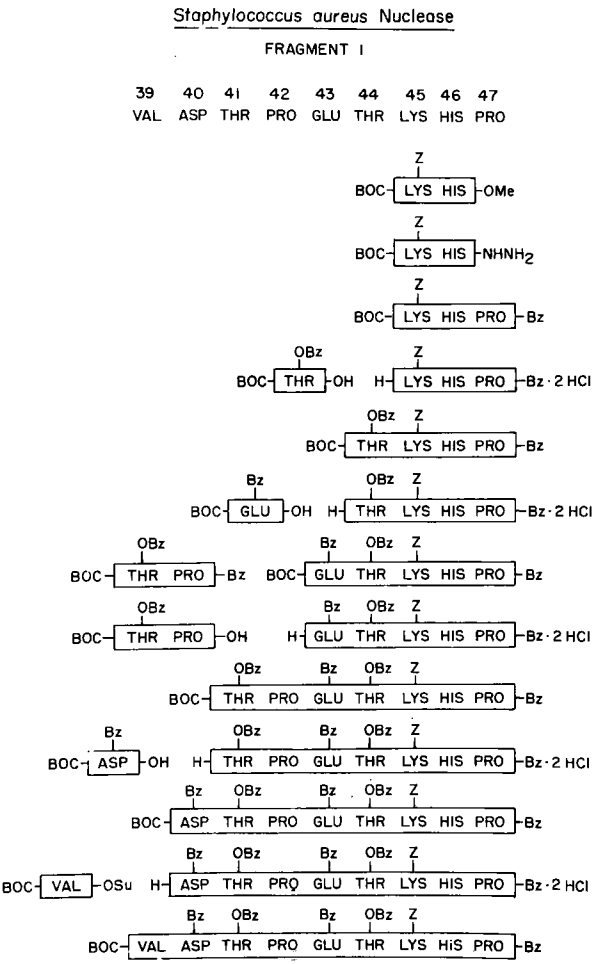
A product of dependably high activity should result from the classical synthetic method of step by step coupling, isolation, purification and characterization of intermediates. However, the synthesis of the P2 peptide would be one of the longest to be attempted classically. Therefore, it would be of considerable interest to compare the properties of the products of the solid phase and classical methods. Some of the low activity of the crude, solid phase P2 could be accounted for by the instability of "native" P2 to the deprotection procedure employing hydrogen fluoride followed by piperidine (13). In the classical scheme it might be wise to avoid deprotection with these reagents. Therefore, the carbobenzoxy moiety has been used to protect the ε-amino group of lysine, eliminating the need for piperidine. The methionine residues at residues 26 and 32 were replaced in the synthesis by norleucine so that deprotection could, in principle, be carried out solely by hydrogenation. The norleucine derivative has previously been shown to be active (14).

The fact that lysine 48 can be removed without loss of activity led to solid phase syntheses beginning at proline 47 (13). In the interest of comparing the two methods, the classical synthesis was also begun at this residue. The overall reaction scheme involves the synthesis of five fragments; fragment 1, from valine 39 to proline 47, fragment 2 from glutamine 30 to leucine 38, fragment 3 from aspartic acid 21 to glycine 29, fragment 4a from lysine 16 to glycine 20 and fragment 4b from lysine 6 to isoleucine 15. It is planned to join the latter two fragments to each other before addition to the main chain.

Figure 5 shows the reaction scheme for the synthesis of the carboxyl-terminal fragment, fragment 1. The tripeptide, BOC-Lys(ε-Z)-His-Pro-OBz, is difficult to crystallize. Stepwise synthesis resulted in the introduction of impurities which made crystallization even more difficult. Therefore, the dipeptide, BOC-Lys(ε-Z)-His-$NHNH_2$, was coupled to proline benzylester by the azide method of Honzl and Rudinger (15) using t-butyl nitrite in an organic solvent. The pure tripeptide was crystallized in 90% yield. The rest of the synthesis was performed in a stepwise fashion with the exception of the dipeptide, Boc-Thr(OBz)-Pro-OH. All of these steps involved dicyclohexylcarbodiimide (DCC) as the activating agent,

except for the amino-terminal residue, valine 39, the addition of which required more stringent conditions.

Figure 5
Summary of the synthesis of fragment 1; residues 39-47.



Initially, several attempts were made to couple residues 30 to 39 with 40 to 47, using the Weygand-Wunsch method of DCC and N-hydroxysuccinimide (16). All of these attempts were unsuccessful. Difficulty was also encountered using the valine monomer. However, the reaction succeeded at 40 C for 16 hours using a 50% excess of the N-hydroxysuccinimide ester. The yield was 82%. Table I shows the amino acid analysis of fragment 1. Removal of the BOC group from the valine residue did not yield a pure product. Better results were obtained by deprotection with HCl in dioxane for one hour. The amounts of starting material remaining and decarbobenzoxylated material, were minimal.

Table I

FRAGMENT I

Residue	Ratio Calculated	Found
Lys	1	0.95
His	1	0.94
Asp	1	0.95
Thr *	2	1.65
Glu	1	1.12
Pro	2	2.11
Val	1	(1)

* The 20 - hour hydrolysates are uncorrected for Threonine destruction.

Figure 6 shows the reaction scheme for the adjacent nonapeptide, fragment 2. The butyl ester used to protect the carboxyl-terminal was removed before the addition of the pentapeptide azide. Again, the yield of apparently homogeneous material using the Honzl-Rudinger azide procedure has been good, averaging between 85% and 90%. Table II shows the amino acid analysis of the hydrogenated material. The arginine value is slightly low. A peak comprising another 7% eluted in the position of lysine and was probably ornithine. In the unhydrogenated material this peak comprises about 15% of the arginine peak.

Figure 6

Summary of the synthesis of fragment 2; residues 30-38.

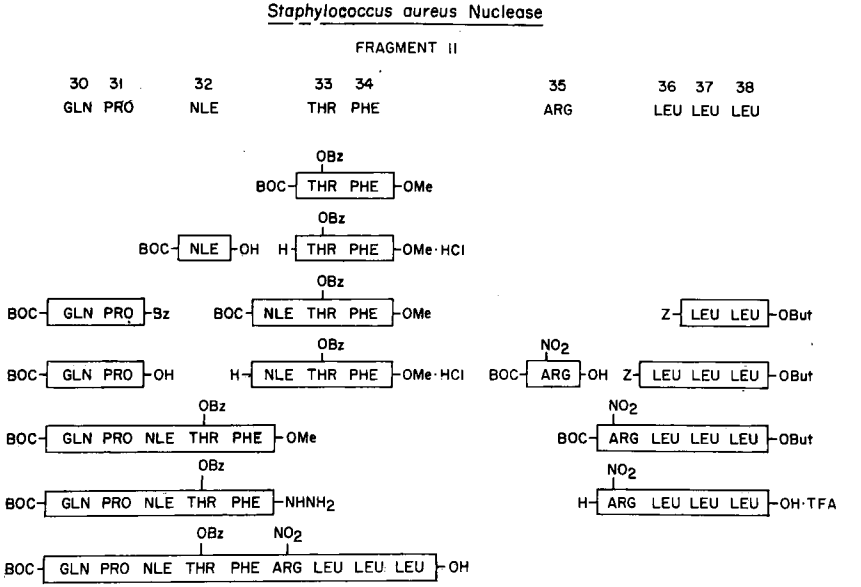


Table II

FRAGMENT II

Residue	Ratio Calculated	Found
Arg *	1	0.88
Thr **	1	0.84
Glu	1	1.08
Pro	1	1.05
Leu	3	2.84
Nle	1	(1)
Phe	1	1.03

* Another 0.08 found in a peak close to that of Lysine and probably due to Ornithine.

** The 20-hour hydrolysates are uncorrected for Threonine destruction.

The two fragments were coupled by the conditions of Weygand and Wunsch using one equivalent of DCC and N-hydroxysuccinimide. There appeared to be a slow, progressive loss of histidine during coupling. This could be due to free-radical formation from the N-hydroxysuccinimide. The reaction was repeated using a 10-fold excess of imidazole as a free-radical trap. After 16 hours reaction time, the mixture was chromatographed on an LH-20 column with DMF. The results, summarized in Figure 7, show absorbancy at 271 mμ, where nitroarginine has a very high extinction. The readings are, therefore, largely a monitor for arginine-containing peptides. The first peak follows the estimated void volume very closely and represents about 60% of the total absorption. The tubes with absorbances having closed circles were analyzed for amino acid content. Tubes 119, 122 and 125 gave excellent analyses. Tube 128 was perhaps 10% high in the amino acids contained in fragment 2. This figure increased in tubes 130, 132 and 135 and declined in tube 138. This indicates that fragment 1 was eluting later than fragment 2, and that the tubes pooled did not contain equal amounts of fragments 1 and 2. The amino acid analysis of tube 125 is given in Table III.

Figure 7

Fractionation of the product of coupling of residues 30-38 and 39-47, using gel filtration on LH-20.

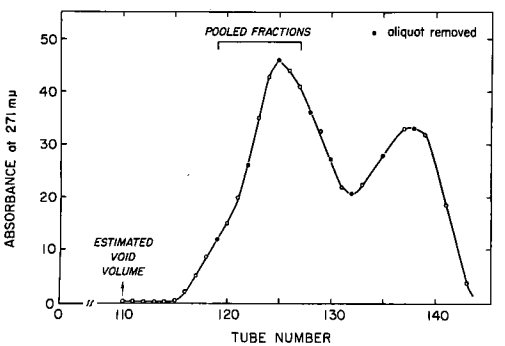


Table III

OCTADECAPEPTIDE

Residue	Ratio Calculated	Found
Lys	1	0.96
His	1	0.88
Arg	1	1.02
Asp	1	1.06
Thr *	3	2.45
Glu	2	2.10
Pro	3	3.10
Val	1	(1)
Leu	3	3.02
Nle	1	0.89
Phe	1	1.08

* The 20-hour hydrolysates are uncorrected for Threonine destruction.

The synthesis of fragment 3 is shown in Figure 8. The reaction scheme is similar to that employed in the synthesis of fragment 2. The glycine ethyl ester is first saponified before the addition of the azide. The yield of apparently homogeneous octapeptide was approximately 65%. The β-benzylaspartic acid was added as the active ester. Some difficulty was encountered in removing the BOC group of the octapeptide with formic acid. Better results were obtained in 4N HCl in dioxane. In general, deprotection of the smaller peptides with formic acid resulted in complete removal of the BOC group with little or no decarbobenzoxylation. The amino acid analysis of this fragment is shown in Table IV.

Figure 8

Scheme of synthesis of fragment 3; residues 21-29.

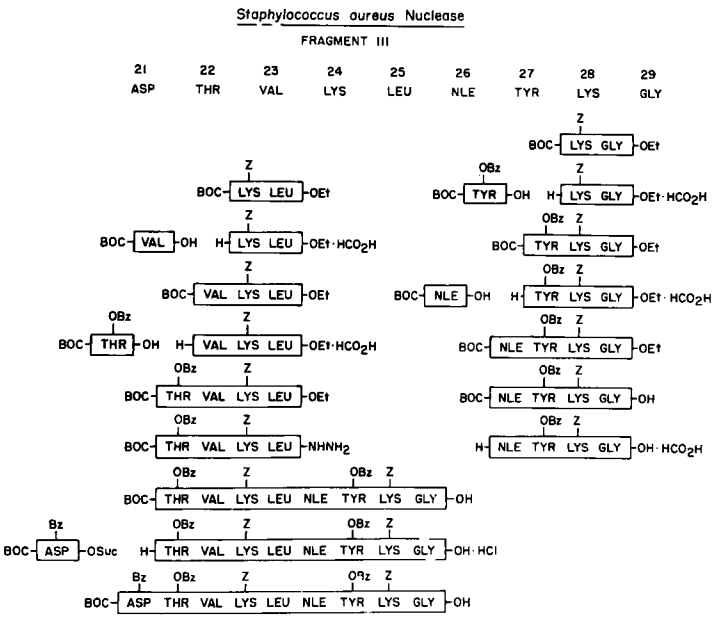


Table IV

FRAGMENT III

Residue	Ratio Calculated	Found
Lys	2	2.02
Asp	1	0.99
Thr *	1	0.99
Gly	1	1.02
Val	1	(1)
Leu	1	1.01
Nle	1	1.04
Tyr **	1	0.96

* The 20-hour hydrolysates are uncorrected for Threonine destruction.
** Contains 10x amount of Phenol to protect Tyrosine from destruction.

Fragment 4a contains the aspartyl-glycine sequence, which has been found to form β-peptide linkages under diverse conditions (17-20). Synthesis and deprotection under mild conditions were considered essential. The aspartic acid β-carboxyl group was protected as the t-butyl ester, which offers steric hindrance to nucleophilic attack. The reaction scheme is shown in Figure 9. The deprotected pentapeptide showed no succinimide carbonyl bands in the infra-red. Only one spot was detected on thin layer chromatography in a solvent used by Ondetti to detect β-aspartyl peptides (17). However, this observation should be tempered with caution. The deprotected pentapeptide is insoluble in all but acidic solvents and there was a trail in this solvent to the origin. An attempt to hydrogenate the pentapeptide for electrophoresis failed due to the insolubility of both the peptide and the catalyst. The amino acid analysis in Table V is in good agreement with that expected.

Figure 9
Scheme of synthesis of fragment 4a; residues 16-20.

Staphylococcus aureus Nuclease

FRAGMENT IV a

16 17 18 19 20
LYS ALA ILE ASP GLY

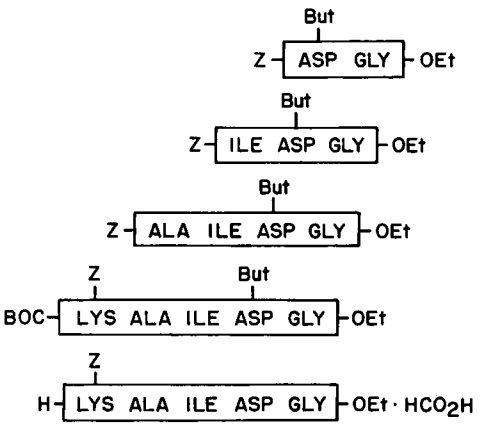


Table V

FRAGMENT IV a

Residue	Ratio Calculated	Found
Lys	1	0.95
Asp	1	(1)
Gly	1	1.02
Ala	1	1.01
Ile	1	1.01

The synthesis of the N-terminal decapeptide is schematically represented in Figure 10. The carboxyl-terminal tetrapeptide was synthesized in a stepwise manner. The amino-terminal hexapeptide was synthesized by two azide condensations, the last one involving a dipeptide with a free carboxyl group. The two large fragments were joined by a DCC condensation. The amino acid analysis is shown in Table VI.

Figure 10

Scheme of synthesis of fragment 4b; residues 6-15.

Staphylococcus aureus Nuclease

FRAGMENT IV b

6 LYS 7 LEU 8 HIS 9 LYS 10 GLU 11 PRO 12 ALA 13 THR 14 LEU 15 ILE

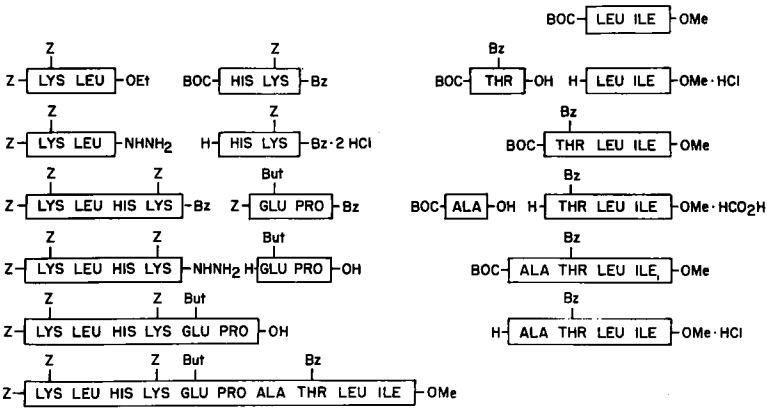


Table VI

FRAGMENT IV b

Residue	Ratio Calculated	Found
Lys	2	2.09
His	1	1.07
Thr *	1	0.93
Glu	1	0.94
Pro	1	0.93
Ala	1	(1)
Ile	1	1.01
Leu	2	2.07

* The 20 - hour hydrolysates are uncorrected for Threonine destruction.

As a precaution the butyl ester was removed prior to hydrazinolysis of the decapeptide. Much difficulty was encountered in this reaction despite a large excess of hydrazine and refluxing in methanol. The method of Maclaren, Savige and Swan (21), using n-butanol at 40 C, did give a product in 80% yield. The hydrazide was analyzed by a modification of the method of Medzihradszky-Schweiger (22) based on the oxidation of the hydrazide to nitrogen by iodine. The amount of hydrazide nitrogen calculated was 1.7%. The amount found was 1.7%. The amino acid analysis is shown in Table VI.

When the final product is obtained in suitable purity, classical synthesis of further analogues can begin. The classical synthesis will, in principle, also yield a product which, in protected form and bearing a carboxyl-terminal azide group, might be suitable for coupling to fragment P3 of Nuclease-T (see Figure 2) to regenerate a semi-synthetic native nuclease molecule.

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SYNTHETIC ASPECTS OF HUMAN β-MELANOCYTE-STIMULATING HORMONE

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We wish to summarize some of synthetic problems which we were concerned in the total synthesis of human β-melanocyte-stimulating hormone (MSH).[1,2] At present, two types of hormones, besides ACTH, are known to be responsible for cell pigmentation as shown in Fig. 1. Species variation of α-MSH is not known, however the structure of β-MSH varies to species to species[3] The reason of the occurrence of two melanocytic principles in the same mammalian pituitary gland is still a mystery. It is reasonable to expect that these hormones may play another important physiological function besides pimgentation. Recently, it has become apparent that in addition to the effect on melanocytes, MSHs elicit other physiological responses ; for example, i) Release of free fatty acid into serum[4]. ii) Thyrotropin-like activity[5]

α-MSH

Pig, Beef
Horse, Monkey Ac.Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val.NH_2

β-MSH

Pig	Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp
Beef	Asp.Ser.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp
Horse	Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Arg.Lys.Asp
Monkey	Asp.Glu.Gly.Pro.Tyr.Arg.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp
Human	Ala.Glu.Lys.Lys.Asp.Glu.Gly.Pro.Tyr.Arg.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp

Fig. 1. Amino acid sequences of MSH

iii) Decrease of the weight of testicle in mice[6]. iv) Occurrence of menstrual bleeding in women with amenorrhoea.[7] v) Stimulation of aqueous flare response in rabbit eyes[8]. vi) Influence of neural transmission[9], vi) Induction of streching and yawning reflex in mammals[10]. vii) Catechol amine-like action[11], etc.

With these considerations, we undertook the systematic synthesis of human β-MSH. The structure of human β-MSH was determined by Harris[12] in 1959. This hormone, as claimed by the author, is the first polypeptide hormone from human pituitary to be characterized in terms of its complete chemical structure. It consists of the octadecapeptide of monkey β-MSH[13] plus an additional N-terminal tetrapeptide unit, Ala-Glu-Lys-Lys.

Main source of the synthetic strategy toward the total synthesis of human β-MSH is the choice of the protecting groups for the ε-amino group of the Lys residue at position 21 and the guanidino function of Arg at position 10 which is adjacent to the Met residue. The synthetic scheme of this peptide hormone is illustrated in Fig. 2.

The formyl group was selected as the ε-amino protection of Lys[14], since most H-Lys(For)-OH peptides known are soluble in water and this property permits to purify every synthetic intermediates by column ion-exchange chromatography. The C-terminal pentapeptide H-Ser-Pro-Pro-Lys(For)-Asp-OH (I)[15] was prepared in the stepwise manner starting from H-Asp-$(OMe)_2$ using either DCC or the active ester

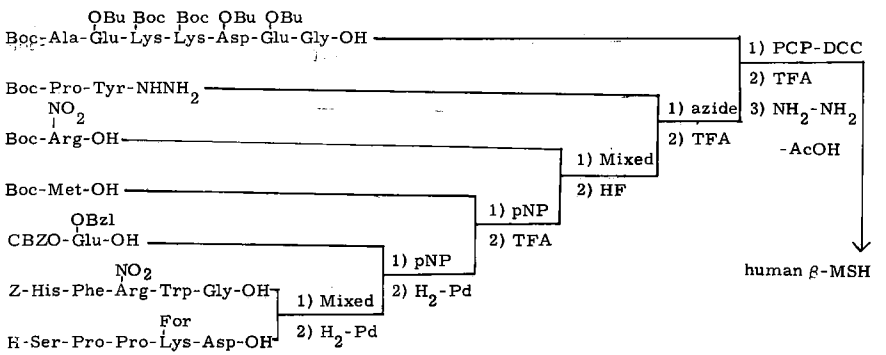


Fig. 2. Synthetic route to human β-MSH

method. Especially CBZO-Ser-OH was coupled with the tetrapeptide, H-Pro-Pro-Lys(For)-Asp-OH, by the PCP method. The methyl ester group of Asp had to be removed at the dipeptide stage, since the peptide bonds of Ser-Pro and Pro-Pro were both found to be very sensitive to base and acid.

Next, the known pentapeptide of α-MSH and ACTH, CBZO-His-Phe-Arg(NO_2)-Trp-Gly-OH, was coupled with the above pentapeptide (I) by the mixed anhydride procedure and the product was subsequently hydrogenated. The yield of the purified decapeptide, H-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH (II), was only 31%. Although other active ester method was examined, any notable improvement could not be achieved.

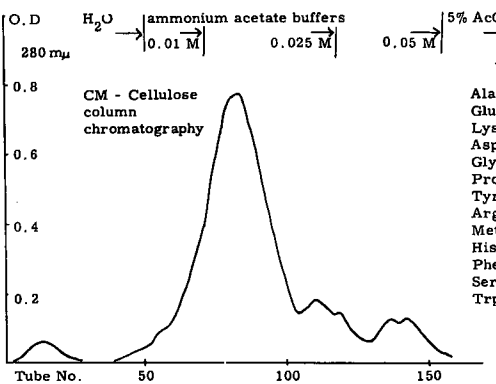
CBZO-Glu-(OBzl)-OH was then coupled with the above decapeptide (II) by the p-NP method and the protecting groups, CBZO and the benzyl groups, were removed by hydrogenolysis. Next, with the resulting undecapeptide, H-Glu-II, Boc-Met-OH was condensed by the same active ester procedure and the removal of Boc from the product was carried out by TFA to give the partially protected dodecapeptide, H-Met-Glu-II. The mixed anhydride method was adopted for condensation of Boc-Arg(NO_2)-OH to the above dodecapeptide, but the concomitant formation of some amount of the urethan could not be avoided[16]. The protecting groups of the resulting product, Boc and NO_2 groups, were then removed by HF according to Sakakibara et al.[17] and the partially protected tridecapeptide, Arg-Met-Glu-II, was purified by column chromatography on CM-cellulose. Boc-Pro-Tyr-OH was condensed with the above tridecapeptide by the azide procedure and the product was treated with TFA to give the partially protected pentadecapeptide, H-Pro-Tyr-Arg-Met-Glu-II[18].

The N-terminal heptapeptide, Boc-Ala-Glu(OBu)-Lys(Boc)-Lys(Boc)-Asp(OBu)-Glu(OBu)-Gly-OH was prepared by the coupling reaction of Boc-Ala-Glu(OBu)-Lys(Boc)-Lys(Boc)-azide with H-Asp(OBu)-Glu(OBu)-Gly-OH and this was converted to the corresponding PCP ester by the DCC-PCP complex according to Kovacs et al.[19] This heptapeptide active ester was isolated in an analytically pure form by chromatography on silica and then allowed to react with the above pentadecapeptide to

give the protected docosapeptide. Treatment of this product by TFA afforded [21-N^{ϵ}-formyllysine]-human β-MSH in 45% yield. This formyl derivative was dissolved in a solution of hydrazine acetate at pH 6.0 and the solution was heated in a boiling water bath for 3 hr under nitrogen atmosphere. Mercaptoethanol was used to prevent possible oxidation of the methionine residue during this treatment. The deformylated product was purified by column chromatography on CM-cellulose. The desired compound was eluted with 0.025 M ammonium acetate buffer as shown in Fig. 3.

The purified material exhibited a single spot on thin layer chromatography and in the field of electrophoresis on paper, it migrated slightly faster to the cathode side than the formyl derivative. Thus the difference between the deformylated product and the formyl derivative could be detected.

Amino acid analysis of an acid hydrolysate of the final product revealed the presence of the constituent amino acids of human β-MSH in ratios predicted by theory except for Trp destroyed by acid. Digestion of the product by AP-M[20] showed that recovery of amino acids, Gly and Ser, attached at the amino function of the Pro residue was somewhat low and only trace of Pro was detected. However the peptide chain attached at the carboxyl function of Pro was digested almost completely. Such a unique property of this enzyme was also pointed out by Jorgensen et al.[21] and Sakakibara et al.[22]



Amino acid analysis

	acid	AP-M
Ala	1.00	1.00
Glu	3.24	2.98
Lys	2.81	3.16
Asp	2.20	1.78
Gly	2.00	1.25
Pro	2.89	0.31
Tyr	0.87	0.92
Arg	2.15	1.79
Met	1.04	0.93
His	1.03	1.02
Phe	1.12	1.00
Ser	0.95	0.41
Trp		0.93

Fig. 3. Elution pattern of synthetic human β-MSH

The MSH activity of synthetic peptides is listed in Fig. 4. The activity of synthetic human β-MSH was 6.2×10^9 MSH units/g. This value is nearly equivalent to that reported by Li et al.[23] in the natural source and by Rittel[24] who synthesized this hormone by the method different from ours. It seems noteworthy that the pentadecapeptide exhibited the highest activity among those listed and elongation of the peptide chain to the N-terminal portion of this peptide caused some decrease in its activity.

	MSH U/g.
Phe.Arg.Trp.Gly.Ser.Pro.Pro	0
His.Phe.Arg.Trp.Gly.Ser.Pro.Pro.Lys.Asp.	1.0×10^6
His.Phe.Arg.Trp.Gly.Ser.Pro.Pro.Lys.Asp.	2.4×10^6
Glu.His.Phe.Arg.Trp.Gly.Ser.Pro.Pro.Lys.Asp.	6.0×10^6
Met.Glu.His.Phe.Arg.Trp.Gly.Ser.Pro.Pro.Lys.Asp.	2.2×10^7
Arg.Met.Glu.His.Phe.Arg.Trp.Gly.Ser.Pro.Pro.Lys.Asp.	1.8×10^8
Pro.Tyr.Arg.Met.Glu.His.Phe.Arg.Trp.Gly.Ser.Pro.Pro.Lys.Asp	2.0×10^{12}
Asp.Glu.Gly.Pro.Tyr.Arg.Met.Glu.His.Phe.Arg.Trp.Gly.Ser.Pro.Pro.Lys.Asp.	2.0×10^9
Lys.Lys.Glu.Ala Synthetic monkey β-MSH	2.5×10^{10}
Asp.Glu.Gly.Pro.Tyr.Arg.Met.Glu.His.Phe.Arg.Trp.Gly.Ser.Pro.Pro.Lys.Asp.	3.4×10^9
Synthetic human β-MSH	6.2×10^9

Fig. 4. MSH activity of synthetic peptides

Looking back over this synthesis, we wish to mention some model experiments which were required for this synthesis. As stated earlier, this peptide hormone contains peptide bonds sensitive to base and acid. Therefore, when the formyl group was adopted for the side chain protection of the Lys residue, the usual mild acidolysis employed for its removal seems still unsatisfactory and much milder conditions should be required. We acquainted with the references of Miyamoto et al.[25] and Lefrancier et al.[26], who removed the formyl group from amino acids by hydrazine hydrate. By modifying this condition, we noticed that Lys could be quantitatively regenerated from H-Lys(For)-OH by treatment of hydrazine acetate at pH 6.0 or by the action of hydroxylamine hydrochloride in pyridine at pH 6.0 as shown in Fig. 5. We applied these conditions to the synthesis of α-MSH from [11-N^{ϵ}-formyllysine]-α-MSH[27] and monkey β-MSH from [17-N^{ϵ}-formyllysine]-monedy β-MSH[28] Independently, Geiger and his associates[29] examined similar deformylation reaction in detail.

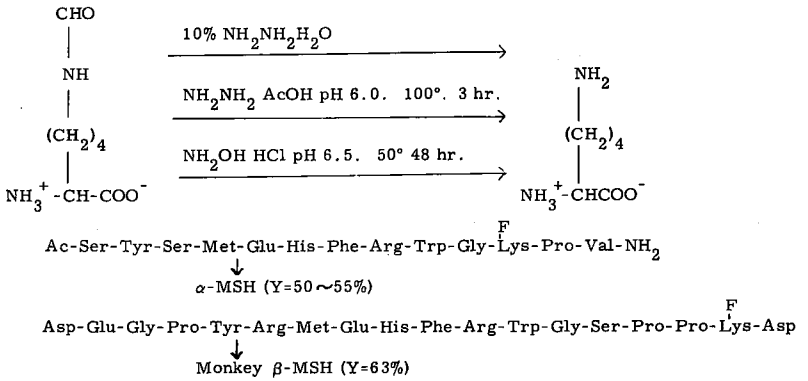


Fig. 5. Deformylation reaction of H-Lys(For)-OH

An another model experiment in which we engaged was the synthesis of H-Arg-Met-OH present in human β-MSH. The protecting group of the guanidino function of Arg had to be considered in connection with the formyl group previously adopted to Lys. When H-Arg(NO_2)-OH is adpoted, this NO_2 group has to be removed either by catalytic hydrogenation in the presence of Met, which poisons the catalyst or by the HF procedure. Along the former line, it was found that CBZO-Arg(NO_2)-Met-OMe could be reduced over a Pd catalyst to H-Arg-Met-OMe in 80% by addition of BF_3 etherate in MeOH[30] This reaction proceeded very slowly in acetic acid. Methanol is the most effective solvent for this purpose but partial methylation takes place during the hydrogenolysis when the free carboxyl group is present. Despite of this finding, this procedure could not be applied for the synthesis of human β-MSH, since conversion of Arg peptide

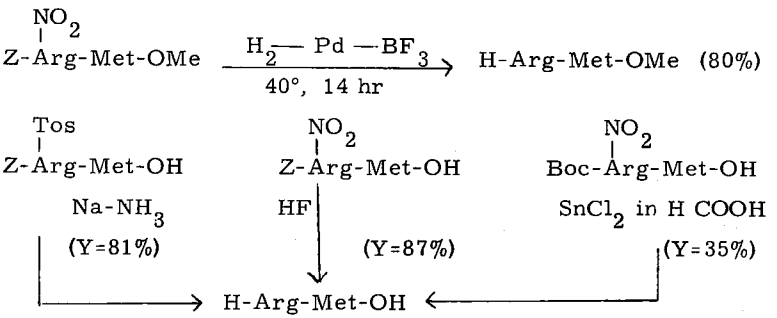


Fig. 6. Synthesis of H-Arg-Met-OH

methyl esters to corresponding hydrazides is impractical. It is known that the guanidino group is labile to the strong basisity of hydrazine. Superior property of the HF procedure was confirmed by the synthesis of H-Arg-Met-OH from Boc-Arg(NO_2)-Met-OH after comparison of two other alternate routes as shown in Fig. 6.[31] The stannic chloride procedure of Noguchi et al.[32] is indeed an interesting one, but gave somewhat low yield in this case. Recently Mazur et al.[33] reported that the tosyl group attached at the guanidino function of Arg could be removed by HF. Therefore, the use of H-Arg(Tos)-OH in a form of Boc-Pro-Tyr-Arg(Tos)-Met-$NHNH_2$ may offer an alternate route to the synthesis of this peptide hormone, since the formyl group resists to the action of HF.

We hope these procedures described above may serve to the synthesis of other biologically active peptides and peptides so far accumulated in our laboratory may serve to clarify this misterious physiological principle which exists in human pituitary gland.

Acknowledgement

The authors express their sincere thanks to Dr. Saul Lande, School of Medicine, Yale University for MSH assays. They are also indebted to Dr. T. Y. Liu, Biology Department, Brookhaven National Laboratory, for his encouragement during the course of this investigation.

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PEPTIDE HORMONE ANALOGS AS MOLECULAR PROBES FOR ENZYME STUDIES

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Certain structural elements of the neurohypophyseal hormones oxytocin and vasopressin can render these molecules resistant to the action of enzymes; e.g., the C-terminal amide group endows neurohypophyseal hormones with resistance to carboxypeptidases(1). Specific conformational features, such as tight packing of the 20-membered cyclic moiety of oxytocin in an antiparallel pleated sheet or cross β-structure(2), also can serve to protect the hormones from enzymic cleavage; a case in point is the failure of several endopeptidases, such as pepsin(3), chymotrypsin C(4) or chymotrypsin A(5), to cleave the peptide bond at the carboxyl side of the tyrosine in position 2 of neurohypophyseal hormones - which on an a priori consideration of the known specificity of these proteolytic enzymes would be a site of enzymic attack. Moreover, the inactivation of neurohypophyseal hormones by certain exopeptidases is either unusually slow or totally prevented; the sluggish action of kidney cytoplasmic leucine aminopeptidase(6) or the inability of liver leucine aminopeptidase(7) to inactivate intact oxytocin and vasopressin may serve as examples.

Nevertheless, all tissues contain enzymes which degrade neurohypophyseal hormones(8), and it is possible that some of these hormone-inactivating processes regulate the duration and intensity of the physiological responses. Assuming that hormone breakdown, like synthesis, is a tightly controlled process, the responsible enzyme(s) could be located in the direct vicinity of the hormone receptors in the target cells and, therefore, not require high substrate specificity. Conversely, the enzymes could be highly specific for one class of peptide hormones and thus their spatial location relative to the receptor within the target cell need not be as stringently specified.

Our initial interest in this general problem has involved a test of the latter hypothesis by a characterization of the specificity of enzymes obtained from various target tissues of neurohypophyseal hormones. We considered the two following approaches: the detection of oxytocin- and/or vasopressin-inactivating principles in various tissues followed by extensive purification and characterization of the highly-purified inactivating enzymes. Since an isolated enzyme is expected to attack the hormone in not more than one or two loci, a direct identification of the products resulting from the hormone-enzyme incubation experiments is possible in most instances. This product identification permits a further delineation of the substrate specificity of the enzyme by using hormone analogs possessing only a single structural change and even lacking biological activity. The disadvantage of this approach is that an assessment of the specificity of the isolated enzyme can be made only after extensive purification and, therefore, much time and energy may well be invested in the isolation of an enzyme of no great interest.

Another approach - which leads to the recognition of non-specific enzymes at an early stage in their purification - is based upon the differential inactivation of hormone and a battery of analogs. Since such a mixture of enzymes is too crude to allow product identification in the peptide digest(except when the hormones are labeled with a radioactive isotope in a specific amino acid residue and when this residue is enzymatically released), the inactivation is followed initially by measuring residual biological activity of the hormone and analogs utilizing a standard pharmacological assay system - hence, the higher the specific biological activity of the analog the more sensitive the method. Inasmuch as more than one enzymic principle is expected to be present at an early stage of enzyme purification, analogs with cumulative structural alterations are required so that the peptide is protected against inactivation by more than one type of enzyme. Thus an ideal analog(not considering for the moment an isotopically labeled analog) would be one which is highly active biologically and yet endowed with resistance to enzymic attack, except at the one locus under scrutiny. The recognition by the above procedure of enzymes which exhibit properties consistent with high specificity for a given class of peptide hormones guides further efforts to purify the enzyme to a degree that allows direct product identification in the hormone enzyme digest.

It was this latter approach which led to the finding in the toad urinary bladder of the carboxamidopeptidase which exhibits a substrate specificity

suitable for a group of peptide hormones possessing a C-terminal carboxamide group(9). Similarly it was found that rat uterus contains an enzyme capable of cleaving the peptide bond between the leucine and the glycinamide residue(10) and, in like manner, that the kidney of the rat contains a highly active enzyme which releases glycinamide from oxytocin(11). The latter finding is interesting because earlier studies suggested that neither aminopeptidase nor redox reactions involving the disulfide bond determined the rate of decay of the antidiuretic response, and that an as yet unrecognized enzyme may play the decisive role in the overall inactivation (12, 13).

The following study of a human uterine extract illustrates the screening technique described above. Uterine tissue, excised from the wall opposite to the placental implantation site, was collected from pregnant women near term but not yet in labor. The tissue sample was thoroughly washed in cold, isotonic saline solution and homogenized in

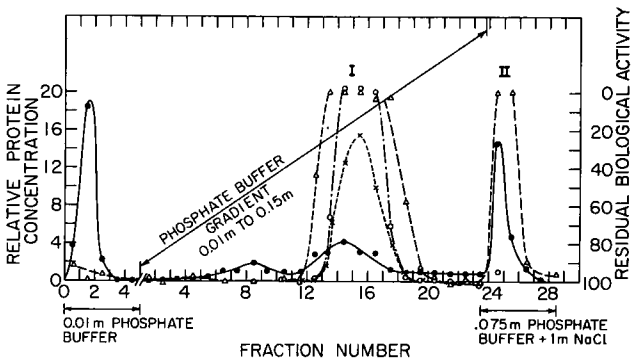
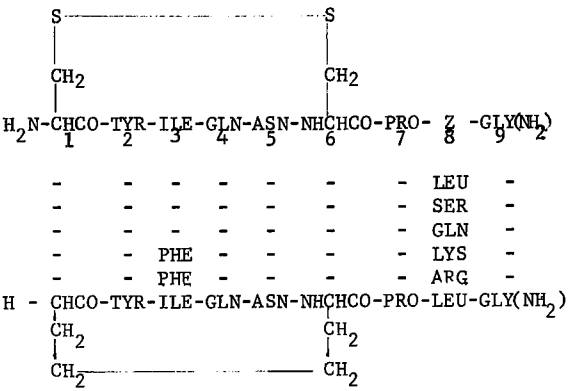


Fig. 1 - Inactivation of neurohypophyseal peptides by soluble fractions of human uterine tissue. The solid line represents the percentage of the total protein applied which is eluted with each fraction. Fractions indicated were assayed in the rat uterotonic assay of Holton(21) as modified by Koida(see in ref. 11) for inactivation of oxytocin(Δ--Δ-), deamino-dicarba-oxytocin(O-·-O) and lysine-vasopressin(X--X). Several additional neurohypophyseal hormone analogs were tested which are not included in this figure (see Table 1).

0.01M phosphate buffer pH 6.5. After removal of insoluble matter by centrifugation the extract was applied to a diethylaminoethyl(DEAE) cellulose column equilibrated with 0.01M phosphate buffer. Elution of the column was carried out as indicated in Fig. 1. Each fraction of the column eluate was assayed for oxytocin-inactivating enzymes by a procedure previously described(10). The enzymic activities associated

Table 1. Neurohypophyseal Peptides Inactivated by Human Uterine Enzyme Fraction I.



	1	2	3	4	5	6	7	8	9	2
Oxytocin[a,b]	-	-	-	-	-	-	-	LEU	-	
[8-Serine]-oxytocin[a,c]	-	-	-	-	-	-	-	SER	-	
[8-Glutamine]-oxytocin[a,d]	-	-	-	-	-	-	-	GLN	-	
Lysine-vasopressin[a,e]	-	-	PHE	-	-	-	-	LYS	-	
Arginine-vasopressin[a,f]	-	-	PHE	-	-	-	-	ARG	-	
Deamino-dicarba-oxytocin[g,h]	H -	CHCO-TYR-ILE-GLN-ASN-NHCHCO-PRO-LEU-GLY(NH2)								

[a]Prepared by the solid-phase method of peptide synthesis according to the general procedure of R.B. Merrifield, Science, 150, 178 (1965). [b]M.Manning, J.Am.Chem.Soc., 90, 1348 (1968). [c]W.H.Sawyer, J.W.M.Baxter, M.Manning, E.Heinicke and A.N.Perks, Gen.and Comp.Endocr., in press. [d]J.W.M. Baxter, T.C. Wuu, M.Manning and W.H.Sawyer, Experientia, 25, 1127 (1969). [e]J.Meienhofer and Y.Sano, J.Am.Chem.Soc., 90, 2996 (1968). [f]J.Meienhofer, A.Trzeciak, R.T.Havran and R.Walter, J.Am.Chem.Soc., in press. [g]Prepared by step-wise method of peptide synthesis according to the procedure of M.Bodansky and V. duVigneaud, J.Am.Chem. Soc., 81, 5688 (1959). [h]T.Yamanaka, S.Hase, S.Sakakibara, I.L.Schwartz, B.M.Dubois and R.Walter, Mol. Pharmacol., in press.

with the two distinguishable zones I and II in Fig. 1 were further characterized by their differential effects on a selected set of synthetic neurohypophyseal peptides. Fraction II inactivated oxytocin and lysine-vasopressin, but had little effect on the activity of crystalline deamino-dicarba-oxytocin, an analog not susceptible to attack by amino-peptidases and reductases involving the disulfide bond. This finding, along with the identification of Tyr and Ileu or Phe as reaction products from oxytocin and lysine-vasopressin, suggests that fraction II contains aminopeptidase activity. Since human pregnancy serum has high levels of aminopeptidase which inactivates oxytocin (8, 14, 15), incomplete removal of blood from the uterine tissue must be considered as a possible source of this enzymic activity.

The enzymic activity eluted from the DEAE cellulose column by the phosphate buffer gradient (fraction I) inactivated all the hormones and analogs listed in Table 1 (some of which are also depicted in Fig. 1) - a finding which cannot be explained by the presence of any single

aminopeptidase, reductase, or endopeptidase previously reported to inactivate neurohypophyseal hormones in human uterine tissue. In addition, the fact that the inactivating activity for all peptides was centered at the same position on the chromatogram in fractions well within the linear salt gradient and not at a point of sharp discontinuity of eluent composition argues for, but does not prove, the presence of a common inactivating principle. Further purification of the enzymic activity of fraction I and ongoing work leading to a direct identification of the hormone digestion products should provide a more definitive answer.

Among the target tissues which we screened in a similar manner was a

Table 2. Differential Effects of Toad Bladder Enzyme on Neurohypophyseal Peptides

Peptide	Cleavage of Peptide Bond Between Residues 8 and 9[a]
Oxytocin[b]	+
[8-Arginine]-vasopressin[b]	+
[8-D-Arginine]-vasopressin[c,d]	-
[8-Lysine]-vasopressin[b]	+
[8-Serine]-oxytocin[b]	+
[8-Glutamine]-oxytocin[b]	+
[8-Phenylalanine]-oxytocin[e,f]	+
[8-Valine]-oxytocin[e,f]	±
[8-Alanine]-oxytocin[c,g]	+
Deamino-oxytocin[e,h]	+
Oxytocinoic Acid[e,i]	-
[8-Arginine]-vasopressinoic Acid[e,j]	-
Deamino-oxytocinoic Acid Methylamide[e,k]	-
Deamino-oxytocinoic Acid Dimethylamide[e,k]	-
[9-Sarcosine]-oxytocin[c,l]	-
[7-Glycine]-oxytocin[c,m]	-

[a] Proteolytic cleavage is denoted +; the lack of cleavage is denoted -. [b] See legend Table 1. [c] See under g in Table 1. [d] M.Zaoral, J.Kolc and F.Sorm, Coll.Czech.Chem.Commun., 31, 382 (1966). [e] See under a in Table 1. [f] J.W.M.Baxter, M.Manning and W.H.Sawyer, Biochemistry, 8, 3592 (1969). [g] R.Walter and V.duVigneaud, Biochemistry, 5, 3720 (1966). [h] B.M.Ferrier, D.Jarvis, J.Biol.Chem., 240, 4264 (1965). [i] B.M.Ferrier and V.duVigneaud, J.Med.Chem., 9, 55 (1965). [j] J.Meienhofer, A.Trzeciak, T.Dousa, O.Hechter, R.T.Havran, I.L.Schwartz and R.Walter, in Proc.Tenth European Peptide Symposium (E.Scoffone, ed.), Abano Terme, Italy, 1968, North Holland Publ. Co., in press. [k] H.Takashima, W.Fraefel and V.duVigneaud, J.Am.Chem.Soc., 75, 6182 (1969). [l] W.D.Cash, L.M.Mahaffey, A.S.Buck, D.E.Nettleton, C.Romas and V.duVigneaud, J.Med.Pharm.Chem., 5, 413 (1962). [m] See ref. 16.

preparation of the toad urinary bladder which, as already mentioned above, likewise contains a neurohypophyseal hormone-inactivating

principle with promise of high specificity for these hormones(9). In this earlier study the enzyme from the toad bladder had been minimally purified. More recently we obtained this enzyme by gradient elution from a DEAE cellulose column followed by dialysis against 0.2M KH_2PO_4, which resulted in the precipitation of the enzymic principle. After redissolving of the precipitate in 0.1M potassium phosphate buffer (pH 7.6) the preparation was allowed to react with the neurohypophyseal peptides listed in Table 2; their reaction products were identified as dansylated derivatives according to the procedure previously described(10). The partially purified toad enzyme was found to cleave the peptide bond between residues eight and nine of neurohypophyseal peptides, resulting in the release of glycinamide. From the data summarized in Table 2 it can be concluded that this enzyme is surprisingly insensitive to structural features of the residue in position 8, provided the L-configuration is maintained. In contrast, the enzyme is sensitive to modifications of the C-terminal primary amide of the hormone. As may have been expected, methylation of the nitrogen atom of the peptide bond between amino acid residues 8 and 9, [9-Sarcosine]-oxytocin, prevents the cleavage of this bond.

Finally, it should be noted that the toad bladder enzyme does not inactivate [7-glycine]-oxytocin, an analog of oxytocin in which the proline residue of the natural hormone has been replaced by a glycine residue(16). This result seemed to suggest that the toad bladder enzyme has structural requirements resembling those of collagenase, i.e., a X-Pro-R-Gly sequence is required for recognition, and the R-Gly peptide linkage is cleaved(17,18). However, caution is required in this interpretation because we later found in preliminary experiments that [7-glycine]-oxytocin resists inactivation by α-chymotrypsin under conditions where oxytocin was readily inactivated. On this basis it may be expected that neurohypophyseal peptides in which the proline residue in position 7 is replaced by glycine such as [7-glycine]-oxytocin, [7-glycine]-lysine-vasopressin(19) or [deamino-dicarba-7-glycine]-oxytocin (20) exhibit protracted biological action.

Acknowledgment. The authors wish to thank Drs. S. Gray and R.T.Havran of this laboratory, and Drs. M.Manning, J.Meienhofer, S.Sakakibara, V. duVigneaud and M.Zaoral for kindly supplying this study with several neurohypophyseal peptides. We are also indebted to Mrs. I.Mintz and Miss M.Wahrenburg for helping in carrying out the oxytocic and pressor assays. This work was supported by United States Public Health Service Grants AM-13567 and AM-10080. One of us(J.D.G.) was the recipient of a Postdoctoral Fellowship AM-40,844 from the National Institutes of Health.

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STUDIES OF ENZYME ACTIVE SITES: SYNTHESIS AND CATALYTIC PROPERTIES OF PEPTIDES INCORPORATING SERINE AND HISTIDINE

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The mechanism of enzyme action, one of the central problems in biochemistry, may be met in many forms, but nowhere is it met as directly as in hydrolytic reactions catalyzed by proteolytic enzymes. The tertiary structure of enzymes plays a very important role in their catalytic activity. The suitable folding brings important amino acids which are required for the activity in closer proximity, even though these amino acids are lying at different distances in the enzyme sequences(1-4).

Considerable attention has been focused recently on the role played by L-histidine and L-serine in the active site of chymotrypsin and other proteolytic enzymes toward the hydrolysis of various esters such as p-nitrophenyl acetate. In chymotrypsinogen, which is the inactive precursor of chymotrypsin, while there are 246 amino acids in the total sequence, the suitable folding of this enzyme brings two histidines from positions 40 and 57 and one serine from 195 position close enough to act as active center responsible for catalyzing the hydrolysis. Trypsin affords the same possibilities where two histidines from positions 29 and 46, and a serine from 183 position act as the active site of the enzyme.

One of the approaches for studying the active site of enzymes is the synthesis and evaluation of the catalytic activity of relatively simple peptides embodying as many as possible of the known features of active sites of proteolytic enzymes(5,6). Katchalski reported the synthesis of poly-L-histidine and copolymers of L-histidine and L-serine as model compounds to investigate the catalytic effect on the hydrolysis of p-nitrophenyl acetate(7). Sheehan and Cruickshank reported an interesting pentapeptide, L-threonyl-L-alanyl-L-seryl-L-histidyl-L-aspartic acid, possessing considerable catalytic activity(8). In order to provide increased flexibility in the molecule, recently Sheehan reported the synthesis of L-seryl-γ-aminobutyryl-L-histidyl-γ-aminobutryl-L-aspartic acid. This pentapeptide exhibited about 50% more catalytic activity toward the hydrolysis of p- nitrophenyl acetate than that of previously reported synthetic peptides(9). As the separa-

tion of serine and histidine in a peptide chain led to relatively more catalytic activity, it was considered worthwhile to investigate the catalytic activity in simple peptides incorporating serine and histidine at different distances in the peptide chains. Recently, we reported the preparation of a relatively more potent esterase model, L-histidyl-glycyl-L-aspartyl-L-seryl-L-phenylalanine(I) (10). The catalytic activity of the pentapeptide I was determined by the liberation of p-nitrophenol from p-nitrophenyl acetate following the procedure used by Sheehan(8,9). The catalytic coefficient for the pentapeptide I was 179 l. mole^{-1} min.$^{-1}$, compared with 147 l. mole^{-1} min.$^{-1}$ for L-seryl-γ-aminobutyryl-L-histidyl-γ-aminobutyryl-L-aspartic acid and 10^4 l. mole^{-1} min.$^{-1}$ for α-chymotrypsin. Our results confirmed the observations of previous workers that the catalytic activity toward the hydrolysis of p-nitrophenyl acetate can be expected from relatively simple peptides incorporating histidine and serine. Although the catalytic coefficient of these peptides, when compared with chymotrypsin is considerably low, it definitely warrants further evaluation of a series of peptides where the molecule can be made more flexible to afford a better interaction between amino acids in the peptide chain and the substrate. Photaki has carried out a similar study on the catalytic activity of peptides incorporating histidine and cysteine (6,11).

Out of a number of peptides that we synthesized in our laboratories, the peptides which incorporate aspartic acid and phenylalanine, in addition to serine and histidine, showed a definite increase in the catalytic activity. It was therefore considered worthwhile to study the role played by specific amino acids in the peptide chains, in order to design better esterase models. In the first studies two tetrapeptides, L-histidyl-L-aspartyl-glycyl-L-serine(II) and L-histidyl-L-glutamyl-glycyl-L-serine(III), were studied for their catalytic activity. These two tetrapeptides are identical in all respects with the exception of monoaminodicarboxylic amino acids. The direct comparison of these tetrapeptides establishes the importance of aspartic acid in these esterase models and would suggest the role played by aspartic acid at the active site of these enzymes. The tetrapeptide II, which incorporates aspartic acid, had the catalytic activity 150 l. mole^{-1} min.$^{-1}$ as compared to the tetrapeptide III, which incorporates glutamic acid and had the catalytic activity 63 l.mole^{-1} min.$^{-1}$. The suggestion that aspartic acid does play an important role in the catalytic activity of these esterase models was further confirmed by the comparison of catalytic activity of two pentapeptides, L-histidyl-L-alanyl-L-aspartyl-glycyl-L-serine(IV) and L-histidyl-L-alanyl-L-glutamyl-glycyl-L-serine(V). These pentapeptides also differ only in the monoaminodicarboxylic amino acid component and, as expected, the pentapeptide IV, which incorporates aspartic acid had a catalytic activity 210 l. mole^{-1} min.$^{-1}$ as compared to the pentapeptide V, which incorporates glutamic acid, and had a catalytic activity 87 l. mole^{-1} min.$^{-1}$.

The increased catalytic activity of peptide esterase models which contain aspartic acid may be justified on the basis that in the case of chymotrypsin, aspartic acid occupies position 194, which is adjacent to postulated active serine at position 195. The same is true of trypsin, in which case, aspartic acid is at position 184 adjacent to active serine at position 183.

The other interesting observation, as it was mentioned previously, was the enhanced catalytic activity of peptides which incorporated phenylalanine. In order to get additional data, a preliminary investigation was carried out by synthesizing different peptide sequences differing only in the phenylalanine residue. The tetrapeptide L-histidyl-glycyl-L-aspartyl-L-serine(VI) had a much lower catalytic activity (83 l.mole^{-1} min.$^{-1}$). The incorporation of phenylalanine in this tetrapeptide increased the catalytic activity to 179 l. mole^{-1} min.$^{-1}$, as in the case of L-histidyl-glycyl-L-aspartyl-L-seryl-L-phenylalanine. For further comparison, a pentapeptide L-histidyl-glycyl-L-aspartyl-L-seryl-L-tyrosine(VII) was synthesized. The pentapeptide,VII,which differs only at the C-terminal amino acid had lower catalytic activity (93 l. mole^{-1} min.$^{-1}$) when compared to pentapeptide I, which incorporates phenylalanine.

The role played by phenylalanine toward the increased catalytic activity in these esterase models is not very surprising when one studies the sequence of amino acids in chymotrypsin and trypsin. In chymotrypsin, phenylalanine occupies position 41, which is next to the active center histidine at position 40. The same is true in the case of trypsin where phenylalanine occupies position 30 which again is next to the active center histidine at position 29. In order to further establish that phenylalanine does play an important role in the catalytic activity of these synthetic peptide esterase models and perhaps in the catalytic activity around the active center of proteolytic enzymes, a hexapeptide L-histidyl-L-alanyl-L-aspartyl-glycyl-L-seryl-L-phenylalanine (VIII) was studied for catalytic activity. The catalytic activity of the hexapeptide VIII was 239 l. mole^{-1} min.$^{-1}$ and this is perhaps the highest activity of a synthetic peptide esterase model reported so far. The comparison of catalytic activity of the hexapeptide VIII with two other hexapeptides which differed only at the C-terminal residue phenylalanine, further lends the support to the hypothesis for the important role played by phenylalanine in the synthetic peptide esterase models. The hexapeptide, L-histidyl-L-alanyl-L-aspartyl-glycyl-L-seryl-L-tyrosine (IX) had catalytic activity 183 l. mole^{-1} min.$^{-1}$ and the hexapeptide L-histidyl-L-alanyl-L-aspartyl-glycyl-L-seryl-L-alanine (X) had catalytic activity 165 l. mole^{-1} min.$^{-1}$. The corresponding pentapeptide, without C-terminal phenylalanine, i.e., L-histidyl-L-alanyl-L-aspartyl-glycyl-L-serine (XI) in line with our expectations had comparatively lower catalytic activity (163 l. mole^{-1} min.$^{-1}$).

The results of catalytic activity determination of some of the peptidespreviously reported and the peptides reported in this work are summarized in Table I and II.

To cite a typical example, the synthesis of two peptides (I and II) used in this study are outlined in Scheme I and II. In order to limit the degree of racemization, the peptide chains were extended from the C-terminal residue of amino acids and all peptide bonds, with the exception for the incorporation of histidine, were formed using the pentachlorophenyl active ester method(12, 13). The coupling of histidine was achieved via the azide method(14). All the protecting groups used for unreacting functionalities during the reaction sequences were acid labile or removable by hydrogenolysis. This

approach excluded the use of the alkali treatment during the whole synthesis; thereby further limiting the degree of racemization and problems of transpeptidation. The elemental analysis of both the peptides I and II, and other peptides, reported in this work were within the experimental tolerance and they were homogenous to paper chromatography and paper electrophoresis under a variety of conditions.

TABLE I

Hydrolysis of p-Nitrophenyl Acetate

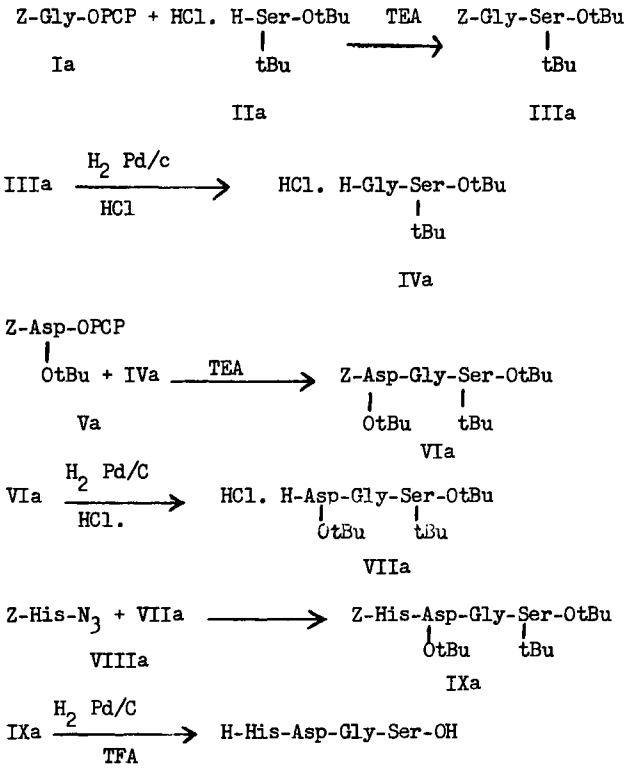
Catalyst	Catalytic Coefficient l./mole/min.
Histidine HCl.	6
H-Gly-His-Ser-OH	15
Copoly His. Ser.	9.7
H-Ser-His-Asp-OH	45
H-Thr-Ala-Ser-His-Asp-OH	92
Seryl-γ-aminobutyryl-histidyl-γ-aminobutyryl-aspartic acid	147
α-Chymotrypsin	104

Catalytic activity of some of the previously reported peptides(9)

TABLE II

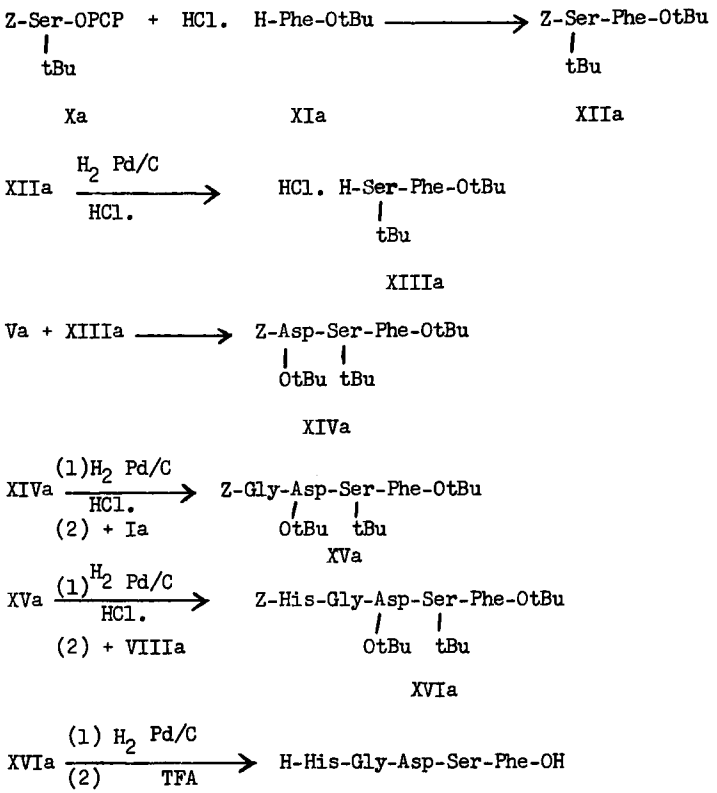
Catalyst	Catalytic Coefficient l./mole/min.
H-His-Gly-Asp-Ser-Phe-OH (I)	179
H-His-Asp-Gly-Ser-OH (II)	150
H-His-Glu-Gly-Ser-OH (III)	63
H-His-Ala-Asp-Gly-Ser-OH (IV)	210
H-His-Ala-Glu-Gly-Ser-OH (V)	87
H-His-Gly-Asp-Ser-OH (VI)	83
H-His-Gly-Asp-Ser-Tyr-OH (VII)	93
H-His-Ala-Asp-Gly-Ser-Phe-OH (VIII)	239
H-His-Ala-Asp-Gly-Ser-Tyr-OH (IX)	183
H-His-Ala-Asp-Gly-Ser-Ala-OH (X)	165
H-His-Ala-Asp-Gly-Ser-OH (XI)	163

Catalytic activity of the peptides reported in this paper.



Scheme I

Synthesis of Histidyl-Aspartyl-Glycyl-Serine



Scheme II

Synthesis of Histydyl-Glycyl-Aspartyl-Seryl-Phenylalanine

$Z = C_6H_5\text{-}CH_2\text{-}O\text{-}C(=O)\text{-}$ $\quad -OPCP = -OC_6Cl_5$

TFA = trifluoroacetic acid $\quad tBu = C(CH_3)_3$

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STERIC HINDRANCE ASSOCIATED WITH TRIETHYLAMINE USE IN PEPTIDE SYNTHESIS BY THE MIXED CARBONIC ANHYDRIDE METHOD

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In an extensive reinvestigation of the mixed carbonic anhydride method (1), we found that yields and racemization were affected by the nature of the tertiary amine used. It was shown that the amine was not merely an "acid acceptor"; it first complexes with the acyl chloride used as reagent, and this then reacts with the acylamino acid or acylpeptide to form the mixed anhydride. Only methylamines of suitable basicity react rapidly and completely, showing that steric effects are important. Consequently we were interested in a recent study by Birr, Lochinger and Wieland (2) of the synthesis of ethyl benzyloxycarbonyl-L-valyl-L-phenylalaninate, in which both amino acid components (Z·Val·OH and H·Phe·OEt) were presumed to be sterically hindered. The yields by the mixed carbonic anhydride procedure, using isopropyl chloroformate and triethylamine, were poor. Johnson and Stock (3) obtained a good yield with isobutyl chloroformate and triethylamine under different reaction conditions (higher temperature, longer time) and a different melting point for the product (148-9° versus 135-6°). The primary purpose in this investigation was to compare N-methylmorpholine (our recommended tertiary base) with triethylamine, so isobutyl chloroformate (our recommended reagent) was also used.

The conditions of Birr et. al. (2 millimoles of reactants, 10 ml. of methylene chloride solvent, -15° bath for mixed anhydride formation, followed by the addition of HClxH·Phe·OEt and tertiary base in 10 ml. of methylene chloride and stirring for an hour with the bath removed) were standard, with increasing times for mixed anhydride formation. Workup was simplified: the methylene chloride solution was washed with 10 ml. of water, 5 ml. of N sodium bicarbonate solution, 5 ml. of water, 5 ml. of N hydrochloric acid, and 5 ml. of water, then dried over sodium sulfate. The resulting solution was concentrated to dryness under vacuum and the crystalline product washed out with petroleum ether and dried. In every case, melting points were in the range 138-140° and TLC (silica gel) of a number of samples gave single spots (R_F 0.6 in 35 benzene-5 acetic acid); analysis (C, H and N) of one sample was good; its rotation was $\alpha]_D^{25}$ -27.2° ± 0.12 (C, 1.60; EtOH) (Johnson and Stock reported $\alpha]_D^{22-26}$ -24° (C, 4.1; EtOH). Activation times and yields with N-methylmorpholine as the tertiary base were: 30 sec, 86%; 60 sec, 88%; 4 min, 87%; 8 min, 85%; 8 min,

85%. With triethylamine as the base: 4 min, 37%; 8 min, 70%; 8 min, 66%; 12 min, 80%; 16 min, 88%. These results are shown in the Figure.

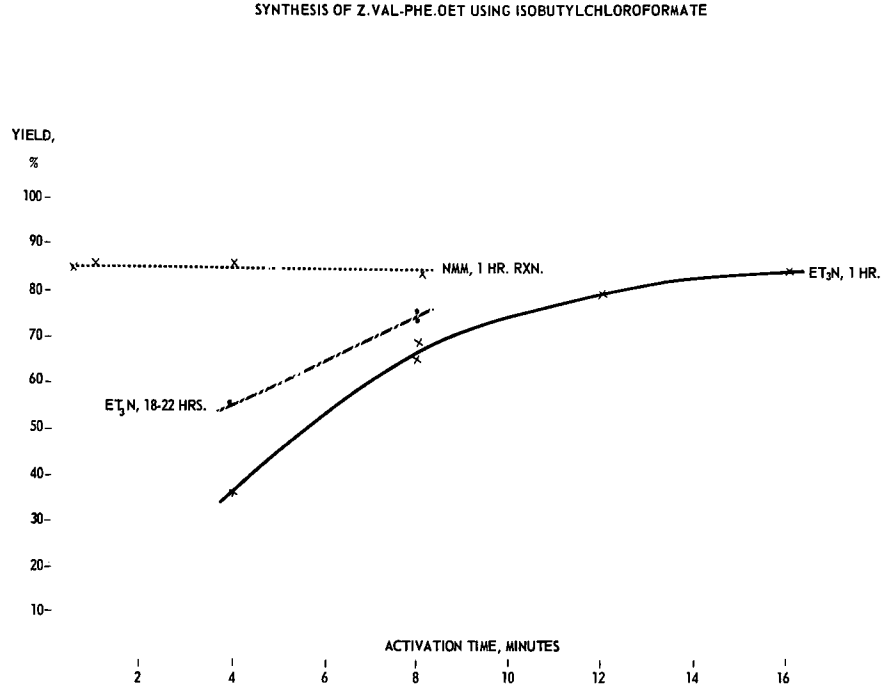

Figure 1

With N-methylmorpholine, yields were not changed by overnight reaction at room temperature, but with triethylamine the yield went from 37% to 56% for a 4 min. activation time, and from 70, 60% to 75, 76% with 8 min. activation times. Possibly some mixed anhydride formation took place slowly.

The synthesis of a less-hindered peptide was briefly studied. Z·Gly-Phe·OEt (4) was obtained in 93% yield, m.p. 91-92°, single spot TLC at R_F 0.3 (35 benzene-5 acetic acid) by a reaction with N-methylmorpholine with 4 min. acitvation time, 1 hr. reaction. Using triethylamine, the yield was 72% at 4 min. activation time, 78% at 8 min.; in both cases the product had a lower m.p. (86-90°) with a trace TLC impurity at R_F 0.5. Thus the major steric hindrance must be associated with triethylamine.

Conclusion: use N-methylmorpholine as the tertiary base in mixed anhydride peptide syntheses.

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LIQUID HCN AS SOLVENT IN PEPTIDE SYNTHESIS

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In a recent study (1) on steric hindrance in solid phase peptide synthesis (2) with active esters (3,4) the marked effect on the rate of acylation of the solvents used was noticed. Since not enough information on such solvent effects seemed to be available from the literature, the influence of some solvents commonly used in peptide synthesis was investigated. The reaction between benzyloxycarbonyl-L-leucine p-nitrophenyl ester (5) with glycine ethyl ester and also with benzylamine was used for comparisons. When the concentrations of the reactants were in the range of practical syntheses the reactions were too rapid for exact measurements. Yet, even a rough comparison showed that the aminolysis of the active ester runs remarkably faster in dimethylformamide than in chloroform. More reliable values could be obtained in dilute solutions (Table I). The ionic mechanism postulated for the aminolysis of active

Table I

Half reaction times (min.) between benzyloxycarbonyl-L-leucine p-nitrophenyl ester and benzylamine

Solvent	$T_{1/2}$
Dichloromethane	>240
Chloroform	200
Ethyl Acetate	10
Dioxane	18
Dimethylformamide	<2
Dimethylsulfoxide	<2
Liquid HCN	12

The reactions were carried out at 20° in 0.01 molar solutions of active ester with 150% excess of benzylamine. Rates were determined by the optical density of the solutions at 267 and 314 mμ.

esters (6) would suggest that polar solvents have a favorable effect on the rate of such reactions. In the search for highly polar solvents, liquid HCN offered itself as potentially valuable in peptide synthesis. However, the observed reaction rate (Table 1) was somewhat disappointing: in this respect liquid HCN, while definitely better than chloroform or methylenechloride, ranks only among solvents like dioxane or ethylacetate and does not show the rate enhancement seen with dimethylformamide or dimethylsulfoxide. Nevertheless, it was interesting to note that liquid HCN is a good solvent not only for the protected amino acid ester used in these experiments, but also for glycine ethyl ester hydrochloride and even for the hexapeptide L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (7), which is insoluble in chloroform, ethyl acetate and dioxane.

The reaction between benzyloxycarbonyl-L-leucine p-nitrophenyl ester (1 m mol) and glycine ethyl ester hydrochloride (1.5 m mol) in the presence of triethylamine (1.5 m mol) in liquid HCN (10 ml) gave, after the removal of the solvent, conventional work-up and recrystallization the protected dipeptide ester benzyloxycarbonyl-L-leucyl-glycine ethyl ester (8), in 66% yield, m.p. 102° $[\alpha]_D^{30}$ -27.6° (c 5, EtOH). The same material was obtained, in a somewhat lower yield, when the reaction was run in chloroform (57%) or in dimethylformamide (56%).

The weak acidic character of HCN could lead to partial protonation of the amino component and thus decrease the rate of acylation. The same property, however, could also diminish racemization, since abstraction of a proton from the α-carbon atom of reactive intermediates of amino acids or peptides is less likely in acidic media. The racemization test (9) involving the coupling of acetyl-L-isoleucine to glycine ethyl ester with dicyclohexylcarbodiimide (10) as condensing agent was applied. The amino component was added as the hydrochloride and an equivalent amount of triethylamine was used to liberate the free amine. The reaction was carried out at ice bath temperature (1/2 hour) and concluded at room temperature (1/2 hour). After evaporation of the solvent, a sample of the residue was hydrolyzed with constant boiling hydrochloric acid at 110° for 16 hours. Amino acid analysis (11) showed that in liquid HCN 4% of the L-isoleucine residue was converted to D-alloisoleucine. In chloroform under identical conditions 8% epimerization was observed, and 22% in the more generally useful solvent dimethylformamide. Hence, liquid HCN could be a desirable solvent for the coupling of valuable large peptides.

The advantages and disadvantages of liquid HCN as a solvent in peptide synthesis need more exploration. In spite of its low boiling point (26°), HCN is not too volatile and can be handled, even without cooling, in open vessels. The dangerous character of HCN need not be an absolute deterrent, since liquid NH_3 (12), HBr (13), and HF (14) already found application in this field.

This work was supported by a grant from the U. S. Public Health Service (NIH No. 1R01 AM 12473-01A1).

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EXCESS MIXED ANHYDRIDE METHOD FOR RAPID SYNTHESIS OF PEPTIDES IN HIGH YIELD AND PURITY WITH MINIMUM PURIFICATION

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Mixed anhydrides have been used for activation in peptide synthesis since their introduction by Wieland(1), Boissonnas(2), and Vaughan(3) in 1951. The use of an excess of mixed anhydrides has generally been avoided because of side reactions that can occur even at room temperature such as decomposition, disproportionation and urethane formation. These side reactions give rise to products which are difficult to separate from the peptides. For these reasons, the use of an excess of mixed anhydrides may give lower rather than higher yields, especially with low molecular weight peptides. Nevertheless, the mixed anhydride method remains quite attractive because of the ease of activation and the lack of racemization during couplings as reported by G. W. Anderson(4).

Weygand et al.(5) have used an excess of symmetrical anhydrides in an effort to improve coupling yields. They removed the anhydride remaining after completion of coupling by treating with a difunctional amine. The resulting amide containing a tertiary amine group was separated from the peptide by extraction with citric acid/H_2O and the amino acid derivative by extraction with $NaHCO_3/H_2O$ while the peptide remained in CH_2Cl_2.

We have found that aqueous bicarbonate will hydrolyze excess mixed anhydride in a homogeneous phase using water miscible solvents such as dimethylformamide and dimethylacetamide. The resulting Nα protected amino acid salts together with side products can be separated from the peptide by water washing. This ability to hydrolyze the mixed anhydride enabled the development of an efficient and simple procedure for peptide synthesis. In this procedure a 1.5 fold excess of Z-amino acid mixed anhydride preformed at -15°C is reacted for approximately 2 hours at -15°C with the amino component. During the formation of the mixed anhydride, we use ~6% excess of the protected amino acid derivative and N-methylmorpholine over isobutyl chloroformate to prevent any excess chloroformate from blocking the amino group. A two-hour reaction period has been sufficient for most couplings. After the reaction is complete, the excess mixed anhydride is hydrolyzed in the presence of bicarbonate/water at 0°C for 1/2 hour. The peptide is precipitated from the reaction mixture with water, is collected

by filtration and is washed thoroughly with water to remove the Z-amino acid K^+ salt and side products. If the peptide does not precipitate upon the addition of water, it is extracted into ethyl acetate and counterextracted with water. The Z group is removed by catalytic hydrogenolysis, and the peptide is then ready to be coupled with the next Z-amino acid. The peptide is never subjected to harsh conditions during deblocking since hydrogenolytic removal of the Z group, as introduced by Bergman and Zervas(6), is one of the mildest deblocking procedures known. Deblocking by hydrogenolysis is usually complete according to our own experience and as noted by Dr. J. S. Morley(7) at the Paris Peptide Symposium. Hydrochloric acid must be added during hydrogenolysis of dipeptides and glutamine(8) peptides to suppress the formation of diketopiperazines and pyroglutamic derivatives.

The crude products obtained using this procedure show high purity as determined by amino acid analyses, thin-layer chromatography in 4 systems, and complete elemental analyses of a series of intermediates. Yields in many couplings have been nearly quantitative and quite reproducible. Using this procedure we have synthesized several different sequences with excellent results. Since purification of the intermediate peptides is usually not necessary, the synthesis cycle is quite short. The procedure is simple enough that one coupling cycle can be completed in one day. Forty grams of the hexapeptide* Z-Tyr(Bu^t)-Ser(Bu^t)-Lys(BOC)-Tyr(Bu^t)-Leu-Asp(Bu^t)-OEt were synthesized in one week with an overall yield of 97%. Obviously this high overall yield required greater than 99% yields at each of the coupling and deblocking steps. The purity of the product is evidenced by good elemental and amino acid analyses and TLC's. Details of the preparation of a nonapeptide including the analysis of the intermediates were reported previously(9) and the results are listed in Table I.

The high purity of the crude products has led us to believe that the coupling and deblocking reactions are essentially quantitative. In addition, the removal of excess reagents and side products is also essentially quantitative. Data presented by G. W. Anderson(4) indicate that racemization does not occur to any significant degree under the conditions we use. We have carried out enzymic digestion of Lys-Tyr-Leu-Asp and Asp-Phe-Val-Gln-Try-Leu synthesized by our method with amino peptidase M. The results indicate a high degree of optical purity. The A.A. ratios were Lys .91, Tyr 1.00, Leu 1.13, Asp 1.08, and Asp 1.0, Phe 1.0, Val 1.0, Gln .6, Try .8, Leu .8, respectively. The lower values of Gln, Leu, and Try can be attributed to cyclization into pyroglutamic peptide. About 20% of the peptide being digested formed pyroglutamyl tripeptide and ~20% further of free glutamine cyclized; therefore, glutamine is 40% low and Leu and Try are 20% low.

The advantages of this procedure are summarized in Table II.

*All derivatives and peptides reported in this paper are of L amino acids.

The general limitations of the mixed anhydride activation obviously apply to our method. The method should not be used with glycine and asparagine residues because of possible side reactions. The glycine amino group can undergo diacylation as reported in the literature. However, glycine usually is a preferred C-terminal during peptide synthesis because peptide fragments with C-terminal glycine can be coupled without fear of racemization. Cyclization to a succinimid type of derivative during activation can occur with asparagine. The synthesis of sequences like Pro-Pro has been reported to give side reactions with mixed anhydride activation(10) because improper opening of the mixed anhydride may give rise to isobutyloxycarbonyl blocked peptides. At present we do not know to what extent this will limit the incorporation of a single proline residue using our procedure. Sometimes, coupling of valine may also give a slight amount of impurity due to urethane formation. However, isobutyloxycarbonyl blocked peptides are permanently blocked; therefore, formation of higher molecular weight components due to elongation of chains does not occur and with many peptides this type of impurity should be readily separable at a later stage.

We believe that our procedure can be successfully employed for the synthesis of most higher oligo peptides. The procedure is rapid and permits the use of relatively inexpensive and easily prepared carbobenzoxy derivatives. The derivatives which we have successfully used are listed below:

Z-Ala, BOC-NO_2-Arg, Z-NO_2-Arg, Z-Gln, Z-im-Bzl-His, Z-Leu, NαZ,-NεBOC-Lys, Z-Phe, BOC-Phe, Z-Ser(OAc), Z-Ser(Bu^t), Z-Thr(Bu^t), Z-Try, Z-Tyr(Bu^t), Z-Val; as C-terminus: Arg(HCl)-OMe, Asp(Bu^t)-OEt, Gln(xanthyl)-OMe, Gly-OEt, Gly-OBu^t, Leu-OMe, Leu-OBu^t, Phe-OMe; as N-terminus: BOC-Asp(OBzl)

In our experience, carbobenzoxy-L-glutamine can be incorporated successfully as a mixed anhydride using an excess. Hydrolysis of Z-Gln mixed anhydride may lead to glutarimid formation. After incorporation of tryptophan as mixed anhydride some impurities were noticed which could, however, be separated from the peptide by recrystallization. While incorporating Z-im-bzl-His, tertiary base was not used since one tertiary amino function is present in the Z-im-bzl-His moiety. Because the sodium salt of Z_3-Arg is insoluble in water, the excess mixed anhydride was removed by precipitating the peptide with cold ether, and filtering and washing the peptide with cold ether.

The peptides listed in Table III have been synthesized with our procedure.

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TABLE I

PEPTIDES OBTAINED WITHOUT INTERMEDIATE OR FINAL PURIFICATION USING EXCESS MIXED ANHYDRIDE METHOD
(M.A. Tilak, Tetrahedron Letters, No. 11, pp 849-854, 1970)

Compound	TLC	MP	Analyses
Z-Leu-Asp(Bu^t)-OEt		Oil	Oil — These peptides were used directly in the synthesis of the peptides below.
Z-Tyr(Bu^t)-Leu-Asp(Bu^t)-OEt		Oil	Oil — " " " " "
Z-Lys(BOC)-Tyr(Bu^t)-Leu-Asp(Bu^t)-OEt	Single Spot		Crude C H N O — AAA Lys 1.06, Tyr 1.00, Leu 1.10, Asp .95 Theory 63.20 8.06 7.67 21.05 Found 63.21 8.19 7.64 20.97 AAA (Amino peptidase digest) Tyr 1.0, Leu 1.13, Asp 1.08, Lys .91. $[\alpha]_D^{23}$ = -25.42, C=3 in DMF
Z-Ser(Bu^t)-Lys-(BOC)-Tyr(Bu^t)-Leu-Asp(Bu^t)-OEt	Single Spot	170-173°C	Crude C H N O — AAA Lys .86, Tyr 1.0, Leu 1.0, Asp .84, Ser .98. Theory 62.59 8.21 7.96 21.22 Found 62.29 8.39 7.78 21.40 $[\alpha]_D^{23}$ = -16.94, C = 1.1 in MeOH
Z-Tyr(Bu^t)-Ser-(Bu^t)-Lys(BOC)-Tyr(Bu^t)-Leu-Asp(Bu^t)-OEt	1 Major 1 Minor Spot	184sint. 194-197°d.	97% Yield based on C-term. Crude C H N O — AAA Lys 1.00, Asp .85, Ser .82, Leu 1.05, Tyr 1.90 Theory 64.07 8.14 7.69 20.08 Found 64.16 7.90 7.64 20.38

TABLE I continued

Compound	TLC	MP	Analyses
Z-Asp(Bu^t)-Tyr(Bu^t)-Ser-(Bu^t)-Lys(BOC)-Tyr(Bu^t)-Leu-Asp(Bu^t)-OEt	Single Spot	209-211°C.	95% Yield based on hexapeptide Crude Theory 63.13 8.09 7.75 21.03 Found 63.16 8.29 8.01 21.27
Z-Ser(Bu^t)-Asp(Bu^t)-Tyr(Bu^t)-Ser(Bu^t)-Lys(BOC)-Tyr(Bu^t)-Leu-Asp(Bu^t)-OEt	Single Spot	215-216°d	Crude C H N O AAA Lys 1.06, Asp 1.90, Theory 62.74 8.18 7.93 21.15 Ser 1.66, Leu 1.13, Found 62.70 8.16 7.93 21.16 Tyr 2.0
Z-Thr(Bu^t)-Ser-(Bu^t)-Asp(Bu^t)-Tyr(Bu^t)-Ser(Bu^t)-Lys(BOC-Tyr(Bu^t)-Leu-Asp(Bu^t)-OEt (9)	Single Spot	205-210°C.	79% Yield based on C-term. (>96% on each step) Crude C H N O AAA Lys 1.03, Asp 1.96, Theory 62.59 8.21 8.02 21.07 Thr 1.06, Ser 1.58, Leu Found 62.34 8.49 8.21 21.30 1.10, Tyr 2.00

TABLE II

Advantages	Limitations
1. Rapid	1. Asparagine requires side-chain protection to prevent cyclization.
2. Quantitative coupling Small excess (1.6:1) required for quantitative coupling; excess recoverable and reusable.	2. Z-Glycine undergoes side reactions (but Gly at C-terminus is preferable and can be used).
3. Peptides usually have high analytic purity without intermediate purifications.	3. Methionine and cysteine have to be N-terminal (because of catalyst poisoning) or may be used with pNO_2-Z, or BOC.
4. Intermediate purification is possible if necessary.	4. Prolyl-proline sequences may give side reactions.
5. Peptide never subjected to harsh conditions.	
6. High yields.	
7. Easy activation and work-up.	
8. NαZ derivatives can be used.	
9. Other protective groups can be used.	

TABLE III

EXAMPLE PEPTIDES

Compound	TLC	MP	Analyses
Z-Gln-Gly-OEt	Single Spot	163-164°C.	Crude C 55.88 H 6.35 N 11.30 O 26.27 Theory C 55.88 H 6.35 N 11.30 O 26.27 Found C 55.66 H 6.31 N 11.33 O 26.20
Z-Ser(Bu^t)-Gln-Gly-OEt(9)	Single Spot	169-171°C.	88% Yield Crude C H N O AAA Ser 1.86, Gln 1.90, Gly 1.88 Theory C 56.68 H 7.14 N 11.02 O 25.17 Found C 56.80 H 7.21 N 11.28 O 25.18 $[\alpha]_D^{23}$ = +3.37, C = 4 in DMF
Z-Try-Leu-OBu^t		Oil	Oil
Z-Gln-Try-Leu-OBu^t	Single Spot recryst.	204-205°C recryst.	81% Crude yield based on C-term. Recryst. C H N O AAA Glu 1.50, Leu 1.50 Theory C 64.23 H 7.16 N 11.06 O 17.70 Found C 64.50 H 7.15 N 10.82 O 17.69
Z-Val-Gln-Try-Leu-OBu^t	Single Spot	181-185°C.	
BOC-Phe-Val-Gln-Try-Leu-OH	Single Spot		AAA Glu .92, Val 1.05, Leu .97, Phe 1.03

TABLE III continued

Compound	TLC	MP	Analyses
Z-Ala-Gln-(xanthyl)-OMe	Single Spot	208-210°C (Lit. 208)	~94% Yield Crude C H N O AAA Ala 2.11, Glu 2.06 Theory 66.04 5.73 7.70 20.53 Found 65.86 5.85 7.63 20.71 $[\alpha]_D^{23}$= 1.54°, C = .25 in DMF
Z_3-Arg-Arg(HCl)-OMe		98-104°	87% Yield Crude C H N O Cl Theory 56.74 6.05 14.31 18.38 4.53 Found 56.56 6.00 14.02 18.40 4.42
Z-Ser(OAc)-Gln-Gly-OEt	Single Spot	198-199°C	83% Yield Crude C H N AAA Ser 1.00, Glu .99, Gly 1.02 Theory 53.50 6.12 11.33 Found 53.31 6.11 11.08
Z-His(imBz)-Ser(Bu^t)-Gln-Gly-OEt	Single Spot	142 sint 166-172°d.	47% Yield (Soluble in H_2O) AAA im. Bz His 1.02, Ser .88, Glu .96, Gly 1.00

Synthesis of Bis-Cystine Peptides

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Manuscript not received

SPECIFIC N^{ϵ}-ACYLATION OF FREE PEPTIDES CONTAINING LYSINE

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Recently, a semisynthetic approach to preparation of the lysine-10-analogue of human β-MSH, was described, in which naturally occurring porcine β-MSH, an octadecapeptide, was reacted with a suitably blocked tetrapeptide azide (1). Since the coupling reaction could occur at three sites, the α- and two ε-amino positions of β-MSH, a low pH was employed to maintain ε-aminos in a protonated, unreactive form, thereby to direct reaction toward the α-position. The desired product was obtained in well characterized form in yields of about four percent, similar to overall yields of purified peptides of equivalent size and complexity prepared by total synthesis. In addition, equal amounts of a mono-N^{ϵ}-acyl product were produced, indicating an N^{ϵ}-reactivity very much higher than that of the α-amino group. The present report describes means to employ high N^{ϵ}-amino reactivity in order to effect specific N^{ϵ}-acylation of free peptides containing lysine.

Free lysine has been the subject of a number of specific derivatization studies. Zervas et al. prepared N^{ϵ}-benzylidine lysine by virtue of product insolubility and rapid precipitation from solution (2). Weygand et al. synthesized N^{α}-trifluoroacetyllysine with trifluoracetic anhydride in trifluoracetic acid (3); in this case, strong acid so repressed N^{ϵ}-ammonium:amino equilibrium that no N^{ϵ}-acylation occurred. Although similar results, N^{α}-trifluoroacetylation, were obtained with a model tripeptide, the desired aim of this study was to develop a technique for direct, reversible N^{ϵ}-blocking, leaving the α-position free for peptide bond formation. LeClerq and Benoiton (4) in a systematic study of conditions for specific acylation of lysine found that nitrophenyl acetate effects N^{ϵ}-acylation at pH 11. No α-acylation was observed with excess reagent and long reaction times. Since both α-and ε-acylation occurs at lower pH, probably at pH 11 there is very rapid N^{ϵ}-aminolysis, while hydrolysis is so much faster than α- aminolysis that none of the latter takes place. These studies were not extended to peptides.

Initial acylation experiments described in the present report were with the tripeptide Ala-Lys-Ala, prepared as shown in fig. 1. This procedure afforded authentic N^{α}-, N^{ϵ}- and $N^{\alpha,\epsilon}$-diacyl products for use as standards. While a mixture of all four compounds can be resolved on carboxymethyl cellulose, fig. 2, more than ion exchange is operant since the free N^{α}-component is retarded more strongly than the free N^{ϵ}-derivative. For analytical purposes, electrophoresis at pH 9.5 was found more convenient, fig. 3, with products detected by ninhydrin after

SYNTHESIS OF Ala-Lys-Ala DERIVITIVES

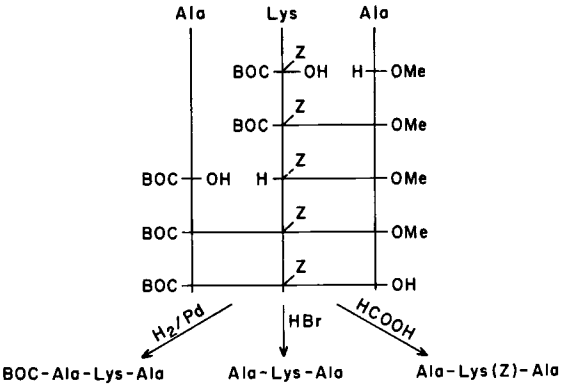

FIGURE 1

RESOLUTION OF Ala-Lys-Ala DERIVITIVES ON CM 52

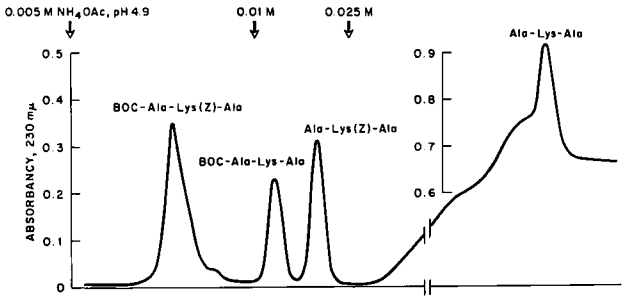

FIGURE 2

ELECTROPHEROGRAMS OF Ala-Lys-Ala DERIVITIVES
BORATE pH 9.5

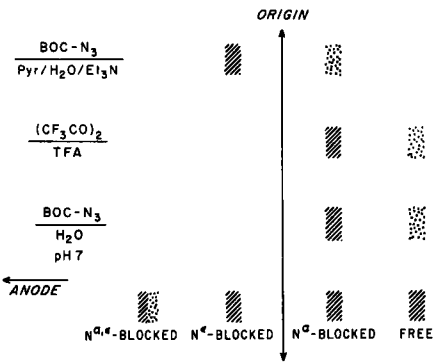

FIGURE 3

acid spray to deblock any diacyl derivative.

The acylating agent employed was t-butyl azidoformate (Boc.N_3), (5) because of its wide general utility in peptide chemistry and relatively low order of reactivity. In all experiments a ratio of 2μl Boc.N_3 per mg peptide was employed. Acylation of the tripeptide at pH 7 in water for 30 minutes yielded the N^{α}-acyl product, fig. 3; no N^{ϵ}-acyl component was detected. These results were in contradiction to previous results with a blocked tetrapeptide azide coupling to β-MSH at a lower pH (1). Exposure of Ala-Lys-Ala in trifluoracetic acid to trifluoracetic anhydride also afforded N^{α}-blocked material as described by Weygand et al. (3), for free lysine, fig. 3. Boc- and Tfa-peptides were characterized further by amino acid analysis and dinitrophenylation, table 1. Observed amino acid ratios confirm the proposed structures.

TABLE 1
Identification of Ala-Lys-Ala Derivatives

	Ala-Lys-Ala	DNP-Ala-Lys(DNP)-Ala	BOC-Ala-Lys(DNP)-Ala	TFA-Ala-Lys(DNP)-Ala	DNP-Ala-Lys(BOC)-Ala
Lysine	1.03 (1)	0.08 (0)	0.02 (0)	0.20 (0)	1.04 (1)
Alanine	1.96 (2)	1.00 (1)	2.00 (2)	2.00 (2)	0.97 (1)

Similar reaction conditions of β-MSH with Boc.N_3 did not produce α-acyl product in good yield, fig. 4. Although smearing indicates formation of some N^{α}-acyl product, very little formed even with prolonged reaction times. At pH 10.5, however, a new major component was produced, ninhydrin positive with a lower anodic mobility than authentic, fully acylated, ninhydrin-negative tri-Boc-β-MSH. The latter compound was prepared by an overnight reaction of β-MSH with Boc.N_3 in pyridine/water/triethylamine 10:10:1. If similar conditions are employed for one hour, a ninhydrin-positive product is formed with mobility similar to that of the pH 10.5 product, fig. 4. Although some tri-Boc-compound is also formed under these conditions, volatility of all reagents can be an advantage.

The desired N^{ϵ}-N'^{ϵ}-diacyl β-MSH was isolated by chromatography on carboxymethyl cellulose as shown in fig. 5. The major component, III, electrophoretically pure at pH 6.5 (insert of fig. 5), was isolated in yields of 50 to 60 percent. Products were characterized by dinitrophenylation and amino acid analysis, table 2. A partial analysis of β-MSH is shown in the first column. Dinitrophenylation removes both lysines and one aspartic acid, indicating the latter to be amino-terminal. A similar reaction with the tri-Boc-compound, column 3, table 2, fails to remove lysine or aspartic acid. Results of dinitrophenylation of the N^{ϵ}-N'^{ϵ}-di-Boc-product are shown in the final column of table 2. Both lysyl-residues are protected and recovered but the N-terminal

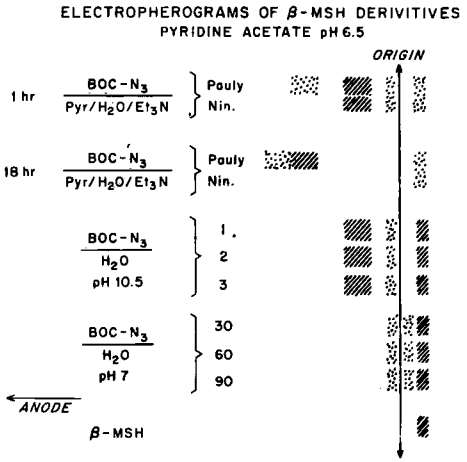

FIGURE 4

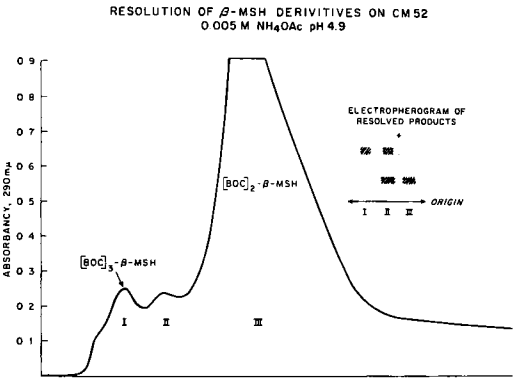

FIGURE 5

TABLE 2
Identification of β-MSH Derivatives

	β-MSH	DNP-β-MSH	DNP-BOC^3-β-MSH	DNP-BOC^2-β-MSH
Lysine	2.09 (2)	0.01 (0)	1.95 (2.0)	1.98 (2.0)
Aspartic	1.95 (2)	1.05 (1)	1.90 (2.0)	1.15 (1.0)
Glutamic	2.05 (2)	1.80 (2)	2.03 (2.0)	1.85 (2.0)
Glycine	2.00 (2)	2.10 (2)	1.95 (2.0)	1.95 (2.0)

aspartic acid is unprotected and lost.

Reaction of Ala-Lys-Ala with Boc.N_3 in pyridine/water/triethylamine also resulted in N^{ϵ}-acylation. The major product exhibited the same electrophoretic mobility as authentic N^{ϵ}-blocked peptide (fig. 3) and only traces of other possible products were observed. Dinitrophenylation and amino acid analysis gave a lysine-alanine ratio of 1:1, indicating that the amino-terminal residue was unblocked (table 1).

The relatively low reactivity of Boc.N_3, coupled with high nucleophilicity of N^{ϵ}-amino groups appears to afford direct preparation of N^{ϵ}-acyl derivatives in reasonable yield and under simple reaction conditions. This technique, designed to allow semisynthetic studies with naturally occurring peptides obtained from tryptic hydrolysates, may also find some utility in totally synthetic methodology as well. The procedure also makes possible stepwise Edman degradation of naturally occurring lysine-containing peptides for purposes of structure-activity studies, since formation of very stable N^{ϵ}-phenylthiocarbamyl derivatives can be reversibly blocked. Finally, specific acylation with stable blocking groups may be useful for sequence determination by subtractive Edman degradation techniques (6). In the usual procedure recoveries of lysine tend to be low, making difficult an unequivocal determination of sequence in lysine-containing peptides. Stable N^{ϵ}-acyl peptide derivatives may also be ideal substrates in solid phase subtractive Edman techniques (7).

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ACKNOWLEDGEMENT

This research was supported by National Institute of Health Grant Number CA 04679 and by American Cancer Society Grant Number P168.

SYNTHESES IN THE MELITTIN FIELD

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Introduction. The structure of melittin, the hemolytic principle of bee venom, was elucidated by Habermann and Jentsch (1) (Fig. 1):

Structure of Melittin

1	2	3	4	5	6	7	8	9	10	11	12	13	14
H-Gly-	Ile-	Gly-	Ala-	Val-	Leu-	Lys-	Val-	Leu-	Thr-	Thr-	Gly-	Leu-	Pro-

15	16	17	18	19	20	21	22	23	24	25	26
Ala-	Leu-	Ile-	Ser-	Try-	Ile-	Lys-	Arg-	Lys-	Arg-	Gln-	Gln-NH_2

Fig. 1

Our first attempt to synthesize melittin was planned via the partial sequences 21-26, 18-20, 15-17, and 1-14 respectively (Fig. 2).

Attempt to the Synthesis of Melittin I

							18		20	21		26		
							Ser-	--	Ile	Lys-	--	--	Gln-NH_2	
				15		17								
				Ala-	--	Ile	Ser-	--	--	--	--	--	Gln-NH_2	
1			14											
Gly-	--	--	Pro	Ala-	--	--	--	--	--	--	--	--	Gln-NH_2	
Gly-	--	--	--	--	--	--	--	--	--	--	--	--	Gln-NH_2	

Fig. 2

This synthesis, however, was not successful due to a Curtius rearrangement by coupling the fragment 18-20 via the azide method to the C-terminal sequence 21-26. This rearrangement was recognized by amino acid analysis which showed an extremely reduced value for isoleucine.

The instability of this azide Z-Ser-Try-Ile-N_3 was proved by keeping the azide in DMF solution at 0° and following the disappearance of the azide band in IR spectroscopy (2). Besides this fragment, the stability of the second tripeptide azide Z-Ala-Leu-Ile-N_3, 15-17, and the stability of other intermediates was also measured (Fig. 3).

Remaining Azide of Different Melittin Peptides at 0° in DMF

	after 3 days	after 10 days
Z-Ala-Leu-Ile-N_3	44 %	-
Z-Ser-Try-Ile-N_3	30 %	0 %
Z-Ser-Try-N_3	70 %	50 %
Z-Ser(tBu)-Try-N_3	80 %	65 %

Fig. 3

These investigations have shown that the tripeptides with C-terminal isoleucine are relatively instable whereas the dipeptides, especially with a protected hydroxyl function, should be stable enough for a successful azide coupling. The checking time of three days was chosen because initial experiments had shown that the azides reacted only slowly with the C-terminal fragments.

Synthesis of Melittin. For the successful synthesis of the C-terminal melittin sequence 15-26, Z-Ile-OTCP was coupled to the C-terminal hexapeptide, thus avoiding a Curtius rearrangement (Fig. 4). After hydrogenation the resulting heptapeptide was condensed with Z-Ser(tBu)-Try-N_3. Purification by counter current distribution and hydrogenation led to the nonapeptide. The next three amino acids in the sequence were coupled via the stepwise method, using the mixed anhydride method for isoleucine and activated ester methods for the other two. The resulting dodecapeptide was hydrogenated and purified by counter current distribution yielding a pure product with the correct amino acid analysis.

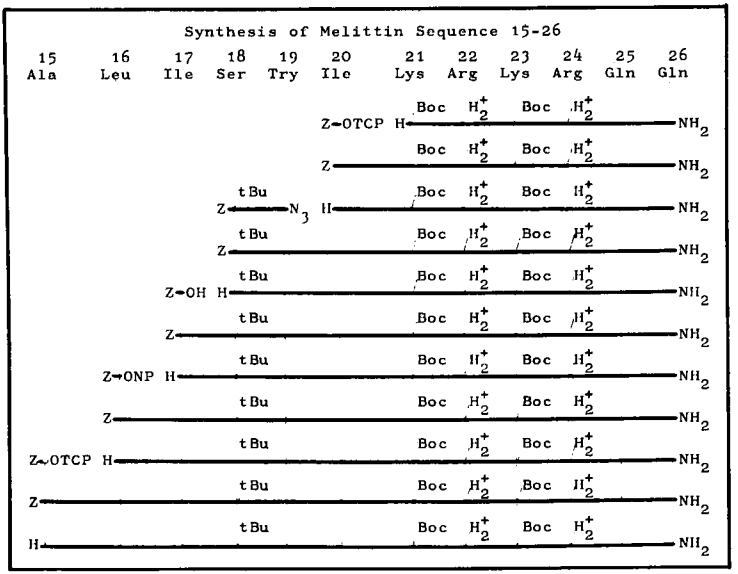

Fig. 4

Finally the sequence 1-26 (Fig. 5) was obtained by condensing the N-terminal tetradecapeptide 1-14, in 1,5 molar

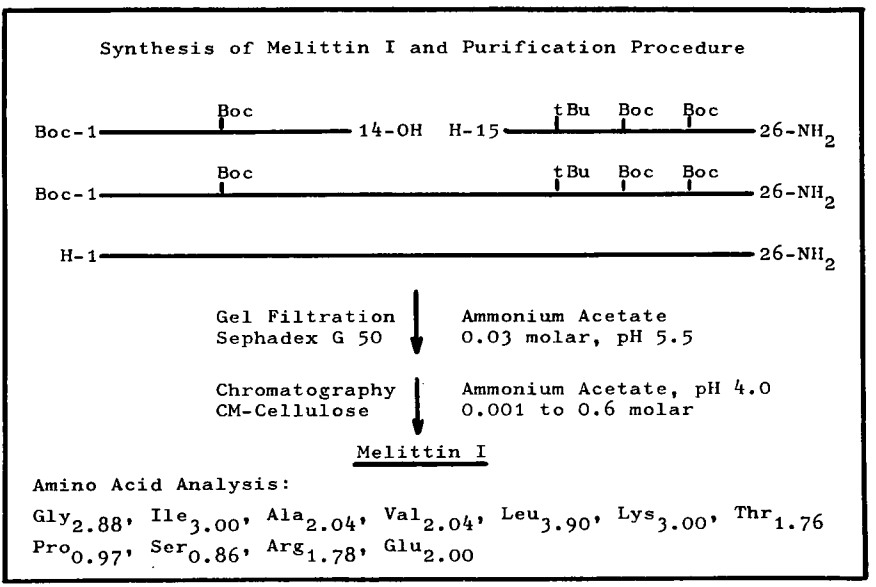

Fig. 5

excess, to the C-terminal fragment via the mixed anhydride method. The partially protected hexacosapeptide was deblocked with trifluoroacetic acid. The excess of fragment 1-14 was removed by gel filtration on Sephadex G 50. The final purification was carried out by CMC-chromatography. The pure hexacosapeptide, with the amino acid sequence published by Habermann and Jentsch for melittin, shows a correct amino acid analysis.

To compare the hemolytic activity of the synthetic melittin with the natural product, and to prove the identity of synthetic melittin, bee venom was separated into its components and pure melittin isolated.

The first separation step was carried out by gel filtration on Sephadex G 50 (Fig. 6). The individual tubes

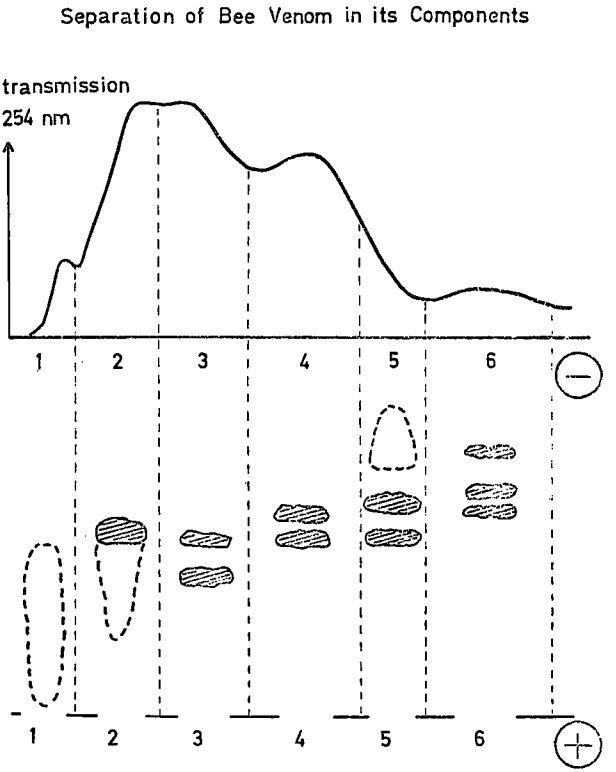

Fig. 6

were pooled and the resulting fractions were checked by electrophoresis. By comparison with an authentic sample of melittin, kindly sent to us by Prof. Habermann, the compound with the faster mobility in fraction 4 was recognized as melittin. Separation from the more slowly migrating compound of this fraction was possible by CMC-chromatography.

For comparison of both compounds, the natural melittin

as well as the synthetic product were checked by paper chromatography in different solvent systems, by paper electrophoresis, disc electrophoresis as well as by enzymatic degradation with different enzymes and finger printing. In no case could differences be found.

In the hemolytic test the synthetic material showed the full activity of natural melittin. For 50 % hemolysis a concentration of 1.2 ug/ml of the synthetic product and 1.4 ug/ml of the isolated melittin was necessary. A concentration of 1.5 ug/ml for natural melittin was also reported by Habermann(3).

Isolation, Structure and Synthesis of N^{α}-Formyl-Melittin. During the isolation of natural melittin the more slowly migrating compound of the melittin fraction was also checked by amino acid analysis. To our surprise this compound also showed an analysis in agreement with the amino acid composition of melittin (Fig. 7).

Amino Acid Analysis of the two Compounds of the Melittin Fraction

Theory	more slowly migrating compound	more rapidly migrating compound
Gly 3	2.94	2.93
Ile 3	3.00	2.99
Ala 2	2.03	1.95
Val 2	2.12	1.99
Leu 4	3.91	4.05
Lys 3	2.95	3.17
Thr 2	1.85	1.76
Pro 1	1.16	0.76
Ser 1	0.79	0.80
Arg 2	1.82	2.01
Glu 2	1.99	2.06

Fig. 7

In this connection it must be mentioned that in 1967 Kreil and Kreil-Kiss (4) reported an enzymatic degradation of a melittin preparation. After digestion with pronase and subsequently with carboxypeptidase a ninhydrin negative compound was isolated and identified as formyl-glycine. The authors assumed that besides melittin, also its N-formyl derivative is present in bee venom. The isolation of this compound, however, was not described. Therefore we investigated whether the more slowly migrating compound could be this N-formylated melittin.

The proof of a blocked N-terminal amino group should be possible by degradation with LAP. The results, however, were not satisfactory, because melittin itself was only partially digestible to an extent of 30-40 %, due to its poor solubility in the pH 8.6 buffer. The digestion rate of 5-10 % of the more slowly migrating compound was therefore not sufficiently significant.

The evidence for a N-terminal formylated melittin, however, was possible by digestion with thermolysine. This enzyme hydrolyses the peptide bond preceding a hydrophobic amino acid. Therefore the hydrolysis of the N-terminal glycylisoleucine bond and the formation of free formyl-glycine could be expected. In fact, after enzymatic degradation and electrophoresis at pH 5 besides neutral and basic products, one acidic compound could be detected. This compound was isolated by elution from the paper. After total hydrolysis only glycine was found and identification as formyl-glycine was possible by gas chromatography and comparison with an authentic sample.

To confirm the structure of a N^{α}-formylated melittin, this compound was synthesized (Fig. 8) via the partial sequences formyl-1-3, 4-14, and 15-26 respectively. After

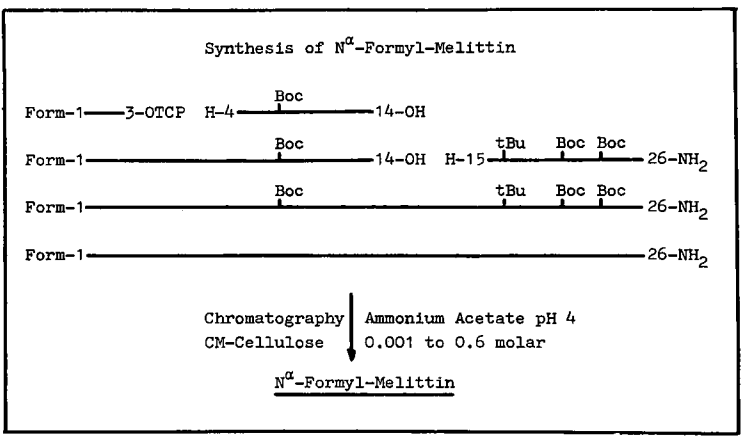

Fig. 8

purification on CM cellulose the pure product was identical with the natural compound in paper electrophoresis. It showed also the same electrophoretic pattern after thermolysine degradation. Also the hemolytic activity was identical with that of the natural compound. In comparison with natural or synthetic unformylated melittin, the biological activity is slightly reduced to 80-90 %.

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Structure and Function of Peptides with α,β-Unsaturated Amino Acids

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