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Solid-phase synthesis of peptide fragments of human growth hormone and related model peptides

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SOLID-PHASE SYNTHESIS OF PEPTIDE FRAGMENTS OF HUMAN GROWTH HORMONE AND RELATED MODEL PEPTIDES

by

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Richard Leslie Noble

ABSTRACT

The central theme of this thesis is the investigation of various aspects of the solid-phase peptide synthesis method during the syntheses of peptide fragments of human growth hormone (HGH) and related model peptides.

The stability of amino acid side-chain protecting groups was tested in a trifluoroacetic acid-dichloromethane solution generally used for deprotection in solid-phase peptide synthesis. Included in the 18 amino acid derivatives tested were (1) side-chain protecting groups in general use and (2) new protecting groups that have been reported but not yet generally employed. The results showed the stability of benzyl protection for aspartic acid, serine, threonine and glutamic acid to be satisfactory. For glutamic acid, 3- and 4-bromobenzyl protection were both more stable than the benzyl protection. For cysteine, the 3,4-dimethylbenzyl protection was shown to be stable enough to withstand many cycles of deprotection; however, this was not so for the 4-methoxybenzyl protection. The benzyl and benzyloxycarbonyl protection for tyrosine and lysine, respectively, were unsatisfactory; however, halogenated derivatives of these protecting groups exhibited the desired degree of stability.

N^{α}-*para*-Methoxybenzyloxycarbonyl [Z(OMe)] amino acid derivatives were synthesized and used in the syntheses of two model peptides and an HGH fragment. Three previously unreported derivatives were synthesized: Z(OMe)-Lys(2-BrZ)-OH, Z(OMe)-Met-*d*-(O)-OH, and Z(OMe)-Tyr(2-BrZ)-OH. A side product peptide was obtained from the synthesis of the methionine-containing model heptapeptide H-Leu-Gly-Arg-Leu-Gly-Met-Phe-OH. It behaved similarly to the sulfonium form obtained from a synthesis of the same peptide which employed N^{α}-Boc protection. It was not obtained when the above methionine sulfoxide derivative was used in place of methionine. The crude peptide product obtained from the synthesis of the HGH octapeptide H-Phe-Lys-Thr-Gln-Tyr-Ser-Lys-Phe-OH with N^{α}-Z(OMe) protection was shown to be of comparable purity to similar material obtained from use of N^{α}-Boc protection.

The carboxyl-terminal cyclic disulfide dodecapeptide of HGH was obtained from two syntheses which employed different side-chain portection for the cysteinyl residues. The linear form of the dodecapeptide was obtained from a synthesis which employed *S*-carbamidomethylcysteinyl residue derivatives. This peptide was identical to peptide material obtained from the cyclic dodecapeptide after reduction and alkylation with α-iodoacetamide. A curve was obtained from the subtraction of the circular dichroism (CD) spectra of the linear dodecapeptide from that of the cyclic dodecapeptide and was proposed to represent the CD band for the disulfide bond of the cyclic dodecapeptide.

ment was purified on the basis of charge by preparative isoelectric focusing on polyacrylamide gel. This fragment exhibited immunoreactivity in complement fixation experiment.

Dedication

This work is dedicated to L., T., B. and N.

Acknowledgment

I wish to thank Dr. C. H. Li for his guidance and constructive criticism of the studies in this work, and moreover, for the years of instruction during my association with the Hormone Research Laboratory. I wish to thank Dr. D. Yamashiro for setting by his own actions an example of the dedicated scientist. I will make this example the standard for my own research. I wish to thank Dr. E. C. Jorgensen for his contributions to this thesis as a committee member.

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CHAPTER 1

INTRODUCTION TO HUMAN GROWTH HORMONE AND SOLID-PHASE PEPTIDE SYNTHESIS

Native Human Somatotropin and Derived Fragments

Twenty years have passed since the first report (Li & Papkoff, 1956) of the isolation of human somatotropin (human growth hormone, HGH). The current procedures used to isolate the highly purified monomer of HGH have been described in a recent review of the chemistry of HGH (Bewley & Li, 1975). Briefly, monomeric HGH is obtained by extraction of freshly frozen pituitary glands, fractionation with ammonium sulfate, chromatography on a cation exchange resin, isoelectric precipitation in the presence of ethanol, exclusion chromatography on Sephadex G-50, and finally, gel filtration on Sephadex G-100. The bioassay most generally used for the growth-promoting activity of HGH is the rat tibia bioassay (Greenspan et al., 1949), in which the dose of HGH is reflected in the width of the proximal epiphysial cartilage of the tibia. The lactogenic activity of HGH is most often assayed by the promotion of pigeon crop-sac growth in either local or systemic tests (Lyons et al., 1960; Nicoll, 1967).

Ten years after its isolation, the first report of the primary sequence of HGH appeared (Li et al., 1966). This sequence was simultaneously revised in 1971 by two independent groups (Li & Dixon, 1971; Niall, 1971). In 1972 a final

revision (Li, 1972) reported the currently accepted primary sequence of HGH, Figure 1.

HGH is a single polypeptide chain of 191 amino acid residues with a molecular weight of 22,124, based on the composition determined by sequence studies. There are two loops in the chain resulting from two disulfide bridges between cysteine residues at positions 53 and 165, and 182 and 189. There is one tryptophan residue at position 86 and three methionone residues at positions 14, 125 and 170. The amino acid composition is:

Lys	His	Arg	Asp	Asn	Thr	Ser	Glu	Gln	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Trp.
9	3	11	11	9	10	18	13	14	8	8	7	4	7	3	8	26	8	13	1

The chemistry of HGH has been actively investigated since its isolation, and some interesting structure-function relationships have resulted. Early studies involved enzyme digestion of the molecule. Quantitative removal of the carboxyl-terminal phenylalanine by treatment with carboxypeptidase resulted in no loss of biological activity (Li et al., 1958). Limited digestion with chymotrypsin (Li, 1957), trypsin (Li & Samuelsson, 1965) and pepsin (Li, 1962) showed that the molecular integrity of HGH may not be required for the growth-promoting activity.

Studies within the last five years have shown that, in fact, the molecular integrity of HGH is not required for biological activity, and have resulted in the isolation and

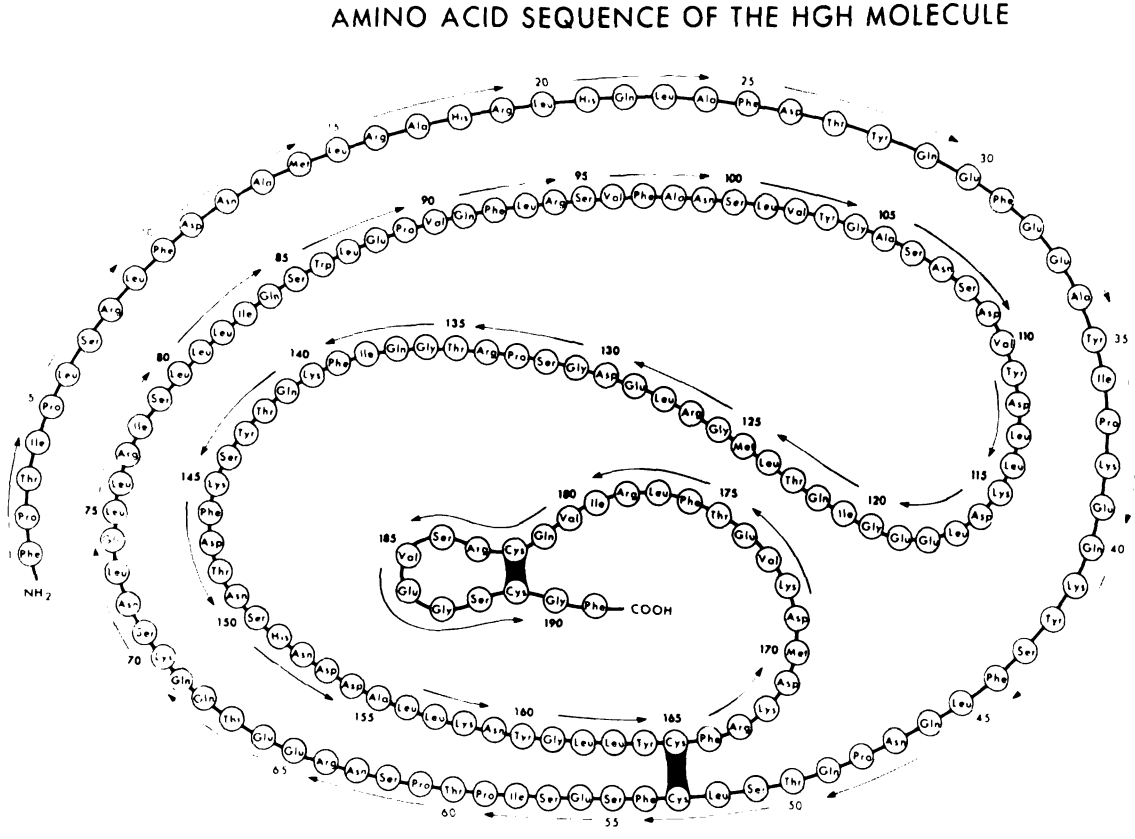


Figure 1. The amino acid sequence of the human somatotropin (human growth hormone, HGH) molecule (Li, 1972).

characterization of biologically active fragments of HGH. In 1971 it was reported (Lewis et al., 1971) that enzymatic degradation of HGH resulted in the loss of a hexapeptide, residues 135-140, and an increase of the lactogenic activity in the crop-sac assay. This supported previous evidence that plasmin digestion of HGH increased the lactogenic activity (Chrambach & Yadley, 1970). A preliminary report (Reagan et al., 1973) indicated plasmin digestion of HGH followed by reduction and carbamidomethylation resulted in the N-terminal 134 residue fragment of HGH and the C-terminal 51 residue fragment. While the N-terminal fragment was reported to be inactive in promoting growth, it was shown to be active in in vitro assays; however, the C-terminal fragment was inactive in all assays.

In 1974 detailed chemical characterization and the biological activities of the two fragments of reduced-carbamidomethylated, human plasmin-digested HGH were reported (Li & Graf, 1974). The highly purified N-terminal 134 residue and the C-terminal 51 residue fragments were isolated in 28% and 22% overall yield, respectively, and although the C-terminal fragment was not as active in all the cases as the N-terminal fragment, both were active in the rat tibia and pigeon crop-sac tests and were immunologically active in the complement fixation assay and radioimmunoassay using antiserum to HGH.

In 1976 it was reported (Li & Bewley, 1976a) that, in comparison to plasmin-digested HGH, the two fragments recombined to restore full biological activity and conformation in the tibia and crop-sac assays as measured by circular dichroism spectra. More detailed conformational studies have been reported (Li & Bewley, 1976b) on these fragments prepared by milder procedures for reduction and alkylation and suggest "... that the retention of full biological activity in these derivatives is intimately associated with the retention of the conformation of native HGH..."

Studies of other biologically active HGH fragments obtained from enzymatic digestion of the native molecule have been reported. The action of a bacterial proteinase resulted in the enhancement of the growth-promoting activity of HGH (Lewis et al., 1976). This same study described a modified form of HGH in which a tripeptide, residues 39-41, was missing and the N-terminal 38 residue fragment remained noncovalently bound to the rest of the molecule in 6 M urea or 10% formic acid. Another study (Keda et al., 1973) described the isolation of a tetradecapeptide fragment, residues 31-44, from a peptic digestion of HGH, and reported the fragment had 500 times the in vitro lipolytic activity of HGH. A hyperglycemic peptide fragment was obtained from a peptic digestion of HGH (Lostroh & Krahl, 1974), but no sequence data were given.

In addition to studies on the effect of enzymatic digestion of HGH and the resulting products, there have been numerous studies on the chemical modification of specific residues in HGH. These have been reviewed (Bewley & Li, 1975). Two areas of investigation should be described in reference to the feasibility of obtaining synthetic fragments of HGH which exhibit biological activity.

A major question was whether or not the disulfide bonds of HGH were required to maintain the structure of the intact molecule and its biological activities. In 1966 the two cystine bridges were reduced and the resulting thiol groups alkylated with α-iodoacetamide to yield the monomer of the S-tetracarbamidomethylated (RCAM) derivative (Dixon & Li, 1966). In 1968 the same derivative was prepared in greater yields by methods which did not use any denaturant (Bewley et al., 1968). The S-tetracarboxymethylated (RCOM) derivative was also prepared by this latter method with α-iodoacetic acid (Bewley et al., 1969). While reduction and alkylation with either α-iodoacetamide or α-iodoacetic acid had reduced the stability of the conformation, the loss of the disulfide bonds did not lead to an abrupt denaturation (Bewley et al., 1969). RCOM-HGH exhibited very little of the growth-promoting activity but retained approximately 50% of the lactogenic activity. Yet, RCAM-HGH retained full biological activity when assayed by the rat tibia and pigeon crop-sac tests, and

was also shown to have growth-promoting activity in human subjects (Connors et al., 1973). This demonstrated that the intact disulfide bonds were not necessary for the biological activities of HGH, and in fact led to the studies of plasmin-treated HGH mentioned above in which the two fragments were separated following reduction and alkylation, and then recombined to give a fully active molecule (Li & Bewley, 1976a). Thus, as was shown for RCAM-HGH and the recombinant, formation of the disulfide bonds would not be a requirement of analogous synthetic products in order to maintain the structure or activity of intact HGH.

The second area of chemical modification potentially resulting in biologically active fragments was the selective cleavage of the peptide chain at the methionine residues by cyanogen bromide (Gross & Witkop, 1962). Having methionine residues at positions 14, 125 and 170, HGH cleaved with cyanogen bromide resulted in 4 fragments consisting of 14, 111, 45 and 21 residues. Those peptides were isolated and reported in the sequence studies (Li et al., 1969). The 111 residue fragment has had a structure revision (Li, 1972) since it was originally reported as a 108 residue fragment. This 111 residue fragment has been isolated from reduced, S-aminomethylated HGH (Nutting et al., 1972) and S-carbamidomethylated HGH-(1-134) (Li, 1975) and has been shown to have biological activity *in vitro* and *in vivo*. Thus, a peptide fragment of HGH, large on the synthetic scale but

much smaller than the native molecule, has been obtained and shown to retain biological activity.

Synthetic Peptide Fragments of HGH

A. Solution methods

The twenty-one peptides of HGH which have been synthesized by solution methods are listed in Table 1. It should be noted that fifteen of the peptides were reported according to the numbering system of the original 188 residue sequence (Li et al., 1966). Four of the peptides no longer represent HGH peptides of the accepted final sequence (Li, 1972), while the other eleven represent peptides contained in the new sequence. At any rate, 72% of the HGH sequence is within the sequences that these peptides represent; namely, residues 1-15, 18-52, 77-92, 95-124, 139-156, and 169-191. These peptides represent a great deal of synthetic effort and could contribute to the structure-function studies of HGH. However, only two of these peptides have been reported in the fully unprotected form (Kovacs et al., 1971; Camble & Cotton, 1975). The HGH-(95-124) peptide was the only fragment submitted to a biological assay, the rat tibia test, and no activity was observed.

B. Solid-phase methods

The peptides of HGH synthesized by the solid-phase method developed by Merrifield (1964) are listed in Table 2,

which represents a survey of the literature to November, 1976. Of the synthetic HGH peptides, five were from syntheses undertaken to study different aspects of the synthetic method rather than to obtain biologically active HGH fragments. These "model" peptides were the following residue sequences of HGH: 17-23 (Yamashiro et al., 1972a), 113-117 (Wang & Kulesha, 1975), 131-134 (Dorman et al., 1972), 139-146 (Yamashiro & Li, 1972; Yamashiro & Li, 1973a), 180-184 (Marshall & Liener, 1970) and 180-191 (Yamashiro et al., 1973a). Unfortunately, the majority of the reports listed were preliminary at best with little or no account of the synthesis, isolation and purification, characterization and biological assay data. Thus it was difficult to (1) judge the synthetic methods used, (2) assess the nature of the final products and (3) be confident that the conclusions drawn about the biological activities reported were true. Nevertheless, biological activities have been reported for at least 11 of the 17 synthetic products.

The short peptide fragments corresponding to sequences in the amino and carboxyl-terminal twenty residues of HGH have been actively studied by Bornstein and his co-workers (1976). They concluded that(1) the insulin-potentiating action of the short amino-terminal HGH peptides, containing the sequence corresponding to residues 6-13, could be related to the well-known hypoglycemic action of HGH and that

(2) the lipolytic, glucagon-releasing, anti-insulin action of the carboxyl-terminal peptide, corresponding to residues 176-191, could be related to identical actions of HGH.

Pankov and his colleagues (Yudaev et al., 1976; Pankov et al., 1976) reported that the peptide, corresponding to residues 31-44 and isolated from both peptic digestion of HGH and synthesis, had lipolytic activity *in vitro* in isolated rat, rabbit and human cells, and *in vivo* in rats and rabbits.

The studies reported in Table 2 of the large synthetic peptides corresponding to sequences in the middle of HGH stemmed from the work of Sonenberg and his co-workers (Yamasaki et al., 1970) who reported the isolation and biological activity of a tryptic fragment of bovine growth hormone in the region of residues 96-133. The most extensive studies in this respect have been those of Chillemi and his colleagues (1976). However, the low levels of activities obtained by their fragments prompted them to regard the biological data as preliminary but still suggestive of the existence of active cores in the HGH sequence responsible for selected effects of HGH. From preliminary reports, other investigators (Niall & Tregear, 1973; Pena et al., 1975) have reached contradictory conclusions about activities in overlapping sequences. An HGH fragment was

synthesized and the N^{α}- amino group was acetylated (Blake & Li, 1973) in order to obtain a material which might not be degraded as rapidly in vivo as an unprotected fragment. This peptide, N^{α}-acetyl-HGH-(95-136), gave a significant stimulation of tibial growth. However, an estimation of the relative potency was not given because the dose-response curves of the peptide and HGH were not parallel.

In conclusion, the synthesis of peptides of HGH by both solution and solid-phase methods has been an active field of investigation. Peptide fragments synthesized by the solid-phase method have been reported to have biological activity in several assay systems. However, for the most part, questions concerning other properties of these peptides, such as conformational and immunological comparisons to HGH, have not been addressed.

The Solid-Phase Method of Peptide Synthesis

Less than fifteen years ago, the solid-phase method of peptide synthesis was introduced by Merrifield (1962). Since then there have been several hundred reported syntheses of peptides by the solid-phase method and lists (complete to November, 1969) of these peptides have been compiled (Merrifield, 1968; Marshall & Merrifield, 1970). Tables of model peptides, peptide hormones, kinins, and other biologically active peptides that were synthesized by the solid-phase method to June, 1971, have been compiled (Meienhofer, 1973).

Solid-phase peptide synthesis has been reviewed previously in twenty-five articles (see Erickson & Merrifield, 1976). Four very thorough reviews of the solid-phase method have appeared as the method has developed (Merrifield, 1967; Merrifield, 1969; Meienhofer, 1973; Erickson & Merrifield, 1976) and the most recent two are highly recommended for both their historical and current coverage of the field.

Although many variations have been investigated, the general solid-phase strategy for the stepwise synthesis of peptides has consisted of: covalent attachment of the protected carboxyl-terminal amino-acid residue to an insoluble support; a cycle of N^{α}-deprotection, neutralization and coupling of the next protected amino-acid residue; repetition of this cycle for each remaining residue; cleavage of the final protected peptide from the solid support; and removal of the side-chain protecting groups.

An advantage of this technique over solution methods has been separation of the soluble reagents and by-products from the insoluble support-bound peptide simply by repeated filtering and washing of the support. In addition, the soluble reagents can be used in excess to force the reactions to completion, and the nature of the incorporation of each residue in the peptide has seemed to be equivalent, unlike the coupling of peptide fragments in solution which depended on

their solubility characteristics. The repetitive cycles, that is, treatment of the peptide resin with reagent solutions and washes, has also meant that the simplified and accelerated stepwise synthesis could be performed and even automated in a single reaction vessel. Elimination of repeated transfers in the isolation and purification of the intermediates has thus avoided the attendant loss of material.

In exchange for the advantages of solid-phase synthesis, certain limitations and disadvantages had to be accepted. Any side-product resulting from either less than quantitative reactions of deblocking and/or coupling in each cycle, or from side reactions, accumulates on the solid support along with the desired product. Secondly, the analytical tests for deprotection and coupling that have been developed were not able to show that these reactions were 100% complete. The concern has been for accuracy at the level of 1 part per 10,000 (0.01%) or less and all of the tests have had the same inherent problems in terms of complete reactions with or elution from the insoluble peptide-resin or side reactions with the putative inert resin. Finally, complete removal of the solid support has been difficult to attain without any degradative effects. Much effort has been made in defining and minimizing these limitations.

The improvements introduced to the solid-phase method as it is generally used have included more stable side-chain protecting groups for the amino acids; a less cross-linked solid support than was originally used; procedures for attachment of the first residue without derivization of the solid support; new reagents for removal of the N^{α}-tert-butyloxycarbonyl (Boc) group, subsequent neutralization, and swelling and shrinking the solid support and peptide chain; new coupling methods; and cleavage conditions which obtained a higher yield and fewer side reactions. These improvements will be discussed in greater detail in the following chapters.

Table 1

HGH Peptides Synthesized by Solution Methods

Synthetic Peptides[a]	References
1-5, 6-9, 10-15, 77-82, 83-88	Li & Chung, 1971
1-10	Beyerman, 1972
18-24, 25-30[b],31-37,89-92-Ala[b]	Danho & Li, 1971a
38-41,42-46,47-52[b,c]	Kovacs et al., 1971
38-45, 46-52[b,c]	Wang & Li, 1971
95-124	Camble & Cotton, 1975
139-146, 147-156	Chillemi, 1975
169-177, 178-184, 185-191	Danho & Li, 1971b

[a] According to revised sequence (Li, 1972), see text.

[b] Glu instead of Gln.

[c] Asp instead of Asn.

Table 2
HGH Peptides Synthesized by the
Solid-Phase Method

Synthetic Peptides[a]	References
1-188[b]	Li & Yamashiro, 1970
180-184[b]	Marshall & Liener, 1970
81-121, 122-153[b]	Chillemi & Pecile, 1971
1-21, 164-188[b]	Bornstein et al., 1971[c]
6-10, 12-21[b]	Beyerman, 1972[c]
131-134[b]	Dorman et al., 1972[c]
17-23	Yamashiro et al., 1972a
88-124,125-156	Chillemi et al., 1972
1-35, 77-102, 87-123, 94-134, 124-155	Niall & Tregear, 1973[c]
139-146	Yamashiro & Li, 1972, Yamashiro & Li, 1973a
180-191	Yamashiro et al., 1973a
N^{α}-Acetyl-95-136	Blake & Li, 1973
1-13, 3-13, 1-15, 1-20, 6-11	Ng et al., 1974[c]
113-117	Wang & Kulesha, 1975
140-191	Blake & Li, 1975
75-120-NH_2	Pena et al., 1975[c]
31-44	Yudaev et al., 1976[c]
176-180, 180-191, 176-191, 177-191	Bornstein, 1976[c]
1-36, 83-130, 96-134, 111-134, 166-191	Chillemi et al., 1976[c]

[a] According to revised sequence (Li, 1972).
[b] According to original sequence (Li et al., 1966).
[c] Preliminary report.

CHAPTER 2

THE STABILITY OF SIDE-CHAIN PROTECTING GROUPS FOR AMINO ACIDS

Introduction

The conditions of the solid-phase method have required the use of side-chain protecting groups for amino acids which bear side-chain functional groups. Since the functional groups may be irreversibly modified by the reagents employed in the deprotection, neutralization and coupling, or may be coupled with the activated amino acid resulting in branching of the peptide chain, the stability of the side-chain protecting groups became increasingly important as the lengths of peptides synthesized by the solid-phase method increased. In addition, it was required that the protecting groups should not give rise to side products at any stage of (and must be removed efficiently at the end of) the synthesis. Finally, the protecting groups had to result in amino acid derivatives that coupled efficiently to the peptide resin.

Some of the studies presented in this chapter were included in a report (Yamashiro et al., 1972b) of new side-chain protecting groups for amino acids used in solid-phase synthesis in conjunction with N^{α}-*tert*-butyloxycarbonyl (Boc) protection. Side-chain protection has been reviewed (Meienhofer, 1973; Erickson & Merrifield, 1976) in greater detail and in conjunction with other N^{α}-protecting groups.

Experimental Section

Methods and Materials. Melting points were determined on a Fisher-Johns block and were uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Thin-layer chromatography (tlc) was run on silica gel in chloroform-methanol (CM), 1:1, chloroform-methanol-acetic acid (CMA), 15:2:1; 1-butanol-acetic acid-water (BAW), 4:1:1. Precoated thin-layer chromatography plates, Quanta-Gram Q1-F, were obtained from Arthur H. Thomas Co., Philadelphia, Pa. Optical rotations were measured through a 20-cm cell (O.C. Rudolph and Sons, Caldwell, N.J.) in a polarimeter (Bellingham and Stanley, Ltd., London) and were obtained from the average of at least ten readings of both the blank and the sample.

Isobutylchloroformate and tert-butylazidoformate were obtained from Pierce Chemical Co., Rockford, Ill. Triethylamine, anisole, dichloromethane and (ethylenedinitrilo)-tetraacetic acid disodium salt were obtained from Eastman Organic Chemicals, Rochester, N.Y. The triethylamine and anisole were distilled before use. Dichloromethane was placed over P_2O_5 for 16 h and then distilled from fresh P_2O_5 before use. Dimethyl sulfoxide, dicyclohexylamine and 4-bromobenzyl bromide were obtained from Aldrich Chemical Corp., Milwaukee, Wis. 3-Bromobenzyl bromide was obtained

from K & K Laboratories, Inc., Hollywood, Ca. Petroleum ether (bp 35-60°) was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. Trifluoroacetic acid was obtained from Halocarbon Products Corp., Hackensack, N.J. Hydrogen fluoride was obtained from Matheson Gas Products, Newark, Ca. L-Amino acids were obtained from Schwarz/Mann, Orangeburg, N.Y. L-Serine(O-benzyl) was obtained from Cyclo Chemical, Los Angeles, Ca. N^{α}-Boc-Ser(OBzl)-OH was obtained from Beckman Instruments, Inc., Palo Alto, Ca.

γ-4-Bromobenzyl Glutamate. To a stirred solution of 51.5 g (255 mmol) of sodium copper glutamate (Ledger & Stewart, 1965) in 426 ml of water, a solution of 64.0 g (257 mmol) 4-bromobenzyl bromide in 853 ml DMF was added. After the mixture was stirred for 24 h at 35-40°, the product was precipitated by addition of 1.3 liters of acetone, collected by filtration and washed with 200-ml portions of water and acetone. The material (ca. 75 g) was suspended in 5.5 liters of water, 75 g of (ethylenedinitrilo)tetraacetic acid disodium salt was added, and the mixture was boiled until a solution was obtained. The product which came out of solution upon cooling was filtered and washed with 200-ml portions of water and acetone: yield, 25 g (79 mmol, 31%); mp 180-181°; tlc(BAW) R_f 0.62; $[\alpha]_D^{24}$ + 1.4° (c 1, 80% aqueous acetic acid).

Anal. Calculated for $C_{12}H_{14}NO_4Br$ (316.15): C, 45.6; H, 4.5; N, 4.4. Found: C, 45.6; H, 4.0; N, 4.4.

N^{α}-Boc-γ-(4-Bromobenzyl)glutamic Acid. γ-(4-Bromobenzyl)-glutamate (18.5 g, 58.5 mmol) was converted to the N^{α}-Boc derivative by the DMSO procedure (Stewart & Young, 1969, pp. 29-30). Briefly, to a stirred suspension of γ-(4-bromobenzyl)glutamate in 250 ml of DMSO, 16.0 ml of triethylamine (117 mmol) and 13.5 ml of Boc-azide (87.8 mmol) were added. After stirring for 23 h at 24°, the solution was diluted with water (840 ml) and washed with two 150-ml portions of ether. The aqueous phase was then cooled and acidified with 50 ml of 3 N HCl to pH 2. The product was extracted with two 200-ml portions and one 100-ml portion of ethyl acetate. The ethyl acetate solution was washed with three 50-ml portions of water and then dried over anhydrous $MgSO_4$. Removal of the drying agent and solvent gave an oil which crystallized from ether-petroleum ether (bp 35-60°) in a cold room (4°) overnight. The product was filtered and washed with ether-petroleum ether, 1:4 (v/v): yield, 19.3 g (46.4 mmol, 79%); mp 91-92°; tlc (CM) R_f 0.69; $[\alpha]_D^{24}$ -10.7° (c 2.01, DMF).

Anal. Calculated for $C_{17}H_{22}NO_6Br$ (416.27): C, 49.1; H, 5.3; N, 3.4. Found: C, 49.3; H, 5.2; N, 3.2.

γ-3-Bromobenzyl Glutamate. A scaled down version of the procedure to obtain γ-4-bromobenzyl glutamate was used with

6.0 g (30 mmol) sodium copper glutamate and 7.5 g (30 mmol) 3-bromobenzyl bromide: yield, 2.28 g (7.21 mmol, 24%); mp 148-149°; tlc (BAW) R_f 0.70; $[\alpha]_D^{24}$ + 26.4° (c 1.11, 80% aqueous acetic acid).

N$^{\alpha}$-Boc-γ-(3-Bromobenzyl)glutamic Acid. A scaled down version of the procedure to obtain N$^{\alpha}$-Boc-γ-(4-bromobenzyl)glutamic acid was used with 1.85 g (5.85 mmol) γ-3-bromobenzyl glutamate, 1.60 ml (11.7 mmol) of triethylamine and 1.35 ml (8.78 mmol) of Boc-azide. The resulting oil, 2.17 g (5.23 mmol), defied crystallization. After dissolving the oil in 6 ml of ether and cooling, 1.04 ml (5.23 mmol) of dicyclohexylamine was added. Removal of the solvent gave an oil which crystallized from 5 ml of ether. The product was filtered and washed with 10 ml of ether: yield, 2.75 g (4.61 mmol, 79%); mp 132.5-134.5°.

N$^{\alpha}$-Boc-S-Carbamidomethylcysteine. S-Carbamidomethylcysteine (Armstrong & Lewis, 1951) (25.0 g, 140 mmol) was converted to the N$^{\alpha}$-Boc derivative by the pH-stat method (Schnabel, 1967) with Boc-azide (ca. 214 mmol) at 45° for 10 h with addition of 4 N NaOH to maintain the pH between 9.4 and 9.8. Dioxane was removed in vacuo, the solution was diluted with 300 ml of water and washed with three 75-ml portions of ether. The aqueous phase was cooled and acidified to pH 3 with

3 N HCl. The product was extracted into 350 ml of ethyl acetate, washed with two 50-ml portions of saturated NaCl, and dried over anhydrous $MgSO_4$. Removal of the drying agent and solvent gave an oil which crystallized on standing: yield, 17.4 g. For analysis, a sample (1.40 g) was recrystallized from ethyl acetate (30 ml): 1.24 g; mp 128-131°; tlc (CMA) R_f 0.3; $[\alpha]_D^{24}$ -15.2°(c 2.00, absolute ethanol).

Anal. Calculated for $C_{10}H_{18}N_2O_5S$ (278.34) : C, 43.2; H, 6.5; N, 10.1. Found: C, 43.3; H, 6.4; N, 9.9.

N^{α}-Boc-Amino Acid Amides. The N^{α}-Boc-amino acids (2.67 mmol) were each dissolved separately in 8 ml acetonitrile, except for Boc-Arg(Tos) which required a 8-ml solution of DMF-acetonitrile, 1:3. To each solution, 0.375 ml (2.67 mmol) of triethylamine was added and the solution cooled to -20° in a dry ice-acetone bath. With stirring, 0.35 ml (2.67 mmol) of isobutylchloroformate (Vaughan & Osato, 1952) was added and the reaction allowed to proceed for 15 min. A solution of 7.25 M NH_4OH (1 ml) was added and the solution allowed to reach room temperature. With the exceptions of the Arg(Tos) and Cys (3,4-Me_2Bzl) derivatives, the products were precipitated by addition of water (8 ml), filtered and washed with 40 ml of ethanol-water, 1:1. After the addition of the 8-ml portion of water, the Arg(Tos) and Cys(3,4-Me_2Bzl) derivatives were separately extracted into 25 ml of ethyl acetate,

washed with three 20-ml portions of water, one 20-ml portion of 5% (w/v) aqueous $NaHCO_3$ and three 20-ml portions of water. The ethyl acetate solution was dried over anhydrous $MgSO_4$. Removal of the drying agent and the solvent resulted in an oil for both derivatives. The characterization of the eleven Boc-amino acid amide derivatives are shown in Table 3.

N^{α}-Acetyl-amino Acid Amide Derivatives. Each N^{α}-Boc-amino acid amide derivative (0.97 to 2.67 mmol) was dissolved separately in 10 ml of trifluoroacetic acid. After 10 min at 24°, the trifluoroacetic acid was evaporated, 5 ml of acetic acid was added and then evaporated. The resulting material was dissolved in 10 ml of pyridine, 1.0 ml (ca. 10.6 mmol) acetic anhydride was added. After 15 min at 24°, the solution was evaporated and the products were crystallized from the absolute ethanol. The N^{α}-acetyl amides of Asp(OBzl), Glu(OBzl), Ser(Bzl), Tyr(OBzl) and Cys(4-MeOBzl) were prepared by Dr. D. Yamashiro. The N^{α}-acetyl amide derivatives were characterized as shown in Table 4.

Stability Test. Each amino acid derivative (ca. 20 mg) shown in Table 5 was dissolved separately in 10 ml of trifluoroacetic acid-methylene chloride, 1:1. After the time period shown in Table 5 for the different derivatives, the solvent mixture was removed, 5 ml of acetic acid was added and then

removed. Then the product was dissolved in 0.5 ml of acetic acid and aliquots (1 µl and 5 µl) were submitted to tlc (BAW) with controls of the untreated material and an HF-treated sample. Estimations of material from the stability test, which ran with R_f of the HF-treated material, were made following detection by chlorine-spray method (Zahn & Rexroth, 1955).

Side-Chain Removal by HF Treatment. Each amino acid derivative (ca. 20 mg) shown in Table 5 was treated separately with liquid HF (5 ml) at 0° for 10 min in the presence of anisole (0.1 ml). After removal of the HF by a stream of nitrogen, the material was dried under reduced pressure over NaOH. After dissolving in 1 ml of acetic acid, 10 and 100-fold dilutions were made. These were the standard tests for side-chain protecting group removal, and were also used as controls for the stability test. Aliquots, 2 µl of the 10-fold dilution and 1 and 3 µl of the 100-fold dilution, were submitted to tlc (BAW) as described for the stability test, allowing estimation for as little as 0.1% removal of side-chain protection.

Results and Discussion

The stabilities of the side-chain groups were studied in solution because of the difficulty in obtaining quantitative data on the loss of protection on the resin support.

It was recognized that the results of experiments in solution differed from those conducted on the solid-phase resin; however, it was assumed that the reactions on the resin were several times slower than in solution.

The stability tests were performed at room temperature in 50% (v/v) trifluoroacetic acid in dichloromethane (Gutte & Merrifield, 1969), a solvent mixture frequently used for removal of N^{α}-Boc groups during solid-phase synthesis. The results of the stability test differed when performed on an amino acid in which only the side-chain was protected or when carried out on the N^{α}-acetylated amide derivative in which the influence of the amino and α-carboxyl groups was removed, similar to the protected residue in a peptide during solid-phase synthesis. Therefore, the majority of the tests were carried out on the N^{α}-acetylated amide of the protected amino acid.

The N^{α}-Boc-amino acid derivatives were prepared by the pH-stat method (Schnabel, 1967) or in dimethylsulfoxide (Stewart & Young, 1969, pp. 29-30). The N^{α}-Boc-amino acids were converted to the amides by activation with the mixed anhydride procedure (Vaughan & Osato, 1952) followed by addition of a solution of ammonium hydroxide. The N^{α}-Boc amide derivatives shown in Table 3 were treated with trifluoroacetic acid to remove the Boc group and then acetylated in pyridine with acetic anhydride. The N^{α}-acetylated amide

derivatives, Table 4, were then used in the stability test, results of which are shown in Table 5.

Gutte & Merrifield (1971) reported that γ-benzyl protection of glutamic acid was not entirely stable and this was confirmed by the stability test of γ-benzyl glutamate which lost 5% of the benzyl protection in 5.5 h. However, the protection was shown to be quite stable when the influence of the amino and α-carboxyl groups was removed, since only 2% protection was lost in 23 h from the N^{α}-acetylated amide. Approximately the same degree of stability was observed for the benzyl protection of aspartic acid, serine and threonine.

More stable protection of the side-chain of glutamic acid was achieved with the 4 and 3-bromobenzyl groups which resulted in 0.8% and less than 0.1% loss, respectively, in 72 h. The former group was used with success in protection of glutamic acid in the solid-phase synthesis of a dodecapeptide fragment of HGH (Yamashiro et al., 1973a).

The S-4-methoxybenzyl (4-MeOBzl) group has been shown to be completely removed from the cysteine residue in liquid HF within 30 min at 0°, which was not the case for the S-benzyl group (Sakakibara et al., 1967). However, the stability of the S-4-methoxybenzyl group to trifluoroacetic acid had not been reported. As can be seen from Table 5, 27% of the protection was lost by trifluoroacetic acid treatment

for 23 h. Since this degree of instability was unsatisfactory for synthesis of very large peptides if used in conjunction with N^{α}-Boc protection, the S-3,4-dimethylbenzyl (3,4-Me_2Bzl) group was tested and found to be quite stable to trifluoroacetic acid with a loss of 0.2% in 23 h. The S-3,4-dimethylbenzyl group was utilized for protection of the cysteine residues in the dodecapeptide synthesis mentioned above.

The S-carbamidomethyl group has been used for the synthesis of peptides in which the desired product was to contain the S-carbamidomethyl cysteine residue (Yamashiro et al., 1973a; Blake & Li, 1975). As shown by the results in Table 5, it was stable to the conditions employed for removal of the N^{α}-Boc group and judged suitable for the synthesis of very large peptides in which the cysteine residue(s) were intended to be in the protected form.

The benzyl protection of the phenolic hydroxyl group in tyrosine was shown to be quite unstable, with a loss of 62% in 23 h. It has been reported also that removal of the benzyl group with liquid HF resulted in a side product, 3-benzyl tyrosine (Erickson & Merrifield, 1972). Both the 3-bromobenzyl (Yamashiro & Li, 1972) and 2,6-dichlorobenzyl (Yamashiro & Li, 1973b; Erickson & Merrifield, 1972) groups were observed to have increased stability with losses of 1.6% and 1.4%, respectively, in 21 h. The 2,6-dichlorobenzyl

protection of tyrosine was employed in the synthesis of human adrenocorticotropin (Yamashiro & Li, 1973b). The benzyloxycarbonyl(Z) group for protection of tyrosine has been reported to be lost at a rate 7 times slower than the benzyl group in the test conditions, and also to be unstable to nucleophiles, because about 75% of the benzyloxycarbonyl group was removed by an excess of diisopropylethylamine-dichloromethane, 1:10 (v/v), within 2 h (Erickson & Merrifield, 1973). However, the 2-bromobenzyloxycarbonyl (2-BrZ) group for protection of tyrosine was about 50 times more stable than the benzyl group, Table 5, and only 5% loss of the protection was obtained after treatment with diisopropylethylamine-dimethylformamide, 1:10 (v/v), for 24 h (Yamashiro & Li, 1973a).

For protection of the side-chain of lysine, the benzyloxycarbonyl group has been shown to be unstable from the test with a 42% loss in 20 h and from the synthesis of a model peptide, decalysylvaline, where branched peptides were detected containing 11-19 lysine residues (Erickson & Merrifield, 1972). When compared to the benzyloxycarbonyl group by the results in Table 5, the 4-bromobenzyloxycarbonyl (4-BrZ) was judged to be 3.5 times more stable and the 2-bromobenzyloxycarbonyl (2-BrZ) group 60 times more stable. Similar values have been reported for the chloro derivatives (Erickson & Merrifield, 1972). The N^{α}-Boc-N^{ε}-(2-bromobenzyloxycarbonyl)lysine derivative was prepared and utilized in the

synthesis of human adrenocorticotropin (Yamashiro & Li, 1973b).

Although side-chain protection of tryptophan was not studied in this work, it should be mentioned that a protecting group for the indole side-chain of tryptophan has been reported (Yamashiro & Li, 1973c). The formyl group was stable to both trifluoroacetic acid and liquid HF, and required an additional treatment, either in 1 M NH_4HCO_3, pH 9 for 24 h, or in liquid ammonia, to effect its removal from the free peptide.

The stabilities of the side-chain protecting groups for histidine, arginine, asparagine and glutamine also were not studied. The reviews already mentioned have presented in great detail the various protecting groups that have been employed for these amino acids in solid-phase synthesis. In this work, no side-chain protection for asparagine or glutamine was used. The protection of the δ-guanidino group of arginine utilized in the syntheses described in the following chapters was the 4-toluenesulfonyl (Tos) group (Schwyzer & Li, 1958) in conjunction with N^{α}-Boc protection (Ramachandran & Li, 1962; Yamashiro et al., 1972a). Histidine, reported to be a "problematic" amino acid for solid-phase synthesis (Meienhofer, 1973; Erickson & Merrifield, 1976), was also the only natural amino acid to racemize during solid-phase synthesis when a urethane protecting group was

used for N^{α}-protection (Jorgensen et al., 1970; Windridge & Jorgensen, 1971). However, when Im-Boc was used for protection of the side-chain of histidine in conjunction with N^{α}-Boc protection, a heptapeptide containing two histidine residues was synthesized (Yamashiro et al., 1972a) and the final product gave quantitative recovery of histidine from a leucine aminopeptidase digest when determined by amino acid analysis (Spackman et al., 1958). Thus, even though detailed racemization studies have yet to be performed, the usefulness of this derivative has been shown and for this reason it has been utilized in the syntheses that follow. The Im-Boc group is removed partially or completely under conditions for N^{α}-Boc deprotection and subsequent acylation of the free imidazole ring by Boc-amino acids during the coupling step and deacylation during the N^{α}-Boc deprotection step have been observed (Yamashiro et al., 1972a).

The thioether group of methionine has been generally unprotected during its incorporation in solid-phase synthesis, and occasionally, when unprotected, scavengers for carbonium ions, such as anisole, have been used to prevent possible S-alkylation by tert-butyl cations present during N^{α}-Boc removal (Sharp et al., 1973; Gutte, 1975). One form of protection reported (Iselin, 1961) is the use of methionine sulfoxide; however, it has been infrequently

employed in solid-phase synthesis (Gutte & Merrifield, 1969; 1971). The results of syntheses of methionine-containing peptides, with and without protection, are discussed in Chapters 3 and 6.

In summary, the stability of some of the side-chain protecting groups used in solid-phase synthesis has been studied, New protecting groups for protection of glutamic acid and cysteine were examined, and for other amino acids the new protecting groups were reviewed.

Table 3

Properties of N^{α}-Boc Amide Derivatives

N^{α}-Boc amides	Yield from N^{α}-Boc amino acid	m.p.[a]	R_f(tlc)[b]
Arg(Tos)	68%	oil	0.75
Asp(OBzl)	69%	160-162.5	0.75
Cys(Cam)	50%	--	--
Cys(4-MeOBzl)	55%	152-154	--
Cys(3,4-Me_2Bzl)	87%	oil	0.80
Glu(O-3-BrBzl)	86%	132-133	--
Glu(O-4-BrBzl)	72%	130-131	0.75
Ser(Bzl)	46%	104-105	0.75
Thr(Bzl)	78%	133-135	--

[a] Uncorrected.

[b] Chloroform-methanol, 1:1 (v/v).

Table 4

Properties of N^{α}-Acetyl Amide Derivatives

N^{α}-Acetyl Amides	Yield from N^{α}-Boc Amide	m.p.[a]	R_f(tlc)[b]	Elemental analyses calculated for C,H,N.O. (M.W.) Theoretical for C,H; found for C, H.
Arg(Tos)	--	oil	0.55	$C_{15}H_{23}N_5O_4S$ (369.44) C, 48.6; H, 6.5; C, 45.5; H, 5.2
Asp(OBzl)[c]	66%	129-130	0.60	$C_{13}H_{16}N_2O_4$ (264.28) C, 59.1; H, 6.1; C, 59.3; H, 5.8
Cys(Cam)	64%	157-158	0.47	$C_7H_{13}N_3O_3S$ (219.26) C, 38.4; H, 6.0; C, 38.4; H, 6.0
Cys(4-MeOBzl)[c]	40%	150-152	0.60	$C_{13}H_{18}N_2O_3S$ (282.36) C, 55.3; H, 6.4; C, 55.4; H, 6.4
Cys(3,4-Me_2Bzl)	33%	147-147.5	0.77	$C_{14}H_{20}N_2O_2S$ (280.39) C, 60.0; H, 7.2; C, 59.6; H, 7.0
Glu(OBzl)[c]	80%	148-150	0.60	$C_{14}H_{18}N_2O_4$ (278.31) C, 60.4; H, 6.5; C, 60.6; H, 6.7
Glu(O-3-BrBzl)	61%	145-146	0.90	$C_{14}H_{17}N_2O_4Br$ (357.20) C, 47.1; H, 4.8; C, 47.0; H, 4.8
Glu(O-4-BrBzl)	84%	191-192	0.90	$C_{14}H_{17}N_2O_4Br$ (357.20) C, 47.1; H, 4.8; C, 46.9; H, 4.8
Ser(Bzl)[c]	64%	170-172	0.60	$C_{12}H_{16}N_2O_3$ (236.27) C, 61.0; H, 6.8; C, 61.2; H, 7.2
Thr(Bzl)	93%	219-220	0.67	$C_{13}H_{18}N_2O_3$ (250.30) C, 62.4; H, 7.3; C, 62.4; H, 6.8
Tyr(Bzl)[c]	60%	191-195	0.65	$C_{18}H_{20}N_2O_3$ (314.37) C, 69.2; H, 6.5; C, 69.2; H, 6.4

[a] Uncorrected.

[b] Chloroform-methanol, 1:1 (v/v).

[c] Prepared by Dr. D. Yamashiro.

Table 5
Stabilities of Amino Acid Derivatives in Trifluoroacetic Acid[a]

Amino Acid Derivatives	Loss of side-chain protection in 50% (v/v) trifluoroacetic acid in methylene chloride, duration of test in hours[b]
Ac-Asp(OBzl)-NH_2[c]	4%, 23
Ac-Ser(Bzl)-NH_2[c]	3%, 23
Ac-Thr(Bzl)-NH_2	5%, 23
Glu(OBzl)	5%, 5.5
Ac-Glu(OBzl)-NH_2[c]	2%, 23
Ac-Glu(O-3-BrBzl)-NH_2	$<$0.1%, 72
Ac-Glu(O-4-BrBzl)-NH_2	0.8%, 72
Ac-Cys(4-MeOBzl)-NH_2[c]	20%, 23 27%, 23[d]
Ac-Cys(3,4-Me_2Bzl)-NH_2	0.2%, 23[d]
Ac-Cys(Cam)-NH_2	$<$0.1%, 28
Ac-Tyr(Bzl)-NH_2[c]	50%, 23 62%, 23[e]
Tyr(Bzl)	55%, 21[e]
Tyr(3-BrBzl)[f]	1,6%, 21[e]
Tyr(2,6-Cl_2Bzl)[c]	1.4%, 21[e]
Ac-Tyr(2-BrZ)-NH_2[g]	1%, 24
Lys(Z)	42%, 20[h]
Lys(4-BrZ)[f]	12%, 20[h]
Lys(2-BrZ)[c]	0.7%, 20[h]

[a] Modified from Yamashiro et al.,1972b to include related studies
[b] Unless otherwise indicated, estimated on tlc in 1-butanol-acetic acid-water,4:1:1(v/v),against measured amounts of HF-treated samples.
[c] Prepared by Dr. D. Yamashiro.
[d] Determined by the Ellman reagent (Ellman, 1959).
[e] Estimated by adsorption at 295 nm in 1 N NaOH-dimethylformamide, 1:1 (v/v).
[f] Yamashiro & Li, 1972.
[g] Yamashiro & Li, 1973a.
[h] Determined by quantitative amino acid analysis.

CHAPTER 3

SYNTHESIS OF N$^{\alpha}$-(4-METHOXYBENZYLOXYCARBONYL)-AMINO ACID DERIVATIVES AND THEIR USE IN SOLID-PHASE PEPTIDE SYNTHESIS

Introduction

The previous chapter dealt with the stabilities of side-chain protecting groups used in conjunction with N^{α}-Boc protection, and the stabilities of such groups to the deprotection treatment of TFA-CH_2Cl_2 (1:1). It should be noted that about 95% of peptides synthesized by the solid-phase method have employed N^{α}-Boc protection, and that the majority of the syntheses used TFA-CH_2Cl_2 (1:1) for removal of the Boc group from the peptide resin. Thus, the use of side-chain protecting groups which are stable to this treatment is quite important. However, cleavage of the peptide chains from the solid support by the deprotection conditions should also be recognized.

In a study (Karlsson et al., 1970) of different mixtures for the removal of the Boc group, it was observed that when Boc-Ala resin was treated for 17.5 h with TFA-CH_2Cl_2 (1:1), 35% of the amino acid was released from the resin. This represented an average of 1% loss per 30 min. In the synthesis of ribonuclease A, an average loss of 1.4% of the peptide chains per synthetic cycle was reported (Gutte & Merrifield, 1971). A loss of 1.9% of the final protected peptide in the synthesis of lysozyme (Sharp et al., 1973) was shown to result from a 30-min treatment of TFA-CH_2Cl_2 (1:1).

Since treatment with TFA-CH_2Cl_2 (1:1) seems to be the most efficient method for removal of the Boc group (Karlsson et al., 1970; Reid, 1976), two approaches to this problem have been investigated. One approach was to develop a more stable attachment to the resin. The other approach was to develop other N^{α}-protecting groups which could be removed under milder conditions. Obviously, milder N^{α}-deprotection conditions would also mean that side-chain protecting groups, such as were presented in Chapter 2, would probably be sufficiently stable for the synthesis of peptides containing more than 100 residues.

Two such groups which have been utilized are the 2-(4-biphenyl)-2-propyloxycarbonyl (Bpoc) group and the 2-(3,5-dimethoxyphenyl)-2-propyloxycarbonyl (Ddz) group. The Bpoc group has been removed either by TFA-CH_2Cl_2 (1:199) (Wang & Merrifield, 1969) or by 0.05 M HCl in chloroform (Blake & Li, 1973). The Ddz group has been removed by TFA-CH_2Cl_2 (1:19) (Birr, 1973).

A third group which has been removed under milder conditions is the 4-methoxybenzyloxycarbonyl [Z(OMe)] group. When first employed in the solid-phase method, the Z(OMe) group was removed with TFA-anisole (1:1.5) (Weygand & Ragnarsson, 1966). Subsequently, 0.9 M HCl in anisole-acetic acid (1:10) has also been used to effect deprotection (Ohno et al., 1971). However, in a thorough study (Ragnarsson et al., 1970) of the behavior of the benzyloxycarbonyl (Z), o-nitro-

phenylsulfenyl (Nps), Boc, Z(OMe) and Bpoc groups toward ten deprotecting reagents, it was shown that the Z(OMe) group was removed from Z(OMe)-Ile-Ala resin to the extent of 98.4% in 5 min and 100% in 10 min with TFA-CH_2Cl_2 (1:12.5), and 81.6% in 5 min and 96.6% in 10 min with TFA-CH_2Cl_2 (1:26). The data indicated that only 8.2% of the dipeptide was cleaved from the resin with a 17.5 h treatment of TFA-CH_2Cl_2 (1:12.5); this would represent an average loss of 0.23% for a 30-min treatment.

This chapter presents the synthesis of N^{α}-Z(OMe)-amino acid derivatives and the synthesis of peptides with these derivatives. Deprotection was effected by use of TFA-CH_2Cl_2 (1:17.2) for 10 min. Two model heptapeptides were investigated because of the concern that there might be modification of unprotected methionine if a scavenger, such as anisole, were not present in the deprotection treatment. The third synthesis was an attempt to prepare a 60-residue fragment of HGH.

Experimental Section

Methods and Materials. In addition to the methods and materials described in Chapter 2, the following were used in the work in this chapter.

The dansyl technique (Gray & Hartley, 1963; Woods & Wang, 1967) was used to identify amino-terminal residues.

Micropolyamide sheets, F1700, were obtained from Carl Schleicher and Schuell Co., Keene, N.H. 5-Dimethylamino-1-naphthalenesulfonyl chloride (Dansyl-Cl) was obtained from Calbiochem, Los Angeles, Ca.

Descending paper chromatography was performed in 1-butanol-pyridine-acetic acid-water (BPAW), 15:10:3:12, on Whatman No. 1 paper obtained from Whatman Inc., Clifton, N.J. Paper electrophoresis was performed on Whatman No. 3MM paper. High-voltage paper electrophoresis was carried out in a high-voltage electrophoresis apparatus (High Voltage Electrophorator Model D, Gilson). Low-voltage paper electrophoresis was carried out in the Durrum electrophoresis cell (Beckman Instruments). The buffers were a formic acid-acetic acid buffer, pH 2.1 (218 ml of 90% HCOOH and 63 ml of glacial acetic acid per liter) and a collidine-acetic acid buffer, pH 6.4 (8.9 ml of collidine and 3.1 ml of glacial acetic acid per liter). The conditions were 400 V for 6 h for low-voltage electrophoresis and 2000 V for 1 h for high-voltage electrophoresis.

Thin-layer chromatography was run in chloroform-acetic acid (CH), 15:1, and 1-butanol-pyridine-acetic acid-water (BPAW), 15:10:3:12.

Carboxymethylcellulose (CMC) was obtained from Carl Schleicher and Schuell Co., and prepared by previously reported procedures (Li et al., 1970). Chromatography on CMC was performed with an initial buffer of 0.01 M ammonium acetate (NH_4OAc) of pH 4.5. A gradient with respect to pH

and salt concentration was effected by introducing the NH_4OAc buffers (as subsequently described) through a 500-ml mixing chamber containing the starting buffer. CMC chromatography of Leu-Gly-Arg-Leu-Gly-Met-Phe was accomplished by elution with 100 ml of the starting buffer (10 ml/fraction), followed by a gradient formed with 240 ml of 0.10 M NH_4OAc, pH 6.7 (4 ml/fraction); then with 40 ml of 0.20 M NH_4OAc, pH 6.7 (4 ml/fraction); and finally with 0.40 M NH_4OAc, pH 6.7. On the other hand, CMC chromatography of Leu-Gly-Arg-Leu-Gly-Met-d-(O)-Phe was performed by stepwise elution with 40 ml of starting buffer (4 ml/fraction), with 240 ml of the 0.10 M buffer and finally with the 0.20 M buffer.

Solid-phase synthesis was performed in Bio-Beads S-Xl (200-400 mesh) resin obtained from Bio-Rad Laboratories, Richmond, Ca. The resin was washed with the various solvents previously described (Gisin & Merrifield, 1972). Chloromethylation was carried out by modification (see below) of the procedure reported in the same paper (Gisin & Merrifield, 1972).

Tetramethylammonium bromide was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J., and was converted to the hydroxide form by the following procedure. Anion-exchange resin, Dowex 1-X4 (obtained from Dow Chemical Corp., Midland, Mich.), was suspended in water and packed into a glass column to a 50-ml bed volume with a capacity of 1 mequiv/ml. The

resin was washed with 300 ml of water, 330 ml of 3 N HCl, water, until pH of eluant was that of water, 1.2 liter of 1 N NaOH, water, again until pH of eluant was that of water, and finally with 250 ml of methanol. Next, a solution of 7.5 g (48.7 mmol) tetramethylammonium bromide in 300 ml of methanol was passed through the column at a rate slow enough that discrete drops of the eluant were formed. This methanol solution and a 50-ml methanol rinse of the column were combined and evaporated to about 20 ml. Methanol was added to make the final volume 50.0 ml and this constituted the stock solution. In order to determine the final concentration, an aliquot of the stock solution was titrated with a standard solution of HCl in the presence of phenolphthalein.

Acid hydrolysis was carried out with constant boiling HCl at 110° *in vacuo* for at least 18 h and generally 24 h. Amino acid analyses (Spackman et al., 1958) were carried out on a Beckman amino acid analyser Model 120B (Beckman Instruments, Inc.).

Picric acid was obtained from Mallinckrodt Chemical Works. Dicyclohexylcarbodiimide and diisopropylethylamine were obtained from Aldrich and distilled before use. 4-Methoxybenzylazidoformate was obtained from Pierce Chemical Corporation.

Enzyme digestions were carried out in a 0.05 M tris-(hydroxymethyl)aminomethane —0.01 M magnesium chloride buffer of pH 8.5. Trypsin was obtained from Worthington Bio-

chemical Corp., Freehold, N.J. Tryptic digestion was carried out with an enzyme to substrate weight ratio of 1:50 for 20 h at pH 8.5 and 37°.

Chloromethylation. A desired lower chlorine content was obtained by the following modification of the procedure reported by Gisin & Merrifield in 1972. Chloromethyl methyl ether (caution -- a carcinogen) was distilled into a flask containing 10 g of resin to a total volume of 120 ml. While under exclusion of moisture, the suspension was stirred for 30 min at 24° and then 30 min at 4°. At 4° over 1 min, a solution of 0.73 ml of stannic chloride in 7.3 ml of distilled hexane was added. After 7 min, the resin was filtered, washed with 480 ml of cold dioxane-water (3:1), 120 ml of dioxane-water (3:1), 150-ml portions of water, dioxane and methanol, and dried in vacuo over P_2O_5: 10.75 g, 0.635 mmol of Cl/g by elemental analysis and 0.501 mmol of Cl/g by the modified Volhard method (Stewart & Young, 1969, pp. 55-56). It should be noted that for six comparisons of chloromethyl resins by elemental analysis and the modified Volhard method, the latter gave for each sample approximately 80% of the chlorine content obtained by elemental analysis.

N^{α}-Z(OMe)-Amino Acids by pH-Stat Method. Alanine (1.336 g, 15 mmol) was suspended in 40 ml of a dioxane-water, 1:1, solution in a 50° water bath. After 3.729 g (18 mmol) Z(OMe)-azide was added, the pH was maintained between 9 and 9.5 for

4 h at 50° with addition of 4 N NaOH (17.8 ml total). By evaporation, the dioxane was removed and the volume reduced to 30 ml. After an addition of 50 ml of water, the solution was washed with three 25-ml portions of ether. The aqueous solution was cooled and 10 g of citric acid was added. Extraction with three 50 ml portions of ethyl acetate was followed by washing of the combined ethyl acetate with two 15-ml portions of water. The ethyl acetate solution was dried over anhydrous $MgSO_4$. Removal of the drying agent and the solvent gave an oil which crystallized at 24°. The crystals were collected with the aid of petroleum ether and gave: 3.653 g (14.42 mmol, 96% yield); see Table 6.

Z(OMe)-Asp(OBzl) was prepared on a 30-mmol scale according to the procedure used for Z(OMe)-Ala. The final product was recrystallized from ether-petroleum ether, 5:8 (see Table 6).

Z(MOe)-Gly was prepared on a 20-mmol scale according to the procedure used for Z(OMe)-Ala (see Table 6).

Z(OMe)-Lys(2-Brz) was prepared from N^{ε}-(2-brz)Lys (Yamashiro & Li, 1972) on a 20-mmol scale according to the procedure used for Z(OMe)-Ala. The oil crystallized at 4° and the final product was collected with the aid of petroleum ether (see Table 6).

Methionine-d-sulfoxide (Met-d-(O); Lavine, 1947) was submitted to the procedures used for Z(OMe)-Ala on an 8-mmol scale (see Table 6).

Z(OMe)-Ser(OBzl) was prepared on a 15-mmol scale according to the procedure used for Z(OMe)-Ala (see Table 6).

N^{α}-Z(OMe)-Amino Acids by the DMSO Procedure. N^{G}-Tosylarginine (3.29 g, 10 mmol) was converted to the N^{α}-Z(OMe) derivative by the DMSO procedure (Stewart & Young, 1969, pp. 29-30). Briefly, to a stirred suspension of N^{G}-tosylarginine (Ramachandran & Li, 1962) in 25 ml of dimethyl sulfoxide (DMSO), 2.59 g (12.5 mmol) of Z(OMe)-azide and 3.85 ml (22.5 mmol) of diisopropylethylamine (DIEA) were added. After stirring 24 h at 24°, the solution was diluted with cold water (125 ml) and washed with two 100-ml portions and one 50-ml portion of ether. The aqueous phase was cooled and acidified with 15 g of citric acid. The product was extracted with three 80-ml portions of ethyl acetate. After drying over anhydrous $MgSO_4$, the drying agent and ethyl acetate were removed and the resulting oil stored overnight at -20°. The oil crystallized at room temperature and the product was collected with the aid of ether: yield, 4.22 g (8.56 mmol), see Table 6.

Z(OMe)-Gln was prepared on a 20-mmol scale by the DMSO method. The oil was crystallized after 16 h at 4° from ethanol-petroleum ether, 3:5 (see Table 6).

Z(OMe)-Glu(OBzl) was prepared on a 10-mmol scale by the DMSO method and obtained as an oil which defied crystallization (see Table 6).

Z(OMe)-Ile was prepared on a 10-mmol scale by the DMSO method. The oil crystallized at -20°. The crystals were collected with the aid of petroleum ether and recrystallized from ether-petroleum ether, 1:4 (see Table 6).

Z(OMe)-Leu was prepared on a 50-mmol scale by the DMSO method and an oil was obtained: 13.5 g, tlc (CM) R_f 0.62. The oil was dissolved in 100 ml of ether and the solution cooled. After dicyclohexylamine (DCA) (9.1 ml, 45.8 mmol) was added, crystals of Z(OMe)-Leu·DCA salt were obtained and recrystallized from chloroform-ethyl acetate-ether, 4:1:8 (see Table 6).

Z(OMe)-Met and Z(OMe)-Phe were both prepared on a 10-mmol scale by the DMSO method. The oils crystallized at 24° and the products were collected with the aid of petroleum ether (see Table 6).

Z(OMe)-Pro was prepared on a 10-mmol scale by the DMSO method and an oil was obtained: 2.6 g, tlc (CM) R_f 0.62. The oil was dissolved in 20 ml ether and the solution was cooled. After DCA (1.86 ml, 9.32 mmol) was added, crystals of Z(OMe)-Pro·DCA salt were obtained and washed with ether (see Table 6).

Z(OMe)-Tyr(2-BrZ) was prepared on a 10-mmol scale by the DMSO method. The oil crystallized at 24° and the material was recrystallized from ethyl acetate-petroleum ether, 5:6 (see Table 6).

Z(OMe)-Val was prepared on a 10-mmol scale by the DMSO method. The oil crystallized at 24° and the material (2.0 g) was recrystallized from ether-petroleum ether, 3:4 (see Table 6).

<u>N^{α}-Z(OMe)-O^{β}-Benzylthreonine by Buffered pH Method.</u> To a stirred suspension of 6.89 g (20 mmol) O^{β}-benzylthreonine benzyl ester hemioxalate salt (Mizoguchi et al., 1968) in 60 ml of methanol in an ice-water bath, 18.2 ml of 4 N NaOH was added. After 2 h, a solution of 2.48 g (29.5 mmol) $NaHCO_3$ in 30 ml of water was added. Then, a solution of 5.18 g (25 mmol) Z(OMe)-azide in 30 ml of dioxane was added, and another 30-ml portion of dioxane was added. This reaction mixture was stirred in a 50° bath for 60 h. After reduction of the volume to about 50 ml, 100 ml of water was added. This solution was washed with two 75-ml portions and one 50-ml portion of ether. The aqueous phase was cooled and acidified to pH 3 by addition of 15 g of citric acid. After extraction with two 100-ml portions and one 50-ml portion of ethyl acetate, the combined ethyl acetate was washed with three 50-ml portions of water. Upon tlc (CH), the ethyl acetate solution was shown to contain three minor components with R_f less than the desired product, R_f 0.53. Addition of 3.42 ml (20 mmol) of DIEA to the ethyl acetate solution was followed by six washes of 10% (w/v) aqueous sodium chloride. Again the ethyl acetate solution was cooled and acidified with 15 g of citric

acid. After washing with three 65-ml portions of water, the ethyl acetate was dried over anhydrous $MgSO_4$. Only single spots on tlc (CM) and (CH) with R_f values of 0.68 and 0.53, respectively, were observed. Removal of the drying agent and solvent gave a solid (3.72 g) which was dissolved in 30 ml of chloroform. Addition of 15 ml of ether and crystallization at 24° resulted in the final product: 1.38 g (see Table 6).

Solid-Phase Synthesis Procedures. Boc-Phe resin was prepared by a modification (Yamashiro & Li, 1973a) of the Loffet method (Loffet, 1971). A sample of the resin deprotected and neutralized gave an amine content by the picric acid method (Gisin, 1972) of 0.436 mmol/g. Boc-Phe resin was placed in a shaker and carried through the schedule in Table 7 for the incorporation of each residue. The protected amino acids employed are shown in Table 6, with the exception that Gln residues of the HGH peptide were coupled as the N^{α}-Boc protected para-nitrophenyl ester (Bodanszky & du Vigneaud, 1959). This required subsequent deprotection with TFA-CH_2Cl_2 (1.22:1) in steps 2 and 3, step 8 repeated 3 times, step 9 as three 2-min DMF washes, step 10 as coupling 10 equiv of Boc-Gln-ONP in DMF (21 ml) for 22 h, and three 2-min DMF washes in step 11.

The Met-heptapeptide (peptide I), Leu-Gly-Arg-Leu-Gly-Met-Phe, synthesis was performed on 502 mg (0.22 mmol Phe) of

Boc-Phe resin. The Z(OMe)-amino acids, 4 equiv, were added to the resin in 4-ml volumes of CH_2Cl_2 for the Met and Leu residues, DMF-CH_2Cl_2 (1:9) for the Gly residues and DMF-CH_2Cl_2 (1:4) for the Arg residue. The 0.61 M DCCI in CH_2Cl_2 (1.44 ml, 0.88 mmol, 4 equiv) was added 5 min later and the coupling reaction allowed to proceed for 2 h. The final protected peptide resin was deprotected by steps 1 through 4 in Table 7 followed by three washes of absolute ethanol-methylene chloride (1:1), 2 min each. After drying under reduced pressure over P_2O_5 for 36 h the yield was 752 mg. Four days after the peptide resin was deprotected, the amine content was measured by the picric acid method and a value of 91.8% of theoretical was obtained. However, the peptide resin remained yellow after normal elution of the picrate. The peptide resin was eluted with 5 ml of 1 N KOH-DMF (1:1) to give a picric acid solution. This solution was made to 10.0 ml with 95% ethanol and an aliquot (1 ml) was diluted 10-fold with 95% ethanol: 38.7% of theoretical amine content (ε_{358nm}=15,930 in this solvent mixture).

The Met-d-(O)-heptapeptide, Leu-Gly-Arg-Leu-Gly-Met-d-(O)-Phe, was synthesized on 1.00 g of Boc-Phe resin. The Z(OMe)-amino acids, 4 equiv, were added to the resin in 7.7-ml volumes of the solvents used for the Met-heptapeptide; however, the Met-d-(O) residue required DMF-CH_2Cl_2 (1:9). The solution of DCCI in CH_2Cl_2 (2.80 ml of 0.61 M, 3.92 equiv)

was added 5 min later and the coupling reaction allowed to proceed for 2 h. The final peptide resin was deprotected as the Met-heptapeptide resin and dried overnight under reduced pressure over P_2O_5 to yield: 1.421 g. One day later the amine content gave a value of 105% of theoretical, but the resin did not have the yellow color that the Met-heptapeptide resin had at this stage.

The HGH peptide was synthesized on 2.00 g (0.872 mmol Phe) Boc-Phe resin, where Phe represented position 146 in HGH. The N^{α}-Z(OMe)-amino acids, 4 equiv, were added to the resin in 20-ml volumes of CH_2Cl_2, except that the derivatives of Arg, Gly, Lys and Thr required DMF-CH_2Cl_2 solutions of 1:4, 1:5, 1:9 and 1:19, respectively. Coupling tests and surviving amino groups were measured by the picric acid method.

Leu-Gly-Arg-Leu-Gly-Met-Phe (I). Deprotected peptide resin (694 mg) was treated with 12 ml of liquid HF for 1 h at 0° in the presence of anisole (0.7 ml). After removal of the HF the dried resin was stirred with TFA (15 ml) for 15 min and filtered. The filtrate was evaporated and reevaporated twice from acetic acid (5 ml) to give an oil (596 mg). The oil was dissolved in 1.5 ml of 0.5 M acetic acid and washed six times with ether (1.5 ml). The aqueous phase was applied to a 2.16 x 28-cm Sephadex G-10 column. Elution with 0.5 M acetic acid, collection of 3.3-ml fractions, and measuring the absorbance of the fractions at 257 and 280 nm resulted in the

delineation of a single peak which eluted between the elution volumes (V_e) of 30 and 53 ml. Isolation of the material in this peak by lyophilization gave 128 mg. This peptide material was subjected to chromatography on carboxymethylcellulose (CMC), Figure 2A, as described above. Two major peaks were detected. The first peak (V_e, 123 ml) had a shoulder on the trailing side which had absorbance at 280 nm. The second peak (V_e, 404 ml) had absorbance at both 257 and 280 nm. Isolation by lyophilization gave 74.5 mg (46.5% yield based on starting Boc-Phe resin) of peptide material from the first peak (peptide I), 17.7 mg from the shoulder and 30.7 mg from the second peak (peptide II).

Amino acid analyses of acid hydrolysates are shown in Table 8. Peptide I and the peptide material from the shoulder were identical on tlc (BPAW) with R_f 0.73 and high-voltage paper electrophoresis at pH 2.1 with R_{Lys} 0.49. Peptide I also ran with R_f 0.55 on tlc (BAW) and with R_{Lys} 0.40 on low-voltage paper electrophoresis at pH 6.4. Peptide II ran with R_f 0.73 and a trace spot with R_f 0.40 on tlc (BPAW), with R_f 0.55 and a trace spot with R_f 0.31 on tlc (BAW), with R_{Lys} 0.47 and three trace spots with R_{Lys} 0.56, 0.62 and 0.66 on paper electrophoresis at pH 2.1, and with R_{Lys} 0.40 and a trace spot with R_{Lys} 0.59 on paper electrophoresis at pH 6.4. Trypsin digestion of peptides I and II were run on paper electrophoresis at pH 2.1 and pH 6.4. The tryptic digestion of peptide I produced two spots with R_{Lys} 0.39 and 0.79 at pH 2.1 and with R_{Lys} 0.12 and 0.61 at pH 0.64. The tryptic digestion of peptide II gave, in addition to the spots seen for peptide I, trace spots with R_{Lys} 0.48, 0.54

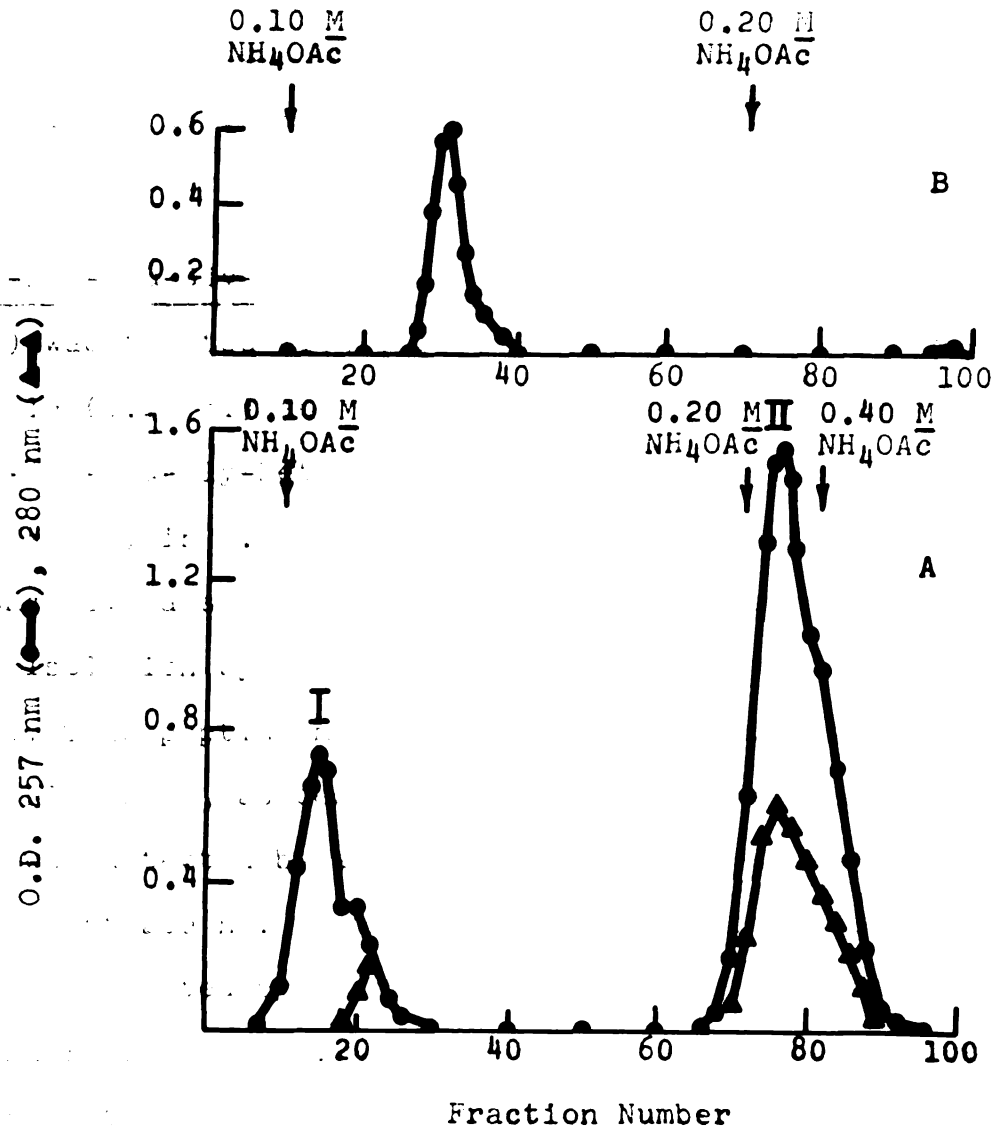
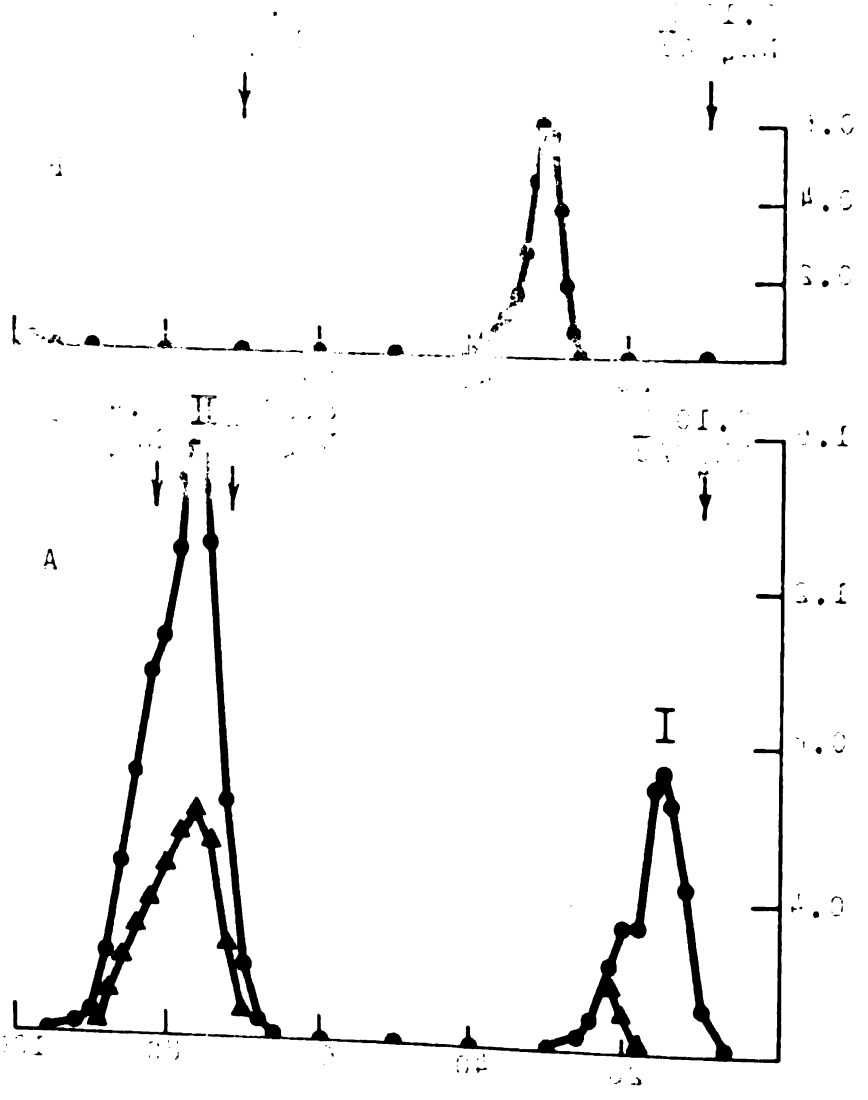


Figure 2. Carboxymethylcellulose chromatography of model heptapeptides: (A) 128 mg of crude Met-heptapeptide with gradient elution, yield (fractions 10-18), 74.5 mg of I, (fractions 19-26), 17.7 mg, (fractions 70-89), 30.7 mg of II; (B) 61 mg of crude of Met-d-(O)-heptapeptide with stepwise elution, yield (fractions 27-35), 52.5 mg. Initial buffer in both cases was 0.01 M NH OAc, pH 4.5.


A
II
I

and 0.65 at pH 2.1 and with R_{Lys} 0.42 at pH 6.4. Both peptides I and II gave a single amino terminal residue, Leu, by the dansyl technique.

Leu-Gly-Arg-Leu-Gly-Met-d-(O)-Phe. Deprotected peptide resin (252 mg) was treated with liquid HF (12 ml) in the presence of anisole (0.25 ml) and worked up in the same manner for Leu-Gly-Arg-Leu-Gly-Met-Phe. Chromatography on Sephadex G-10 (1.37 x 40 cm) in 0.5 M acetic acid with collection of 3.2-ml fractions gave a single peak which eluted between 22 and 45 ml. Isolation by lyophilization gave 61.0 mg of peptide material. This peptide material was submitted to chromatography on CMC, Figure 2B, as described above. A major peak (V_e, 124 ml) was detected by absorbance at 257 nm and showed no absorbance at 280 nm. A trace peak with elution volume of 395 ml was detected by absorbance at 257 nm (no 280 nm absorbance) and represented 2.75% of the major peak based on total absorbance at 257 nm. Isolation by lyophilization of the peptide material in the major peak gave: 52.5 mg, 84% overall yield.

This peptide material ran with R_f 0.56 on tlc (BPAW), with R_f 0.40 on tlc (BAW), with R_{Lys} 0.52 on high-voltage paper electrophoresis at pH 2.1 and with R_{Lys} 0.40 on low-voltage paper electrophoresis at pH 6.4. Tryptic digestion followed by paper electrophoresis showed two spots with

R_{Lys} 0.39 and 0.79 at pH 2.1 and with R_{Lys} 0.12 and 0.61 at pH 6.4. Amino acid analysis of an acid hydrolysate gave

Arg	Met-(O)	Met-(O_2)	Gly	Met	Leu	Phe
0.94	0.27	0.04	2.00	0.62	2.10	1.00

A single amino terminal residue, Leu, was obtained by the dansyl technique.

HGH-(139-146). The octapeptide, HGH-(139-146), was synthesized as described in the synthetic procedures section and Table 7. After Phe_{139} was incorporated, an aliquot (693 mg) of peptide resin was dried under reduced pressure over P_2O_5 to yield 253 mg. This material was treated with liquid HF (10 ml) in the presence of anisole (1.0 ml) and worked up in the same manner as described for the heptapeptides above. Chromatography on Sephadex G-10 (2.16 x 28 cm) in 0.5 M acetic acid and collection of 3.4-ml fractions resulted in the detection of a single peak with elution between 31 and 51 ml. Isolation by lyophilization gave a crude peptide product (III): 68.0 mg, 102.6% overall yield based on starting Boc-Phe resin. This peptide material was compared by tlc, paper electrophoresis and paper chromatography to the corresponding material from Sephadex G-10 (IV) and the final product (V) from a synthesis (Yamashiro & Li, 1972) which employed N^{α}-Boc protection and side-chain protection for Lys and Tyr of 4-Brz and 3-BrBzl, respectively. On tlc (BPAW), V (170 µg) gave a single spot with R_f 0.35, IV (135 µg) gave a major spot

with R_f 0.35 and two trace spots which trailed with R_f 0.47 and 0.20, III (135 µg) gave a major spot with R_f 0.35 and two discrete spots with R_f 0.47 and 0.20. High-voltage paper electrophoresis of the same sample amounts at pH 6.4 resulted in a single spot with R_{Lys} 0.64 for V, a major spot and three trace spots with R_{Lys} 0.64, 0.48, 0.37 and 0.12, respectively, for IV, and a major spot and two trace spots with R_{Lys} 0.64, 0.40 and 0.12, respectively, for III. Paper chromatography (BPAW) of about 70 µg of each peptide gave a single spot with R_f 0.28 for V, a major spot with R_f 0.28 and two minor spots with R_f 0.47 and 0.15 for III and IV, but with more trailing in IV.

HGH-(130-146) Peptide Resin. The synthesis in the previous section was continued with the incorporation of the subsequent residues of the HGH sequence according to the aforementioned procedures (Table 7). Coupling tests by the picric acid method during the synthesis gave the following results: 100% coupling of Lys_{145}, 99.89% coupling of Thr_{142}, 99.96% coupling of Lys_{140}, 99.97% coupling of Phe_{139} and 99.80% coupling of Arg_{134}. The surviving amine content during the synthesis was also determined by the picric acid method and gave the following results: 86.0% of theoretical amine content after deprotection and neutralization at Phe_{139}, 77.9% at Arg_{134} and 64.7% at Ser_{132}. Since 35% of the theoretical amine

content had been lost in the incorporation of 15 residues, the synthesis which was to result in a 60-residue HGH peptide fragment was abandoned.

Results and Discussion.

A total of seventeen N^{α}-Z(OMe)-amino acid derivatives were prepared by two methods (Table 6). Generally, the DMSO method was more convenient; however, the pH stat method resulted in final products that were easier to purify and obtained in higher yields. Only the Z(OMe)-Arg(Tos) derivative differed from a previously reported preparation, but it was used satisfactorily in the two model heptapeptide syntheses.

The first synthesis with N^{α}-Z(OMe) protection was of the model heptapeptide, Leu-Gly-Arg-Leu-Gly-Met-Phe (Met-heptapeptide, I). This model peptide was chosen because of the success of a similar model heptapeptide, Gly-Ala-Arg-Gly-Ala-Trp-Gly (Trp-heptapeptide). The Met-heptapeptide was synthesized on Boc-Phe resin which was prepared in a manner that did not introduce any quaternary ammonium sites on the resin. The coupling method employed was that which is generally used in solid-phase peptide synthesis (Table 7).

The final partially protected peptide resin had an amine content that was 91.8% of theoretical. It is doubtful that this value represents an average of 1.64% cleavage of the

peptide from the resin by the 10-min TFA-CH_2Cl_2 (1:17.2) treatment. An additional elution of picric acid was obtained by further elution of the peptide resin sample by a stronger basic pH solution. In addition, since care had been taken so that the formation of quaternary ammonium sites on the resin was avoided, this indicated that a more positively charged group than the N-terminal amino group had been incorporated into the peptide resin during the synthesis. Following removal of the tosyl group and the resin, the peptide was submitted to chromatography on Sephadex G-10 and then carboxymethylcellulose (CMC, Figure 2A). Two main peaks were detected. The first peak had absorption at 257 nm, but not at 280 nm. The second peak had absorption at both wavelengths. The two peptide materials were isolated and had a weight ratio of first peak to second peak material of 2.4:1.

The first peak material was the desired Met-heptapeptide, I. It was isolated in 46.5% overall yield based on starting Boc-Phe resin. I ran as a single spot on tlc in two solvent systems and on paper electrophoresis at two pH values. Tryptic digestion of I gave the expected two spots on paper electrophoresis at two pH values. Amino acid analysis of an acid hydrolysate gave the expected values (Table 8).

Peptide II seemed to be easily converted into peptide I. Although II ran as a more positive material on CMC chromatography, II behaved on tlc and paper electrophoresis

essentially the same as I. Amino acid analysis of an acid hydrolysate of II was identical to I (Table 8). Although the nature of this side-product peptide was not investigated, its behavior resembled that of the tert-butylsulfonium form of I isolated from a synthesis which employed N^{α}-Boc protection (see Chapter 6 ; Noble et al., 1976). It could be speculated that II is the para-methoxybenzyl sulfonium form of I. However, II appears to have arisen during the solid phase synthesis, because the identical sequence (Chapter 6) did not give this result.

The second model heptapeptide, Leu-Gly-Arg-Leu-Gly-Met-d-(O)-Phe (Met-d-(O)-heptapeptide) was synthesized in the same manner as the Met-heptapeptide, except Z(OMe)-Met-d-(O) was used instead of the Met derivative. The amine content, measured by the picric acid method, of the partially protected final peptide resin indicated a quantitative survival of peptide chains. However, there were no additional groups which retained picric acid after the normal elution of the picrate used in the method. After removal of the tosyl group and the resin, the peptide material was submitted to chromatography on Sephadex G-10 and then CMC (Figure 2B). Only one major peak was detected and it had no absorption at 280 nm. Isolation of the peptide material from this peak gave the Met-d-(O)-heptapeptide in 84% overall yield.

The Met-d-(O)-heptapeptide ran as a single spot on tlc in two solvent systems and on paper electrophoresis at two pH values. After tryptic digestion of this model peptide, only the two expected spots were observed on paper electrophoresis at two pH values. Amino acid analysis of an acid hydrolysate gave the expected values considering the known conversion of methionine sulfoxide to methionine during acid hydrolysis.

Therefore, protection of Met resulted in the elimination of the side-product peptide obtained without protection and almost a two-fold increase in the yield of the final product.

The next synthesis utilizing N^{α}-Z(OMe) protection was the attempt to synthesize HGH-(87-146). The same procedures used for the model heptapeptides were employed in this synthesis with the exception that the Gln residues were incorporated as the N^{α}-Boc p-nitrophenyl ester.

At the octapeptide resin stage, the partially protected resin had an amine content which was 86% of theoretical. An aliquot of the octapeptide resin was treated with HF in the presence of anisole to remove the side-chain protecting groups and the resin. After gel filtration on Sephadex G-10, the peptide material was isolated and compared to peptide material from a synthesis which employed N^{α}-Boc protection (Yamashiro & Li, 1972). Both octapeptides were obtained after gel filtration on Sephadex G-10 and compared by tlc, paper chromatography and electrophoresis. Qualitatively, the octapeptide

from the 'Z(OMe) synthesis' was purer at this stage of purification.

With this reassurance, the synthesis was continued on the remainder of the octapeptide resin. After the incorporation of four more residues, the amine content had dropped from 84% of theoretical to 78% and after 2 more residues the value had dropped to 65% of theoretical. The synthesis was stopped after the incorporation of Asp_{130}, because of this loss of surviving amino groups. Possibly the loss was due to alkylation of the N-terminal amino group by 4-methoxybenzyl cations and that was the reason for the presence of anisole in the earlier work mentioned above.

The use of N^{α}-Z(OMe) protection for the synthesis of methionine-containing peptides has been shown to result in a side-product during the synthesis unless the thioether group of methionine is protected. Although the nature of this side-product was not investigated, it behaved like the t-butyl sulfonium form of the same methionine-containing heptapeptide isolated from a synthesis which employed N^{α}-Boc protection. In addition, for an unknown reason, there was an alarming loss of surviving amino groups during an attempted synthesis of a HGH fragment which employed N^{α}-Z(OMe) protection.

Table 6A

Properties of N^{α}-Z(OMe)-Amino Acid Derivatives

N^{α}-Z(OMe)-Amino Acid	Method	Yield	mp	tlc R_f in (CM), (CH), (BAW)[a]	$[\alpha]_D^{24}$
Ala	pH stat	96%	78-80° (78-79°)[b]	0.70,0.43,--	
Arg(Tos)	DMSO	86%	143-144° (128-130°)[c]	--,0.10,--	+1.23°(c4,DMF) -4.5°(c1.3,MeOH)[c]
Asp(OBzl)	pH stat	73%	95-97° (96-97°)[d]	0.60,0.45,--	
Gln	DMSO	19%	142-144° (143-144°)[e]	0.45,0.07,0.68	
Glu(OBzl)	DMSO	19%	oil	0.57,0.32,--	
Gly	pH stat	99%	96-98° (96-98°)[f]	0.60,0.25,--	
Ile	DMSO	48%	63-66° (63-65°)[f]	0.65,0.52,--	
Leu·DCA	DMSO	52%	159-160° (156-160°)[f]	0.62,0.48,--	
Lys(2-BrZ)	pH stat	100%	104-105°	0.65,0.48,--	-4.37°(c1.28,MeOH)
Met	DMSO	68%	74-75° (74-75°)[f]	0.68, --,--	
Met-d-(O)	pH stat	60%	92-93°	0.45,0.08,--	+32.2°(c1.01,DMF)
Phe	DMSO	81%	88-90° (90-91°)[f]	0.82,0.62,--	
Pro·DCA	DMSO	83%	147-150° (147.5-149°)[g]	0.58,0.33,--	
Ser(OBzl)	pH stat	100%	73-74° (72-73°)[d]	0.60,0.46,--	
Thr(OBzl)	Buffered pH	19%	134.5-135°	0.68,0.53,--	+17.4°(c2,MeOH)
Tyr(2-BrZ)	DMSO	37%	121-122°	0.58,0.48,--	-8.21°(c1.05,MeOH)
Val	DMSO	40%	63.5-65.5° (62-63°)[d]	0.62,0.50,--	

[a] Single spots on tlc determined by chlorine spray. [b] Klieger, 1969; [c] Yajima et al., 1973; [d] Weygand & Nintz, 1965; [e] Schröder & Klieger, 1964; [f] Nagasawa et al., 1973; [g] Weygand & Hunger, 1962.

Table 6B

Properties of N^{α}-Z(OMe)-Amino Acid Derivatives

N^{α}-Z(OMe)-Amino Acid	Elemental Analysis Calculated for C,H,N,O (M.W.) Theoretical for C,H,N; Found for C,H,N
Arg(Tos)	$C_{22}H_{28}N_4O_7S$ (492.55) C, 53.7; H, 5.7; N, 11.4; C, 53.0; H, 5.7; N, 11.3
Lys(2-BrZ)	$C_{23}H_{27}N_2O_7Br$ (523.38) C, 52.8; H, 5.2; N, 5.4; C, 52.8; H, 5.2; N, 5.3
Met-d-(O)	$C_{14}H_{19}NO_6S$ (329.37) C, 51.1; H, 5.8; N, 4.3; C, 51.1; H, 5.8; N, 4.2
Thr(Bzl)	$C_{20}H_{23}NO_6$ (373.41) C, 64.3; H, 6.2; N, 3.8; C, 64.1; H, 6.2; N, 3.8
Tyr(2-BrZ)	$C_{26}H_{24}NO_8Br$ (558.38) C, 55.9; H, 4.3; N, 2.5; C, 55.9; H, 4.4; N, 2.6

Table 7

Schedule of Solid-Phase Peptide Synthesis

Employing N -Z(OMe) Protection

Step	Reagent and Operation	Time (min)
1	CH_2Cl_2 (2 times)	2
2[a]	TFA-CH_2Cl_2, 1:17.2 (1 time)	2
3[a]	TFA-CH_2Cl_2, 1:17.2 (1 time)	10 or 15[b]
4	CH_2Cl_2 (3 times)	2
5	Dioxane-CH_2Cl_2, 1:1 (3 times)	2
6	CH_2Cl_2 (3 times)	2
7	DIEA-CH_2Cl_2, 1:19 (1 time)	5
8[a]	CH_2Cl_2 (6 times)	2
9[a]	Z(OMe)-Amino acid (4 equiv)[c], (1 time)	5
10[a]	DCCI (4 equiv)[d], (1 time)	120
11[a]	CH_2Cl_2 (3 times)	2
12	Absolute ethanol-CH_2Cl_2, 1:1 (3 times)	2

[a] Modified for incorporation of Gln residues in the HGH peptide, see text. Also, employed TFA-CH_2Cl_2, 1.22:1 to deprotect Boc-Phe resin in all three syntheses.

[b] 10 min for heptapeptides and 15 min for HGH peptide.

[c] See text for solvent volumes and composition.

[d] See text for volumes of 0.61 M DCCI in CH_2Cl_2.

Table 8

Amino Acid Analyses of Peptides from CMC Chromatography[a]

Amino Acid	Theoretical	Acid Hydrolysate		
		I	Shoulder	II
Arg	1	1.05	1.02	1.12
Gly	2	2.12	2.25	2.17
Met	1	1.02	1.05	1.06
Leu	2	2.12	2.01	2.17
Phe	1	1.00	1.00	1.00

[a] See Figure 2A: fractions (10-18) represent peptide I; fractions (19-26), shoulder; fractions (70-89), peptide II.

CHAPTER 4

SYNTHESIS OF THE CARBOXYL-TERMINAL DODECAPEPTIDE OF HUMAN GROWTH HORMONE -- THE CYCLIC DISULFIDE PEPTIDE AND A LINEAR FORM

Introduction

One of the two disulfide bridges in the HGH molecule is present in a small loop near the C-terminal phenylalanine. Previous studies showed that the disulfide bridges are unnecessary for the biological activity of the hormone since the reduced S-tetracarbamidomethylated HGH is as active as the native hormone (Dixon & Li, 1966; Bewley et al., 1969). Recently it has been shown that selective reduction and carbamidomethylation can be carried out on the disulfide bridge in the small loop without effecting the other disulfide bridge of the plasmin-modified form of HGH from which the hexapeptide comprising residues 135 through 140 has been removed (Bewley, 1976; Li & Bewley, 1976b).

It was decided to synthesize the cyclic C-terminal dodecapeptide of HGH which contains one of the two disulfide bridges and to use the new 4-bromobenzyl side-chain protecting group for glutamic acid. Also, in order to compare the 3,4-dimethylbenzyl and the 4-methoxybenzyl side-chain protecting groups for cysteine, the cyclic dodecapeptide was synthesized twice. Finally, synthesis

of the S,S'-dicarbamidomethyl dodecapeptide would permit comparison of the reduced and carbamidomethylated cyclic dodecapeptide. These synthetic dodecapeptides could be compared to the identical sequence which has been obtained from human chorionic somatomammotropin (HCS) by cyanogen bromide cleavage at Met_{179} (Li et al., 1973). From this last comparison, the question might be settled as to whether the chirality of the disulfide bridge as it exists in the native protein is attained in the synthetic peptide.

Experimental Section

Methods and Materials. In addition to the methods and materials described in the previous two chapters, the following were used in the work in this chapter.

The paper electrophoresis buffer of pH 3.7 was a pyridine-acetate buffer of pyridine-acetic acid-water, 1:10:289.

Performic acid oxidation was performed as previously described (Li et al., 1970). The 30% H_2O_2 was obtained from Mallinckrodt Chemical Works. The Ellman test for sulfhydryl groups was carried out as previously described (Ellman, 1959) and the 5,5'dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Aldrich. The sodium borohydride-DTNB method for detection of disulfide groups on paper was performed as described (Maeda et al., 1970).

Partition chromatography (Yamashiro, 1964) on Sephadex G-25 (Yamashiro et al., 1966) was performed on a 1.92 x 68.9-cm column. The distribution coefficient, K, was measured on ca. 0.5 mg peptide samples by dissolving in 0.50 ml portions of both the upper and lower phases of the system being tested. Aliquots (0.10 ml) of each phase were submitted to Folin-Lowry analysis (Lowry et al., 1951) and the ratio of the value for the upper phase to the value of the lower phase was the K of the system. The system, 1-butanol-pyridine-0.2 N NH_4OH with 0.1% acetic acid (5:3:12), gave a K of 0.281 and was employed for the partition chromatography of [Cys(Cam)^{182,189}]-HGH-(180-191) on Sephadex G-25. The chromatogram was delineated by Folin-Lowry analysis.

Leucine aminopeptidase (LAP, Worthington Biochemical Corp.) digestion was performed with an enzyme to substrate weight ratio of 1:65 for 26 h at 37° in the pH 8.5 buffer (see Chapter 3). Reduction and alkylation with α-iodoacetamide was performed as previously reported (Bewley & Li, 1969). Dithiothreitol (DTT) and α-iodoacetamide were obtained from Calbiochem.

Loss of S-Carbamidomethylcysteine during Enzyme Digestion. S-Carbamidomethylcysteine (12.5 mg) was dissolved in 10 ml of the pH 8.5 buffer (see Chapter 3). An aliquot (1.0 ml) was heated at 37° for 24 h and resulted in: 40.4% Cys(Cam)

present by quantitative amino acid analysis; tlc (BAW) a ninhydrin-positive, chlorine-positive spot with R_f 0.19, identical to a control sample of S-carbamidomethylcysteine, and a ninhydrin-negative, chlorine-positive spot with R_f 0.48. The cyclization of S-carbamidomethylcysteine to a thiazine derivative has been reported (Press, 1967; Bradbury & Smyth, 1973).

para-Nitrophenyl N^{α}-Boc-S-Carbamidomethylcysteinate. N^{α}-Boc-S-carbamidomethylcysteine (5.0 g, 18 mmol) and p-nitrophenol (10.0 g, 72 mmol) were dissolved in 15 ml DMF and cooled to -10°. At 4°, over a period of 30 min, DCC (4.11 g, 19.5 mmol) dissolved in 5 ml of DMF was added. After 18 h at 4° and 7 h at 24°, the mixture was filtered at 4° and the solid washed with 50 ml of DMF. Removal of solvent in the combined filtrate and washings gave an oil which crystallized from ether: yield, 6.2 g; mp 145°. Recrystallization from DMF-water (3:2) which was performed by Dr. D. Yamashiro gave 5.36 g, mp $161-162^{\circ}$. Further recrystallization of 1.0 g from ethyl acetate gave 0.83 g, mp $162-164^{\circ}$, tlc (BAW) R_f 0.65, $[\alpha]_D^{24}$ -41.5° (c 2.01, DMF).

Anal. Calculated for $C_{16}H_{21}N_3O_7S$ (399.42): C, 48.1; H, 5.3; N, 10.5. Found: C, 48.0; H, 5.6; N, 10.5.

Solid-Phase Synthesis Procedure. Boc-Phe resin was prepared by the triethylamine procedure (Merrifield, 1964). A sample, 20.6 mg, was refluxed in propionic acid -- 12 N HCl (Scotchler et al., 1970) for 18 h, filtered and washed with acetic acid. The combined filtrate and washes were evaporated to dryness. The solid was dissolved in 5.00 ml water and an aliquot was submitted to quantitative amino acid analysis along with a known amount of phenylalanine. The value for substitution obtained was 0.43 mmol Phe/g.

A. HGH-(180-191) with N^{α}-Boc-S-(4-Methoxybenxyl)cysteine. Boc-Phe resin (1.00 g, 0.43 mmol of Phe) was treated by the schedule of synthesis shown in Table 9 for the introduction of each residue. In all but steps 10 and 11, volumes of 35 ml were used. N^{α}-Boc protection was used throughout with the following side-chain protecting groups: Ser, Bzl; Glu, 4-BrBzl; Arg, Tos; and Cys, 4-MeOBzl. For introduction of the glutamine residue, the three washings in step 9 of Table 9 were replaced by DMF, Boc-glutamine p-nitrophenyl ester (4.29 mmol, 10 equiv) in 15 ml of DMF was added in step 10 followed by an 18-h coupling time, and step 11 was replaced with washings of two 35-ml portions of DMF. The DCCI-mediated couplings were performed as follows: in step 10, 1.72 mmol (4 equiv) of the appropriate Boc-amino acid in 15 ml of CH_2Cl_2 for 10 min; and in step 11, 1.71 mmol DCCI in 4.10 ml of CH_2Cl_2 for 2 h. The

valine residues were incorporated by employing 2.58 mmol (6 equiv) of N^{α}-Boc-valine in 15 ml of CH_2Cl_2 in step 10 and by addition of 2.57 mmol DCCI in 6.15 ml of CH_2Cl_2 in step 11; for the arginine residue, Boc-Arg(Tos) was dissolved in 16.5 ml of DMF-CH_2Cl_2 (1:9) for step 10, and a 4-h coupling time was used in step 11. Final weight of the finished protected peptide resin was 1.63 g.

Protected peptide resin (761 mg) was treated with 15 ml of liquid HF for 1 h at 0° in the presence of 1.0 ml of anisole. After removal of the HF at 0° by a stream of nitrogen for 15 min, the peptide-resin mixture was dried under reduced pressure over NaOH for 30 min and then stirred with TFA (15 ml) for 15 min and filtered. The filtrate was evaporated _in vacuo_, and the oily residue was stirred with 75 ml of 0.18 _M_ acetic acid and washed with two 25-ml portions of ethyl acetate. The aqueous phase was made up to 500 ml with water. The Ellman test (Ellman, 1959) for sulfhydryl content on an aliquot indicated 190 μmol (47.2% of theoretical) of sulfhydryl groups to be present in the total crude product. The pH was adjusted to 8.1 with 1 _N_ NH_4OH (_ca._ 14 ml) and, over a period of 30 min with stirring, 0.01 _N_ $K_3Fe(CN)_6$ (20.0 ml) was added. The Ellman test on an aliquot taken 15 min after the end of the titration indicated _ca._ 6.7 μmol of sulfhydryl groups remaining. To the solution, 20 ml (wet volume) of AG3-X4A

resin (chloride form) was added and, after stirring for 15 min, the mixture was filtered and the filtrate lyophilized. The resulting mixture was subjected to gel filtration on Sephadex G-25 (2.5 x 133 cm) in 0.5 M acetic acid (Figure 3). The peptide material in the major peak (V_e of peak, 472 ml) as detected spectrophotometrically at 240 nm was isolated (93 mg) and submitted to chromatography on CMC (Figure 4). A major peak was detected (V_e, 60-80 ml) and isolation by lyophilization gave 66.5 mg of cyclic HGH-(180-191) (ca. 26% yield based on starting Boc-Phe resin); tlc (BPAW) R_f 0.28; paper chromatography (BPAW) R_f 0.25; $[\alpha]_D^{24}$-34.9° (c 0.62, 0.1 M acetic acid).

Paper electrophoresis in pyridine-acetate buffer (pH 3.7, 400 V, 4 h) showed one ninhydrin-positive, chlorine positive spot at R_{Lys} 0. Rechromatography of a sample (6.0 mg) on Sephadex G-25 (2.21 x 61.5 cm) in 0.5 M acetic acid (Figure 5) gave a single symmetrical peak (V_e, 160 ml). Paper electrophoresis in collidine-acetate buffer (pH 6.4, 400 V, 4 h) showed one ninhydrin-positive, chlorine-positive spot at R_{Lys} 0.10. Amino acid analysis of an acid hydrolysate gave $Gly_{2.00}Glu_{1.99}Val_{1.98}Ser_{1.77}Arg_{0.96}Phe_{0.90}$ and half-$cystine_{1.96}$.

The cyclic HGH-(180-191) could also be detected after paper chromatography (BPAW) by the sodium borohydride-DTNB method (Maeda et al., 1970).

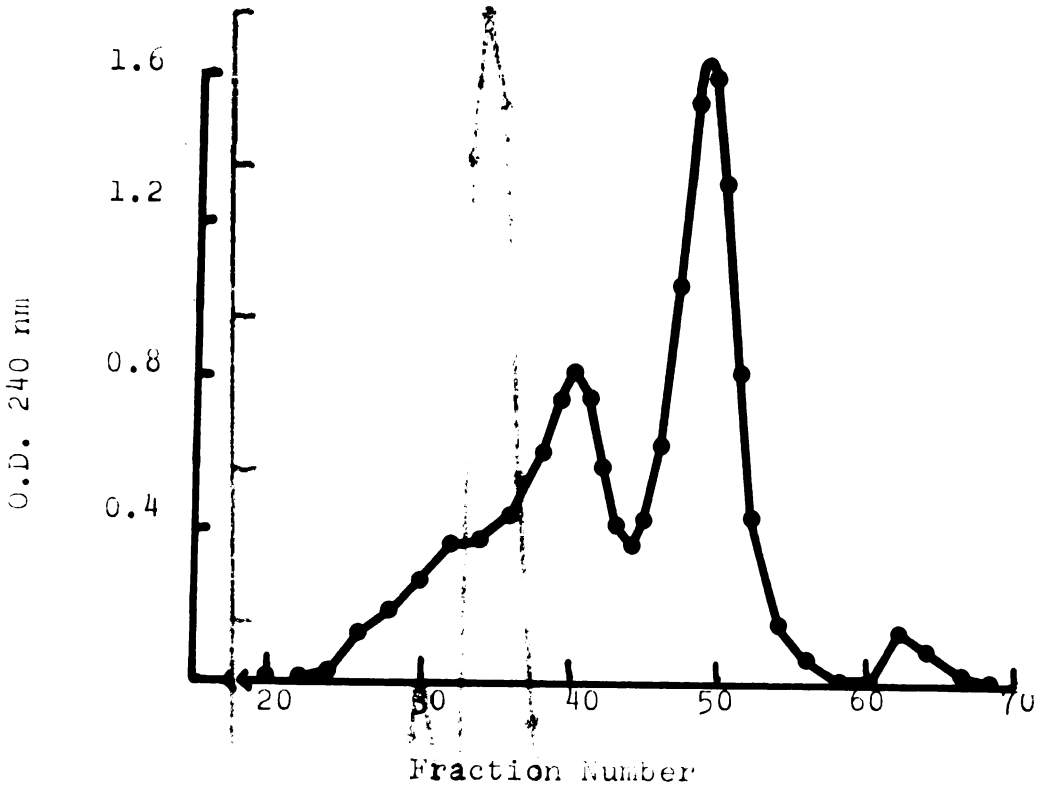
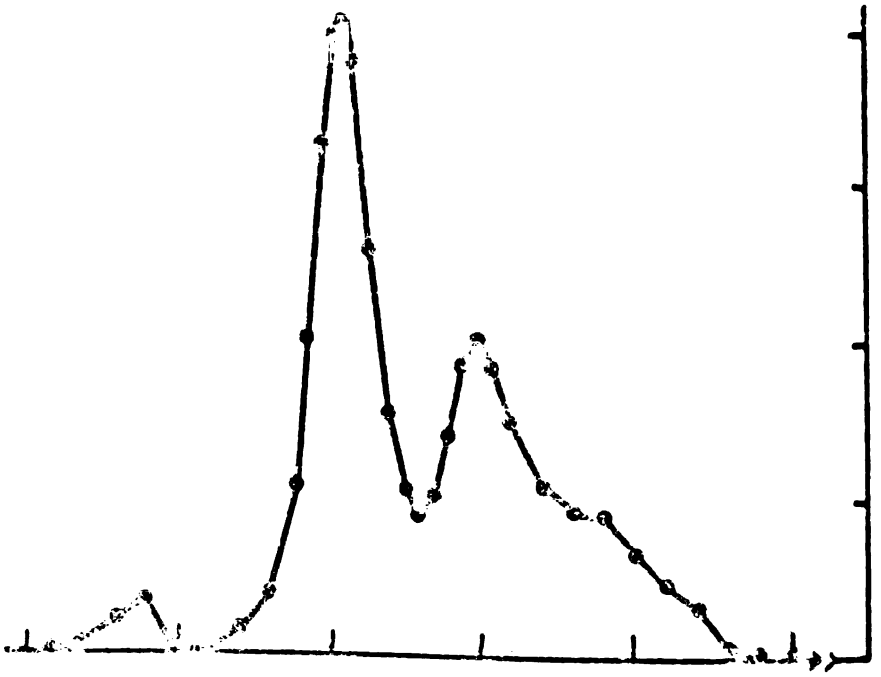


Figure 3. Sephadex G-25 (2.5 x 133 cm) in 0.5 M acetic acid, 20 min/fraction at 26.9 ml/h flow rate, yield (fractions 45-52), 93.1 mg.



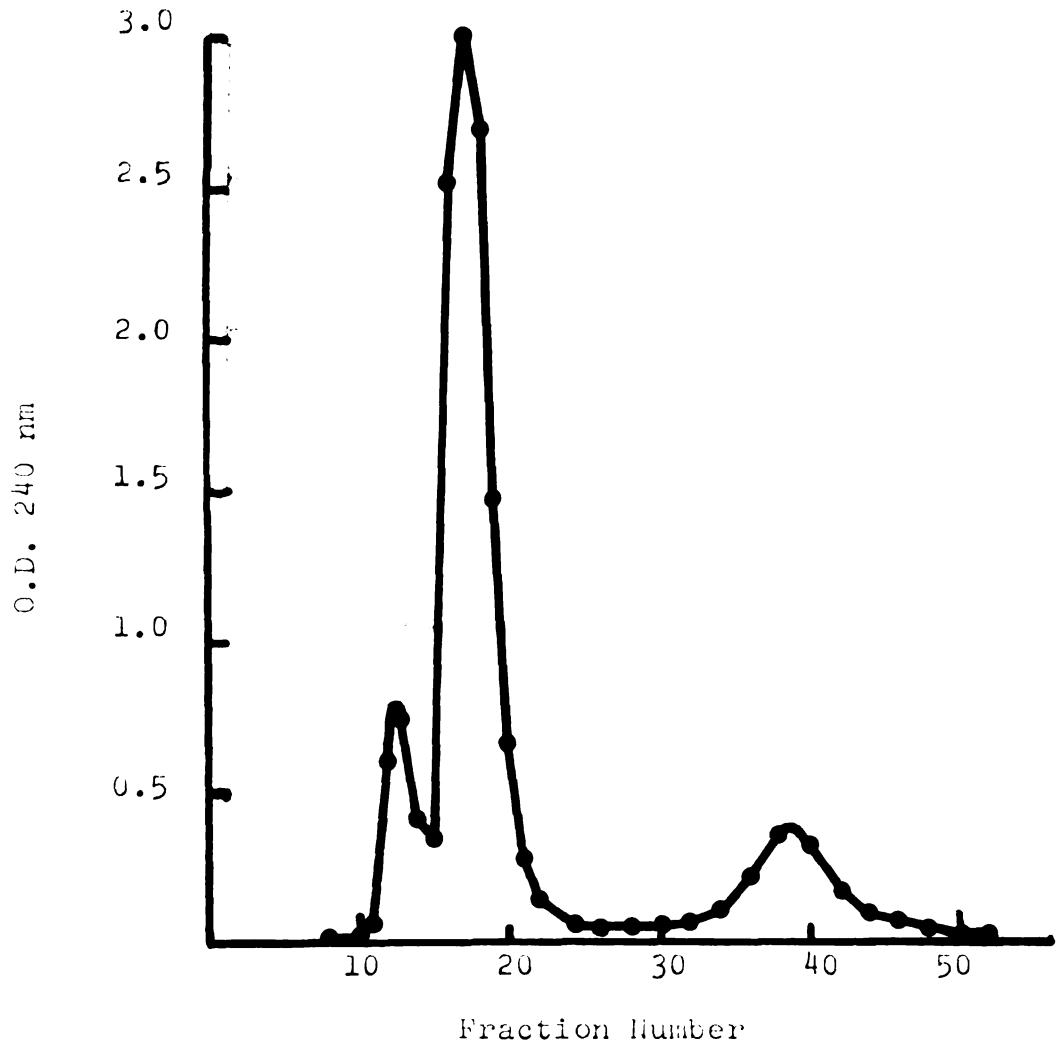
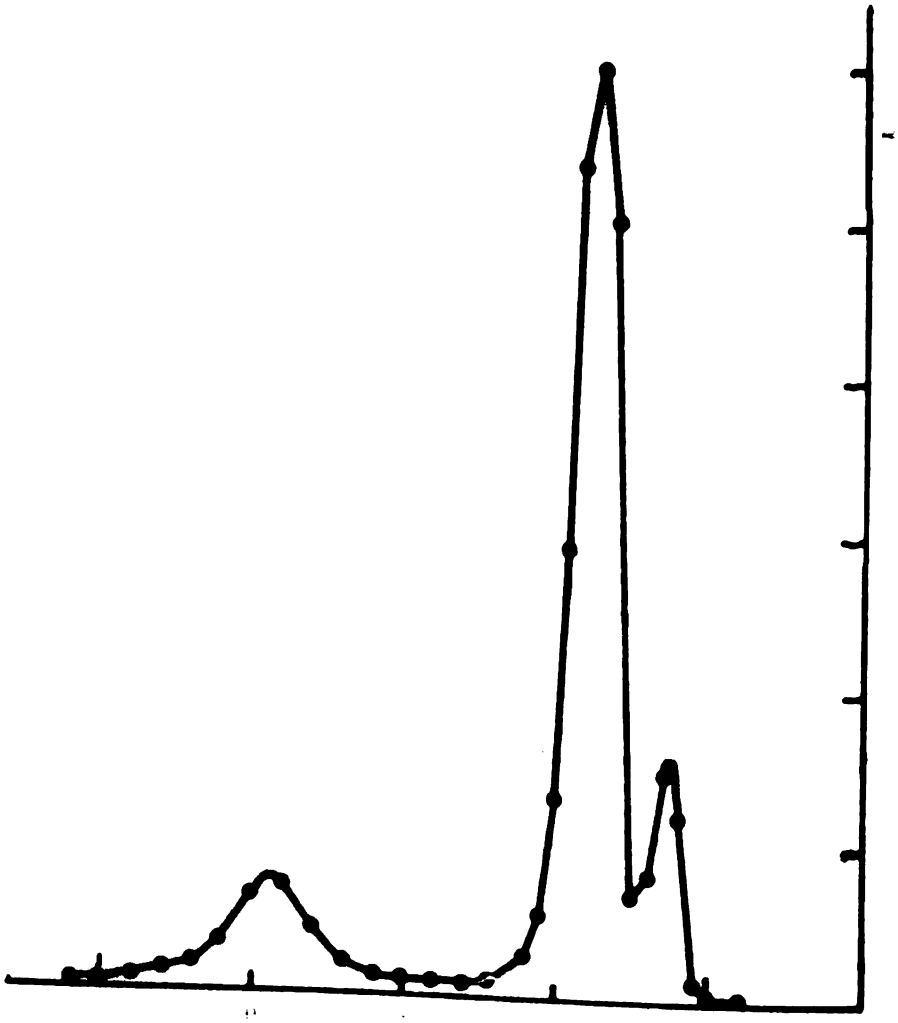


Figure 4. Carboxymethylcellulose chromatography (0.96 x 58 cm) in 0.01 M ammonium acetate, pH 4.5 with 4.0 ml/fraction of 93 mg, yield (fractions 16-20), 66.5 mg.



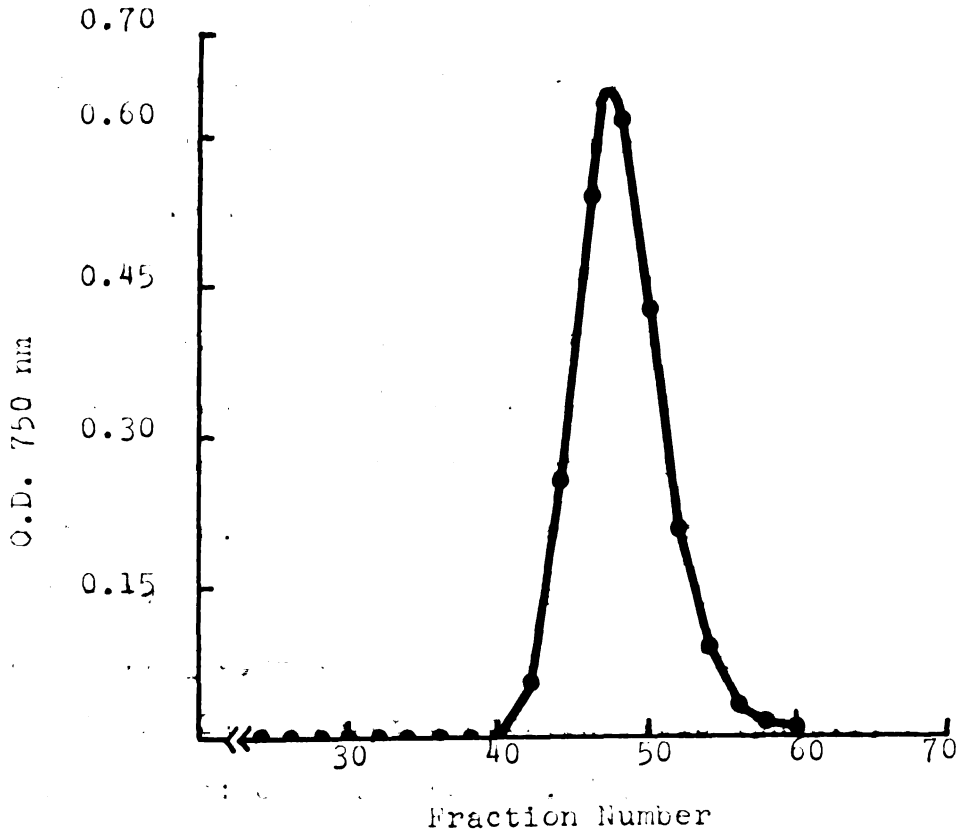
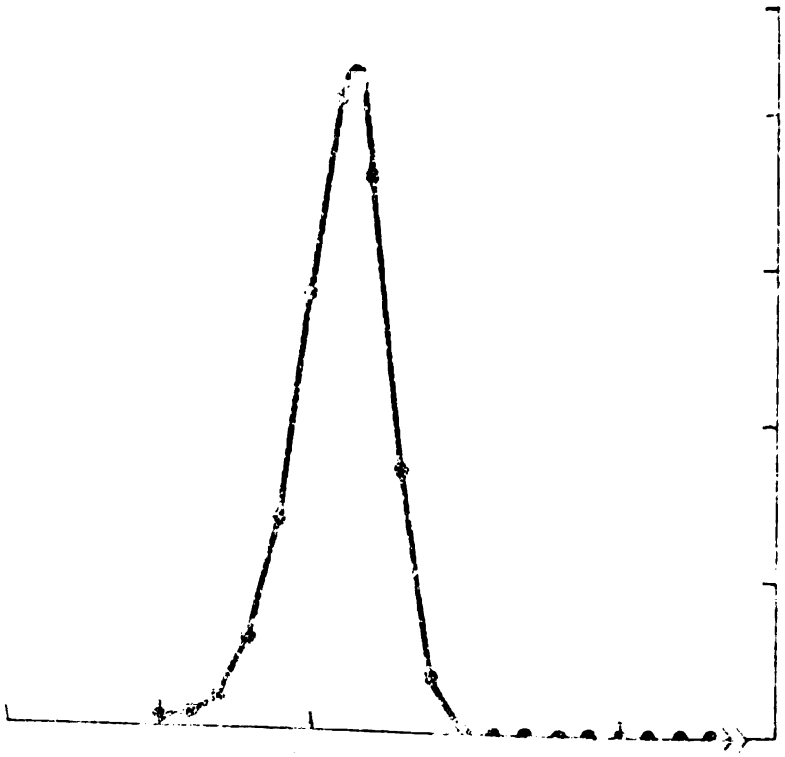


Figure 5. Rechromatography on Sephadex G-25 (2.21 x 61.5 cm) in 0.5 M acetic acid, 3.40 ml/fraction, of cyclic hGH-(180-191) (6.0 mg) from CMC chromatography.



Performic oxidation was performed on a 1.0 mg sample. The resulting lyophilized material was dissolved in 0.5 M acetic acid and an aliquot (one-fourth) run on paper electrophoresis at pH 6.4 as above; one ninhydrin-positive spot ran with R_{Glu} 0.43.

The monomeric nature of the cyclic HGH-(180-191) was established by the partial dinitrophenylation method (Battersby & Craig, 1951) in which a monomer of the cyclic HGH-(180-191) should give two products while a dimer would give three products. A sample of the cyclic HGH-(180-191) (1 mg) was dissolved in 100 µl of 80% aqueous DMSO and treated with 5 µl of 0.1 M 2,4-dinitrofluorobenzene in 75% aqueous dioxane for 4 min at 24°. Paper electrophoresis (pH 2.1, 2 kV, 1h) gave two spots: one with R_{Lys} 0.37 (ninhydrin and Pauly-positive) corresponding to the starting material, and a second at the origin (yellow, Pauly-positive and ninhydrin-negative). Further dinitrophenylation resulted in a decreased amount of unreacted starting material and an increase in the spot at the origin with no appearance of a third spot.

B. HGH-(180-191) with N^{α}-Boc-S-(3,4-dimethylbenzyl) cysteine.

Boc-Phe resin (0.97 g, 0.415 mmol of Phe) was placed in a Beckman Model 990 peptide synthesizer and submitted to the same schedule as in the aforementioned synthesis (Table 9), except that chloroform was replaced by dichloromethane. The only change in side-chain protecting groups was the use

of 3,4-dimethylbenzyl protection for cysteine. Final weight of the finished protected peptide resin was 1.50 g.

A portion (682 mg) of protected peptide resin was treated with 15 ml of liquid HF for 1 h at 0° in the presence of anisole (1.0 ml). The crude product was worked up as above. The sulfhydryl group contents before and after oxidation with 0.01 N $K_3Fe(CN)_6$ were 170 (45.7% yield) and 4.5 μmol, respectively. The major peptide material after gel filtration on Sephadex G-25 (Figure 6) as above (V_e of peak, 465 ml) was isolated (71.0 mg). An aliquot (36.5 mg) of the product was then submitted to chromatography on CMC (Figure 7) as described above. A major peak was detected (V_e, 52-72 ml) and isolation by lyophilization gave 24.8 mg (ca. 20% yield based on starting Boc-Phe resin) of cyclic HGH-(180-191), $[\alpha]_D^{24}$ -33.1° (c 0.60, 0.1 M acetic acid). This material was identical to the preparation described in Part A on paper electrophoresis, tlc (BPAW), and paper chromatography. Amino acid analysis of an acid hydrolysate gave: Gly 2.00, Glu 1.97, Val 1.94, Ser 1.81, Arg 0.93, Phe 1.03, and half-cystine 1.87.

[Cys(Cam)^{182,189}]-HGH-(180-191). Boc-Phe resin (1.00 g, 0.43 mmol of Phe) was carried through the same schedule of synthesis as described above for the synthesis employing 4-methoxybenzylcysteine with the following exceptions

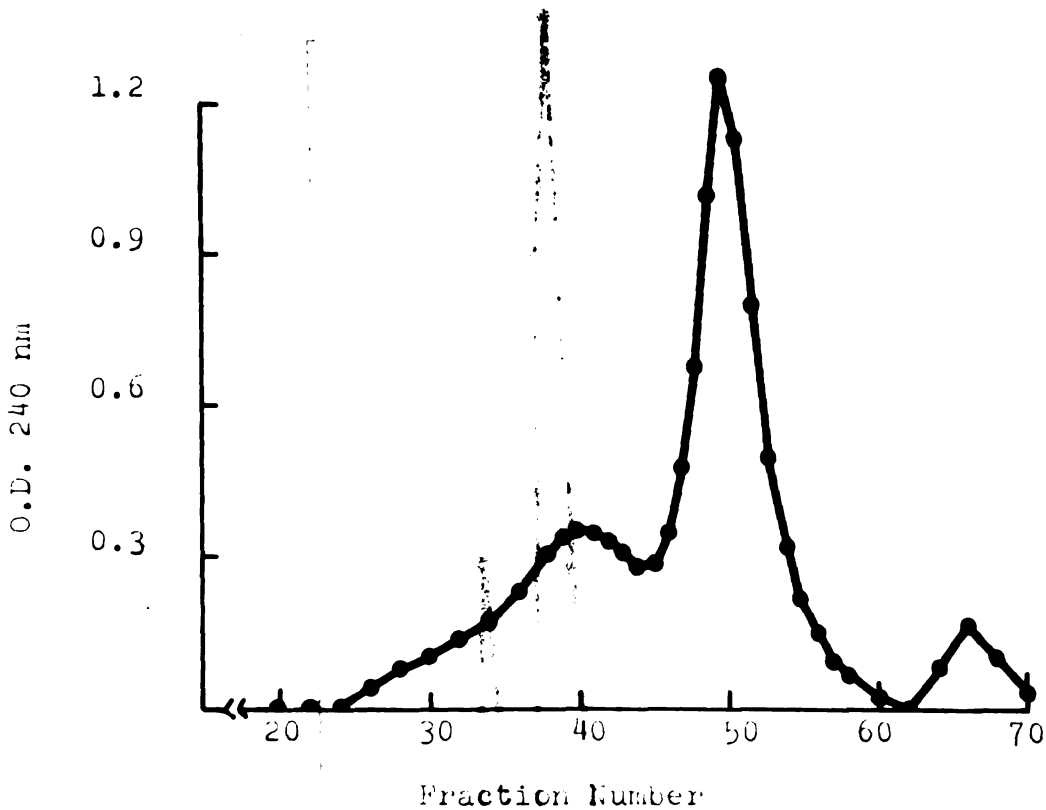
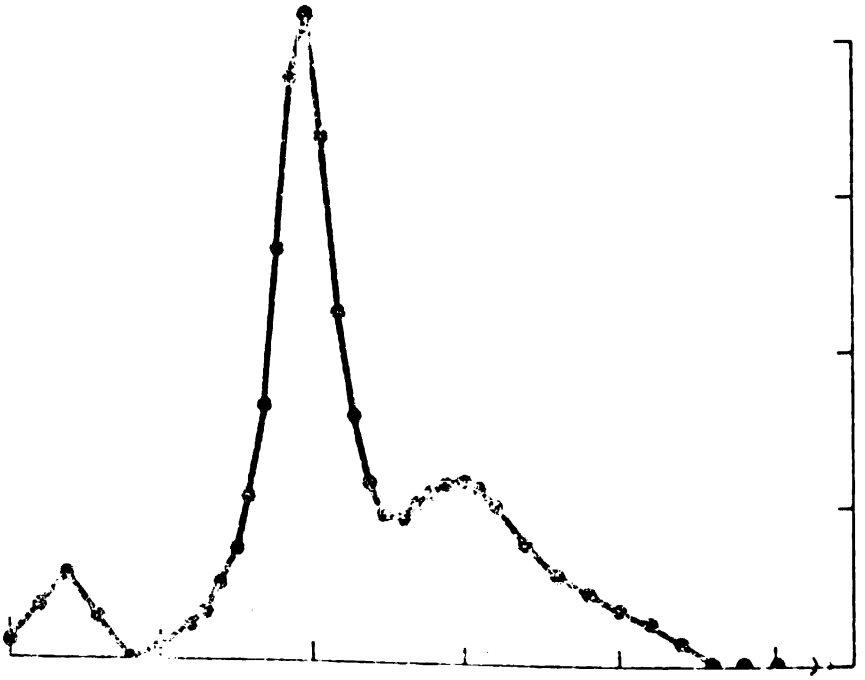


Figure 6. Sephadex G-25 chromatography carried out on the same column and under the same conditions as in Figure 3, yield (fractions 47-54), 71.0 mg.



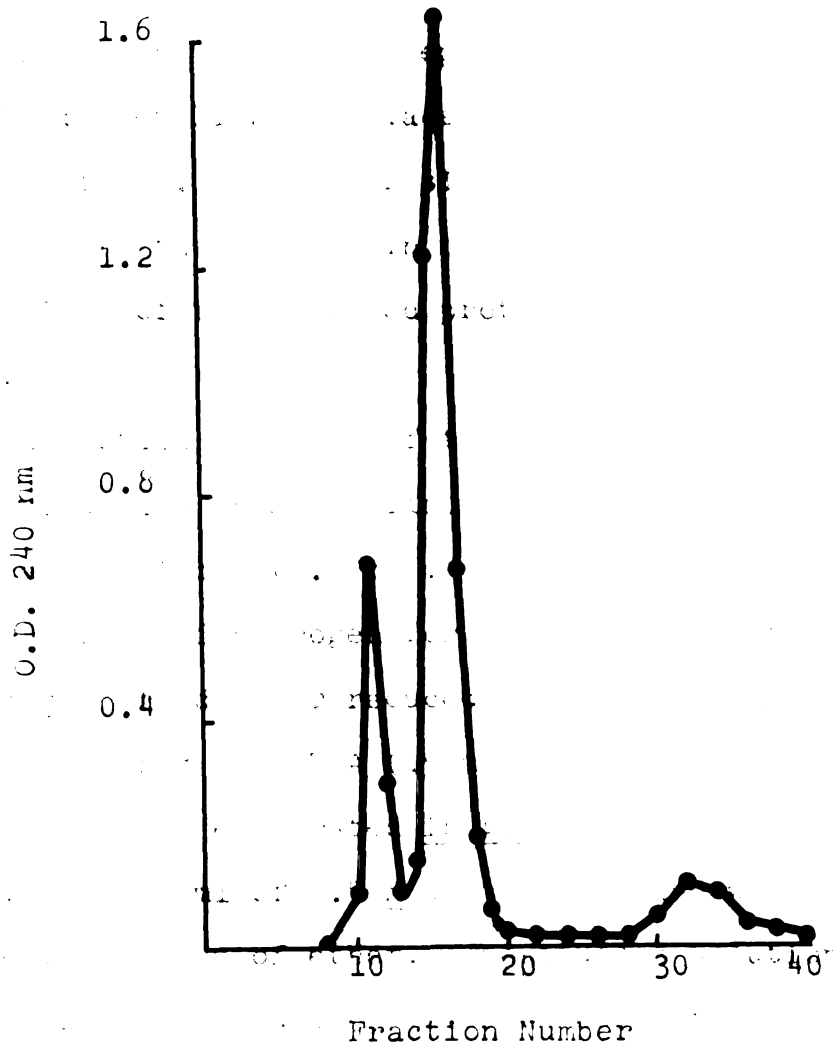
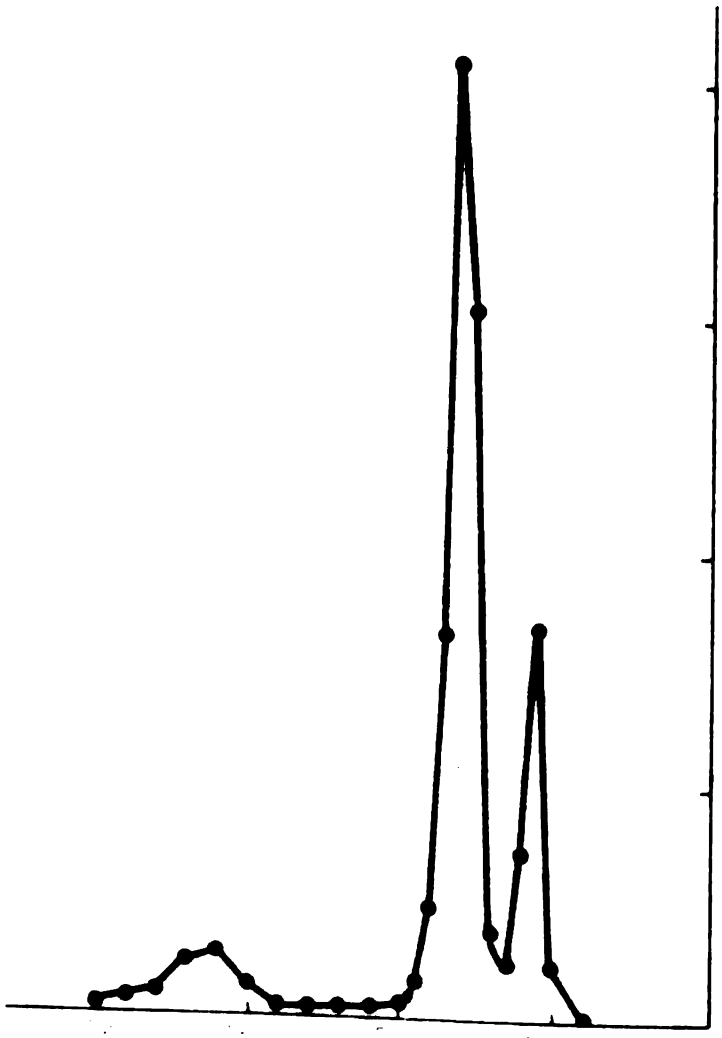


Figure 7. Carboxymethylcellulose chromatography (0.96 x 58 cm) of 36.5 mg carried out under the same conditions as in Figure 4, yield (fractions 14-18), 24.8 mg.



for the incorporation of the Cys(Cam) residues; the three washings in step 9 were replaced by DMF; 3.86 mmol (9 equiv) N^{α}- Boc-S-carbamidomethylcysteine p-nitrophenyl ester in 15 ml of DMF was added in step 10 followed by an 18-h coupling time; step 11 was replaced by washing with two 35-ml portions of DMF. The other side-chain protecting groups employed were the same as in the aforementioned syntheses. Final weight of the finished protected peptide resin was 1.52 g.

A portion (760 mg) of the protected peptide resin was treated with 15 ml of liquid HF for 1 h at 0° in the presence of anisole (1.0 ml). After removal of the HF at 0° by a stream of nitrogen for 15 min, the peptide-resin mixture was dried under reduced pressure over NaOH for 30 min and then stirred with TFA (15 ml) for 15 min and filtered. The filtrate was evaporated in vacuo, and the oily residue dissolved in 10 ml of 0.5 M acetic acid and washed with two 10-ml portions of ether. The aqueous solution was lyophilized and the resulting material subjected to gel filtration on Sephadex G-25 (Figure 8) as described above, and the peptide material in the major peptide (V_e of peak, 446 ml) was isolated (167 mg). An aliquot (52.2 mg)was then subjected to partition chromatography on Sephadex G-25 as described above, and a single symmetrical peak with R_f 0.15 was obtained (Figure 9). The isolated material (40.9 mg) was then submitted to the chromatography on CMC.

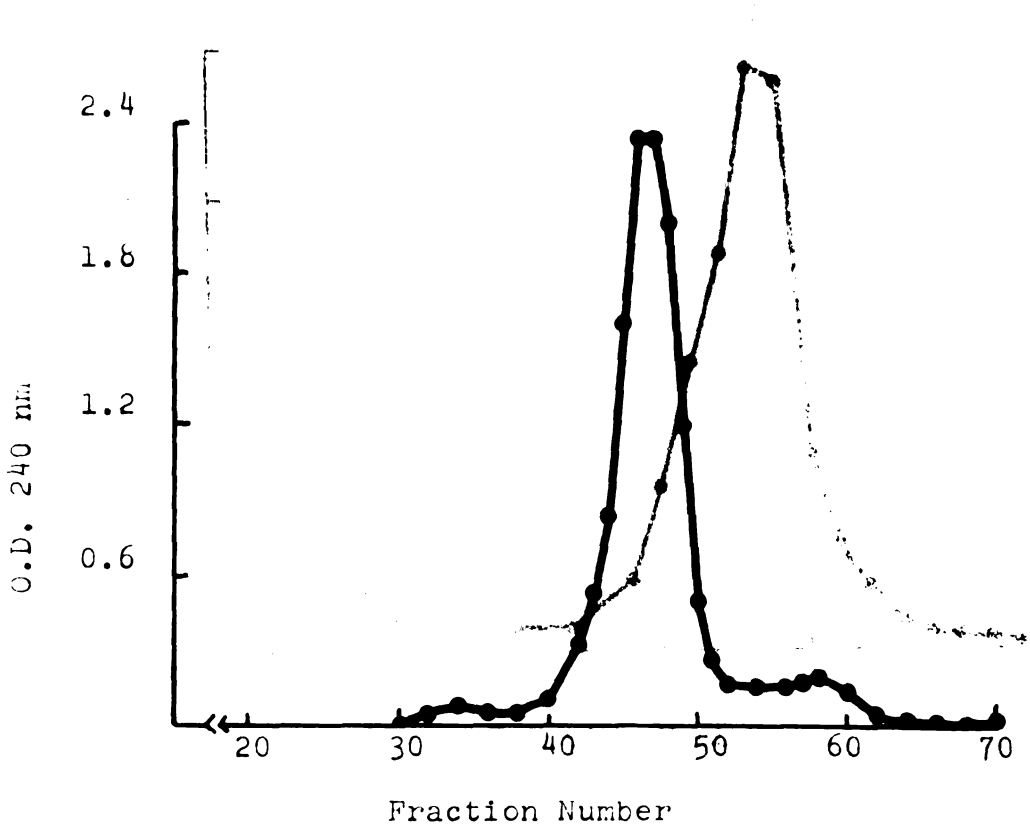
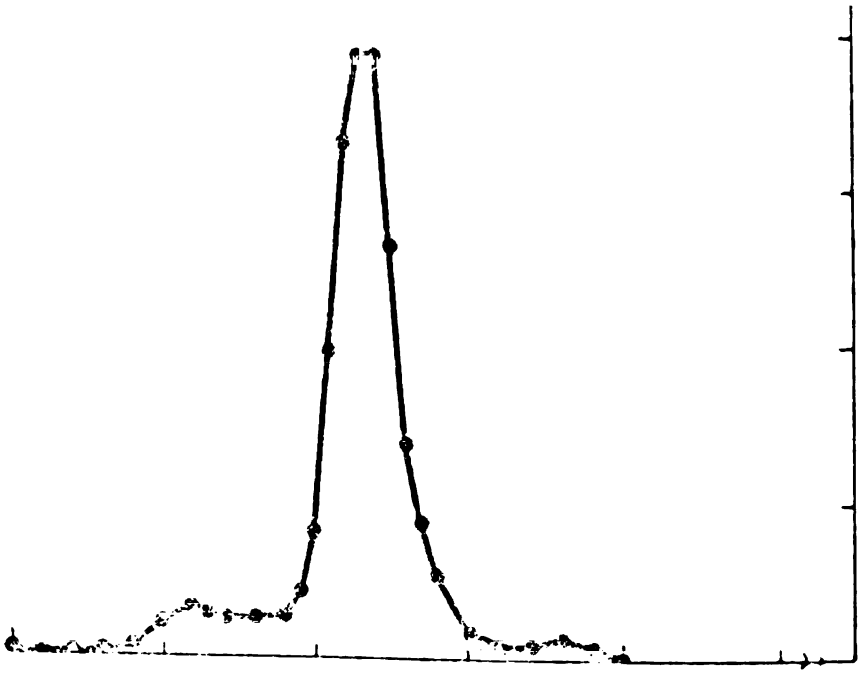


Figure 8. Sephadex G-25 (2.5 x 133 cm) in 0.5 M acetic acid under the same conditions as in Figure 3, 228 mg, yield (fractions 44-50), 167.1 mg.



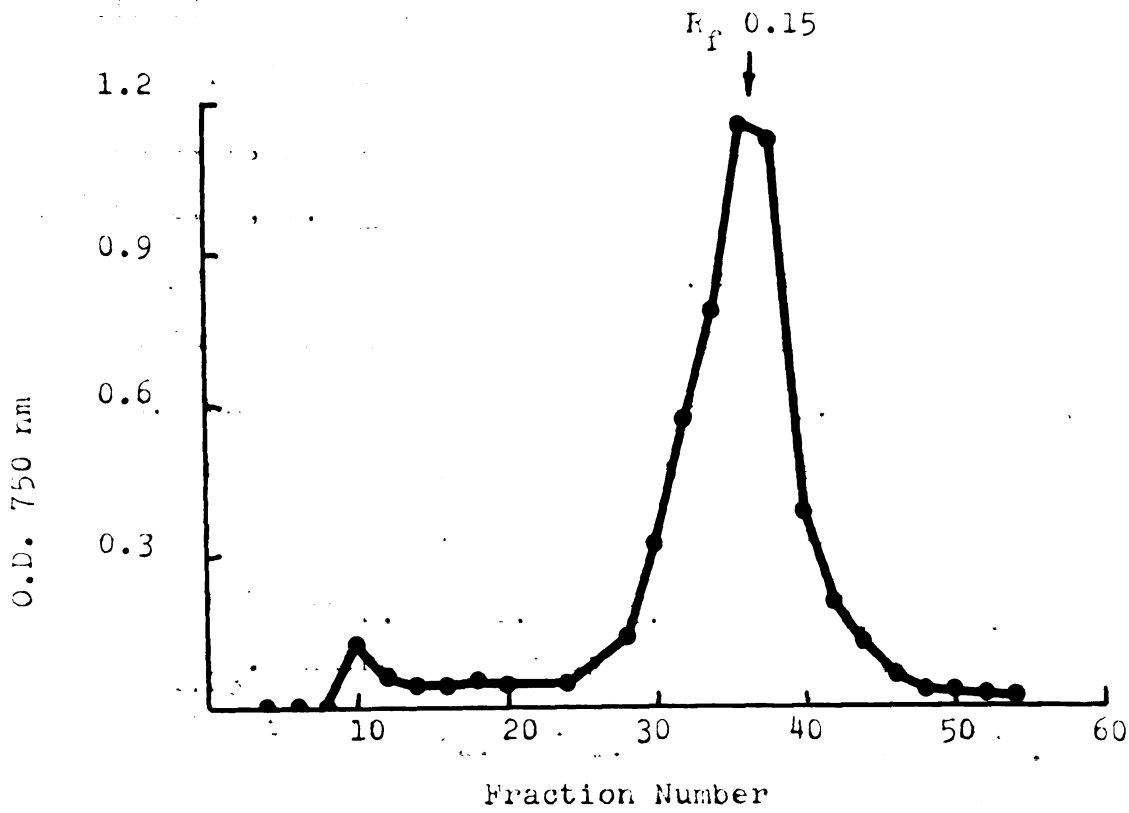
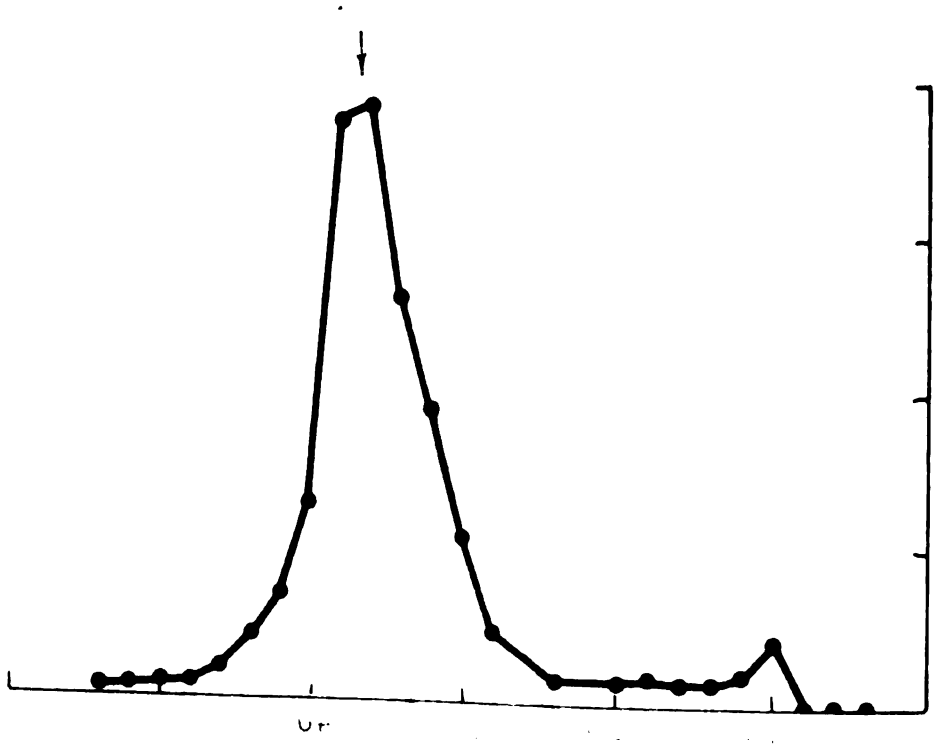


Figure 9 . Partition chromatography on Sephadex G-25 (1.92 x 68.9 cm) in 1-butanol-pyridine-0.2 N NH_4OH with 0.1% acetic acid (5:3:12), 12.1 ml/ fraction; 52.2 mg, yield (fractions 30-40), 40.9 mg.



One peak was detected (V_e, 51-63 ml) and isolation by lyophilization gave 27.1 mg (ca. 29% yield based on starting Boc-Phe resin) of [Cys(Cam)^{182,189}]-HGH-(180-191): tlc (BPAW) R_f 0.22, paper chromatography (BPAW) R_f 0.10, $[\alpha]_D^{24}$ -49.3° (c 0.46, 0.1 M acetic acid).

On paper chromatography (BPAW), no spot was observed by the sodium borohydride-DTNB method for sulfhydryl group detection. On paper electrophoresis at pH 3.7 and pH 6.4, the peptide migrated as a single spot at the same rate as the cyclic HGH-(180-191). Amino acid analysis of an acid hydrolysate gave $Gly_{2.00}Glu_{2.05}Val_{2.12}Cys(Cm)_{2.06}Ser_{1.80}Arg_{1.00}Phe_{1.03}$. Amino acid analysis of a 24-h leucine aminopeptidase digest gave $Gly_{2.00}Glu_{1.00}(Ser + Gln)_{2.80}Val_{2.10}Cys\ (Cam)_{0.82}Arg_{0.96}Phe_{0.99}$. When an authentic sample of S-carbamidomethylcysteine was treated under conditions for enzyme digest (described above), ca. 60% of the compound disappeared as judged by amino acid analysis and quantitatively accounted for the low value obtained on [Cys(Cam)^{182,189}]-HGH-(180-191).

Conversion of cyclic HGH-(180-191) to [Cys(Cam)^{182,189}]-HGH-(180-191). A sample (30.8 mg) of cyclic HGH-(180-191) was dissolved in 0.01 M NH_4HCO_3 (6.0 ml) of pH 8.3 under conditions previously described (Bewley & Li, 1969). Reduction with dithiothreitol (DTT; Cleland, 1964) (37.6 mg) was followed by treatment witth α-iodoacetamide (449.9 mg).

The solution was subjected to gel filtration on Sephadex G-10 (2.2 x 25 cm) in 0.01 M NH_4HCO_3 of pH 8.3. The peptide material (V_e of peak, 48 ml) was isolated (35.8 mg) and submitted to chromatography on CMC. One peak was detected in the position expected for [Cys(Cam)^{182,189}]-HGH-(180-191) (V_e, 49-65 ml) and isolation by lyophilization gave 24.8 mg (ca. 74%): tlc (BPAW) R_f 0.22 and paper chromatography (BPAW) R_f 0.10 identical with those of a sample [Cys(Cam)^{182,189}]-HGH-(180-191) from the previous section; $[\alpha]_D^{24}$ -50.7° (c 0.402, 0.1 M acetic acid). Amino acid analyses of an acid hydrolysate and a leucine aminopeptidase digest gave $Gly_{2.00}Glu_{2.05}Val_{1.99}Cys(Cm)_{2.19}Ser_{1.98}Arg_{0.95}Phe_{1.02}$ and $Gly_{2.00}Glu_{0.97}(Ser + Gln)_{2.95}Val_{1.90}Cys(Cam)_{0.93}Arg_{1.02}Phe_{0.99}$, respectively.

Circular Dichroism . Circular Dichroism (CD) spectra (Figure 10) were taken on a Cary Model 60 spectropolarimeter equipped with a Model 6002 Circular Dichroism attachment according to procedures outlined previously (Bewley et al., 1972). No data was used at dynode voltages greater than 420 V. All spectra, including baselines, were scanned twice. A mean residue weight (MRW) of 105.8 was used for the cyclic dodecapeptide HGH-(180-191) and 115.5 for the dodecapeptide [Cys(Cam)^{182,189}]-HGH-(180-191). Mean residue ellipticities, $[\theta]_{MRW}$, were calculated according

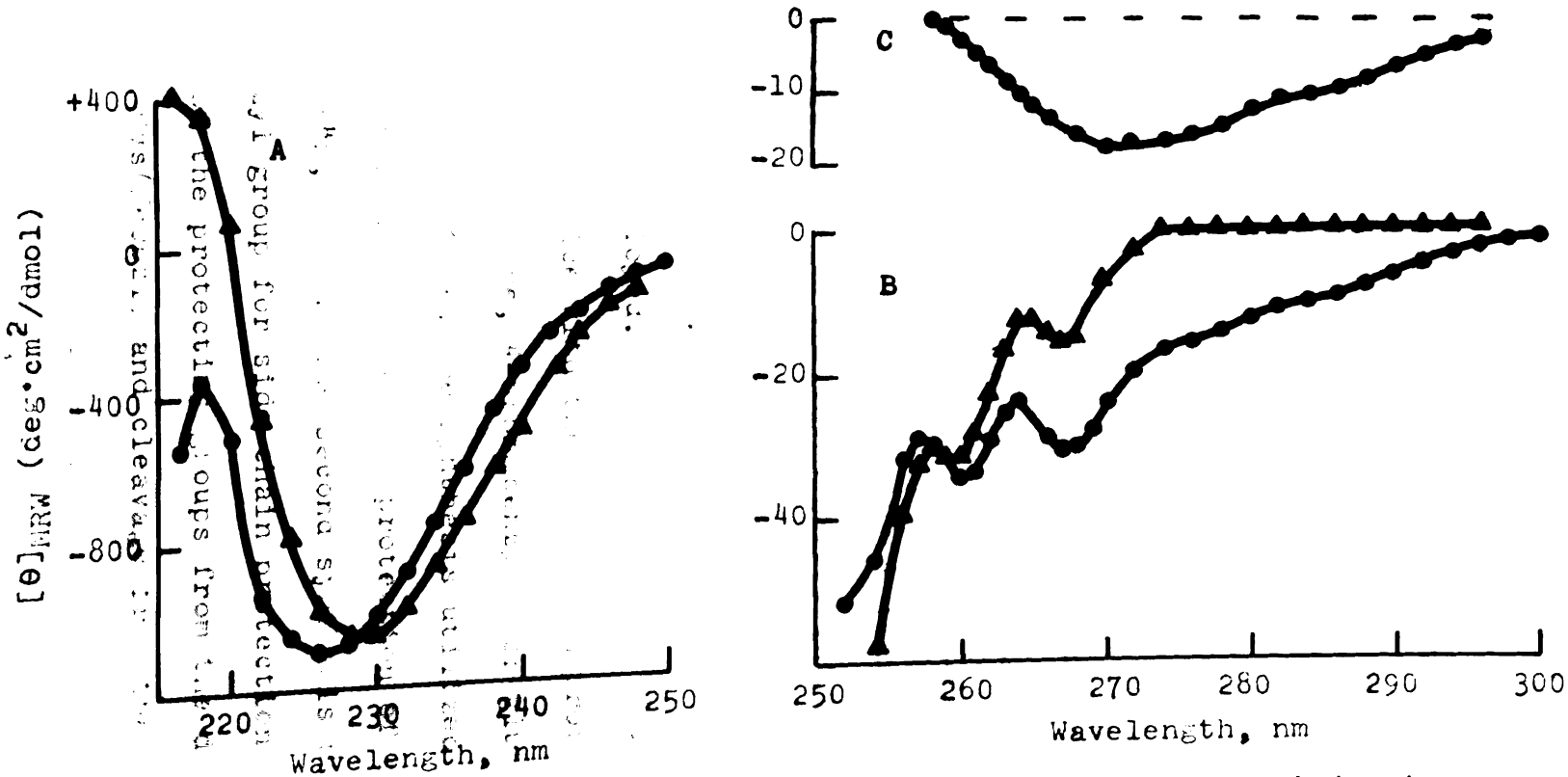
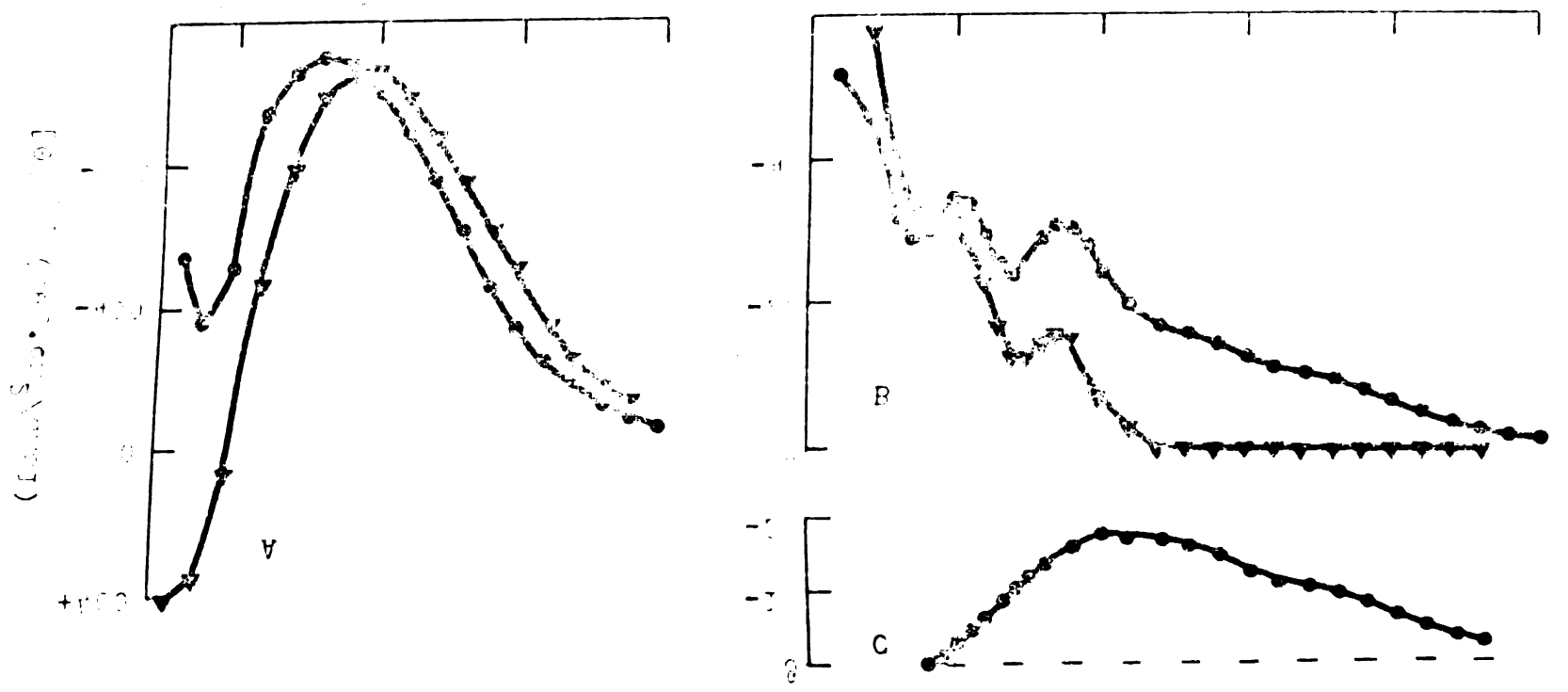


Figure 10. Circular dichroism spectra of cyclic HGH-(180-191) (●—●) and [Cys(Cam)182,189]-HGH-(180-191) (▲—▲) in the region of amide band absorption (A) and side-chain absorption (B). Difference CD band in region of side-chain absorption (C).


A
B
C

to $[\theta]_{MRW} = \theta \cdot MRW\ (c \cdot l)^{-1}$, where θ is the observed ellipticity, c is concentration in g/l, l is length in cm and MRW as above. Both dodecapeptides were dissolved separately in 0.1 M acetic acid at a concentration of 1.75 g/ml for the cyclic HGH-(180-191) and 1.73 g/ml for the [Cys(Cam)^{182,189}]-HGH-(180-191).

Results and Discussion

The dodecapeptide HGH-(180-191) which contains a disulfide bridge between cysteine residues 182 and 189 was synthesized by the solid-phase method. The Boc-Phe resin was prepared by the triethylamine procedure (Merrifield, 1964) because this work was initiated before introduction of the modified Loffet procedure for attachment (Loffet, 1971; Yamashiro & Li, 1973b). The standard DCCI-mediated coupling method was employed. While the cyclic dodecapeptide was synthesized twice with the side-chain protecting groups of benzyl for serine, 4-bromobenzyl for glutamic acid and tosyl for arginine, one synthesis utilized the 4-methoxybenzyl group for side-chain protection of cysteine (Zahn & Hammerström, 1969). The second synthesis used the 3,4-dimethylbenzyl group for side-chain protection of cysteine.

Removal of the protecting groups from the dodecapeptide which contained Cys(MeOBzl) and cleavage from the solid support were performed with hydrogen fluoride (Sakakibara et al., 1967; Lenard & Robinson, 1967; Mazur & Plum, 1968).

Since air oxidation of the disulfhydryl dodecapeptide was unsuccessful although reduced HGH is oxidized by air with ease (Bewley & Li, 1970), oxidation was accomplished by the use of ferricyanide as previously employed for deaminooxytocin (Hope et al., 1962). Purification by gel filtration on Sephadex G-25 (Figure 3) was followed by chromatography on carboxymethylcellulose (Figure 4). Cyclic dodecapeptide HGH-(180-191) was isolated in highly purified form as indicated by thin-layer and paper chromatography, paper electrophoresis, gel filtration on Sephadex G-25 (Figure 5) and amino acid analysis. Evidence that the synthetic product was a monomer was obtained by partial reaction of the single amino group with 2,4-nitrofluorobenzene (Battersby & Craig, 1951) and examination of the products by electrophoresis.

The dodecapeptide which employed the 3,4-dimethylbenzyl group for the side-chain protection of cysteine was synthesized and worked up in the same manner as the first synthesis. The cyclic dodecapeptide HGH-(180-191) was obtained (Figures 6 and 7) in highly purified form and was in complete identity with the preparation from the first synthesis.

The linear dodecapeptide [Cys(Cam)^{182,189}]-HGH-(180-191) was synthesized in the same manner as the cyclic

dodecapeptide, except that S-carbamidomethylcysteine residues were incorporated by the use of Boc-Cys(Cam) p-nitrophenyl ester. The linear dodecapeptide was deprotected and cleaved from the resin by hydrogen fluoride. Purification by gel filtration on Sephadex G-25 (Figure 8), partition chromatography on Sephadex G-25 (Yamashiro et al., 1966) (Figure 9), and chromatography on CMC gave the linear dodecapeptide [Cys(Cam)^{182,189}]-HGH-(180-191) in highly purified form as judged by thin-layer and paper chromatography, paper electrophoresis, and amino acid analyses.

The disulfide bridges of HGH have been reduced with dithiothreitol and alkylated with α-iodoacetamide (Dixon & Li, 1966; Bewley et al., 1969). Thus, an additional check of the synthetic procedures would be the conversion of the cyclic dodecapeptide to the linear dodecapeptide [Cys(Cam)^{182,189}]-HGH-(180-191) by the above procedures. After this conversion was accomplished and followed by purification by gel filtration on Sephadex G-10 and chromatographed on CMC, the final product was found to be identical with the linear dodecapeptide synthesized with S-carbamidomethylcysteine.

The CD spectra of the cyclic dodecapeptide HGH-(180-191) and the dodecapeptide [Cys(Cam)^{182,189}]-HGH-(180-191) are shown in Figure 10. The two negative bands at 267-268 and 259-260 nm have been assigned to phenylalanine (Bewley & Li, 1970). It is evident that the significant difference

in the region of side-chain absorption (Figure 10 B) between the two spectra reaches a maximum around 272-274 nm. Simple subtraction of the spectrum of S,S'-dicarbamidomethyl dodecapeptide from that of the cyclic disulfide dodecapeptide generates a broad negative band shown in Figure 10 C. This band is very similar in shape to the bands attributed to the disulfide dichroism in L-Cystine and also to the synthetic C-terminal cyclic undecapeptide of ovine pituitary prolactin (Yamashiro et al., 1975). However, the spectral position is red-shifted from the position around 260 nm of the prolactin undecapeptide and a similar curve for the C-terminal disulfide bridge in plasmin-modified HGH, to that of the other disulfide bridge in plasmin-modified HGH between cysteine residues 53 and 165 around 272-274 nm (Bewley, 1976). Speculation as to the significance of environment and/or dihedral angle as a cause of this spectral shift should wait until the identical sequence from HCS has been isolated from cyanogen bromide cleavage and investigated in similar studies.

Nevertheless, it is proposed that the optical activity of the disulfide bridge for the synthetic cyclic dodecapeptide is represented by the band in Figure 10 C.

Table 9

Schedule of Solid-Phase Peptide Synthesis for HGH-(180-191) Peptides

Step	Reagent and Operation	Time (min)
1	CH_2Cl_2 (2 times)	2
2	TFA-CH_2Cl_2, 1.22:1 (1 time)	1
3	TFA-CH_2Cl_2, 1.22:1 (1 time)	15
4	CH_2Cl_2 (3 times)	2
5[a]	$CHCl_3$ (3 times)	2
6[a]	DIEA-$CHCl_3$, 1:19 (1 time)	2
7[a]	DIEA-$CHCl_3$, 1:19 (1 time)	5
8[a]	$CHCl_3$ (3 times)	2
9[b]	CH_2Cl_2 (3 times)	2
10[b]	Boc-amino acid (4 equiv)[c], (1 time)	10
11[b]	DCCI (4 equiv)[c], (1 time)	120
12	CH_2Cl_2 (3 times)	2
13	EtOH (3 times)	2
14	CH_2Cl_2 (3 times)	2

[a] $CHCl_3$ replaced by CH_2Cl_2 in synthesis with Boc-Cys(3,4-Me_2Bzl).

[b] Modified for incorporation of Gln and Cys(Cam) residues, see text.

[c] Employed 6 equiv for Val residues.

CHAPTER 5

STUDIES ON THE GENERATION OF TRIFLUOROACETYLATED PEPTIDES DURING SOLID-PHASE SYNTHESIS

Introduction

When the amino group fails to participate in either the deprotection or coupling reactions of step-wise solid-phase synthesis, the peptide is a failure sequence. A failure sequence that subsequently participates in the synthetic reactions is a deletion sequence, in that one or more residues of the target sequence are missing. The failure sequences which do not again participate in the synthetic reactions are terminated sequences. Terminated sequences can arise from either steric hindrance of the peptide chain in the solid support or a side-reaction which covalently blocks the amino group.

Studies of the effect of solvent and resin cross-linking on the synthesis of the heptadecapeptide angiotensinylbradykinin (Merrifield, 1967) indicated that both steric hindrance and covalent blockage of the amino group were the causes of only 20% incorporation of the last five residues. Of the four syntheses outlined, no evidence of chain termination was observed in the synthesis of angiotensinylbradykinin which employed HCl-dioxane for deprotection instead of HCl-acetic acid, triethylamine-chloroform for neutralization instead of triethylamine-dimethylformamide, and a 1%

cross-linked resin instead of a 2% cross-linked resin for the solid support. Terminated sequences from a synthesis of antamanid were shown to be acetylated peptides (Brunfeldt et al., 1972). In this same report, studies were described which quantitated the acetic acid remaining after washing and neutralization in both an amino acid resin and teflon which has been used to coat the reaction vessel of some commercial instruments.

Chain termination also has been observed in solid-phase syntheses which used trifluoroacetic acid-dichloromethane for deprotection. However, no direct evidence has been reported that the trifluoroacetyl group was responsible for chain termination. The synthesis of Ac-Tyr-$(Pro)_{10}$-Tyr resulted in terminated peptides (Bush et al., 1972). By a treatment which removes trifluoroacetyl groups from N^{α} and N^{ε}-amino groups, these terminated peptides were deblocked and separated into a series of CF_3CO-$(Pro)_n$-Tyr peptides. From the syntheses of six angiotensin-converting enzyme inhibitors (Ondetti et al., 1971), terminated peptides were isolated and reported to be trifluoroacetylated peptides, however no characterization of the peptides were given.

By solution methods, a detailed study demonstrated that if trifluoroacetic acid or anion was present during the dicyclohexylcarbodiimide-mediated coupling it could compete and result in trifluoroacetylation (Fletcher et al., 1973). This made the quantitative removal of trifluoroacetic

acid mandatory for this type of coupling. When the coupling conditions were changed by use of 1-hydroxybenzotriazole, the trifluoroacetylation was greatly reduced.

This chapter describes coupling studies on the solid-phase in the presence of trifluoroacetic acid and the amounts of trifluoroacetylated peptides produced. Additional studies presented are the behavior of trifluoroacetylated peptides on the solid-phase and in solution, evidence of trifluoroacetylation which occurred during the synthesis of the 54-residue peptide fragment described in Chapter 7, and attempts to quantitate trifluoroacetylation during the synthesis of a model peptide on the 54-residue peptide resin.

Experimental Section

Methods and Materials. The methods and materials in this chapter have for the most part been described in the preceding chapters except for the following two instances:

Schiff Base Amine Test. Detection of the amine content of a peptide resin by the Schiff base method (Esko et al., 1968) was carried out in the following manner. To a weighed sample of N^{α} deprotected peptide resin in a Pasteur pipette with a glass wool plug in the tip, a solution of 1.0 M 2-hydroxy-1-naphthaldehyde in CH_2Cl_2 was added and shaken for 18 h at 24°. The resin was washed with about 25 ml of CH_2Cl_2. Then a solution of 1.0 M benzylamine in CH_2Cl_2 was added and the suspension shaken for 20 min. The solution was collected in

a 10-ml volumetric flask by filtration through the glass wool plug and the volume was made to 10.0 ml with rinses of the benzylamine solution which were passed through the sample. The absorbance at 420 nm was obtained and the amine content calculated with the value of ε_{420}=6050.

Palladium Catalyst. To 517 mg of palladium chloride in a 600-ml beaker, 0.5 ml of formic acid was added and heated on a hot plate, then 50 ml of concentrated HCl was added and the solution brought to a boil. Next, 100 ml of water was added and KOH was added carefully until the solution was approximately pH 9. Formic acid was added until the pH was about 4 and the solution filtered on sintered glass followed by copious water and methanol washes. The suspension in methanol constituted the palladium catalyst (which burned if allowed to dry -- caution).

Synthesis of CF_3CO-Val-Phe-Gly Resin. Boc-Gly resin prepared by the modified Loffet procedure (Yamashiro & Li, 1973b) gave 0.56 mmol/g by the picric acid method. The Boc-Gly resin (0.9 g, 0.50 mmol Gly) was placed in a Beckman Model 990 peptide synthesizer and subjected to the schedule shown in Table 7 with the following exceptions: 10-ml volumes used in all steps except 9 and 10; TFA-CH_2Cl_2 (1.22:1) in steps 2 and 3; ethanol-CH_2Cl_2 (1:2) in step 5; DIEA-CH_2Cl_2 (1:9) in step 7; 2.1 mmol (4.2 equiv) of Boc-

amino acid in 10 ml of CH_2Cl_2 in step 9; 4.2 ml of 0.465 M DCCI in CH_2Cl_2 (1.95 mmol) for 15 min followed by addition of 3.2 ml of 0.625 M DIEA in CH_2Cl_2 (2.0 mmol) for 15 min in step 10. After Boc-Val had been incorporated, the cycle was repeated through step 8, step 9 was eliminated, and step 10 consisted of a 12-min treatment with 15 ml of trifluoroacetic anhydride-CH_2Cl_2 (1:99). The cycle was completed and the peptide resin dried under reduced pressure over P_2O_5 for 16 h to yield 1.035 g of CF_3CO-Val-Phe-Gly resin.

An amine content determination by the picric acid method showed no amine present. Also, a 1-h treatment of a 50-mg sample with 3 ml of 1 M piperidine in 95% ethanol-CH_2Cl_2 (1:1) resulted in no detectable amine groups. The same result was obtained from a 1-h treatment of a 51-mg sample with 5 ml of 0.2 N tetramethylammonium hydroxide in DMF-CH_2Cl_2 (2:3). However, 0.12% of the theoretical amine content resulted from a 16-h treatment of another 51-mg sample with 5 ml of 1 M hydrazine in DMF. Treatment of a 50-mg sample with 25 ml of refluxing liquid ammonia for 1 h followed by evaporation of the ammonia and washes of ethanol, CH_2Cl_2 and ethanol, again resulted in no detectable amine groups.

Isolation of CF_3CO-Val-Phe-Gly. A portion (501 mg) of the above peptide resin was treated with liquid HF (10 ml) at 0^o for 30 min. After the HF was removed by nitrogen, the

peptide-resin mixture was dried under reduced pressure over NaOH for 30 min, stirred with 15 ml of TFA for 15 min and filtered. The filtrate was evaporated to an oil. After reevaporation from acetic acid, the oil crystallized: 79 mg; tlc (CM) a chlorine-positive spot with R_f 0.70 and a trace ninhydrin-positive spot with R_f 0.05; tlc (CH) chlorine-positive spot with R_f 0.15 and a trace ninhydrin-positive spot at the origin. The ninhydrin-positive material was removed by dissolving a sample of the peptide (10.1 mg) in 30 ml of ethyl acetate and washing with three 10-ml portions of 0.05 M acetic acid. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and filtered. Removal of the solvent gave 11 mg (88% overall yield): tlc (CM) R_f 0.68, tlc (CH) 0.15.

Amino acid analysis of an acid hydrolysate gave $\underset{0.94}{\text{Val}}\ \underset{1.00}{\text{Gly}}\ \underset{0.90}{\text{Phe}}$. The dansyl technique was carried out on a 1.21 μmol sample and an estimated 1.5% of the theoretical amount of valine was detected. The method for removal of the trifluoroacetyl group with $NaBH_4$ (Weygand & Frauendorfer, 1970) was performed on a 0.60 mg sample. The sample was suspended in 100 μl of ethanol and 5 μl of 2 N NaOH. After 5 μl aliquots were spotted for tlc controls, the solution was divided into two: one was a control and to the other 1.0 mg $NaBH_4$ was added. At the end of 1 h at 24°, an aliquot of each solution was spotted

for tlc. By tlc (CM) and (CH), the ninhydrin-positive free tripeptide (R_f 0.38 and origin, respectively) was the only material in both solutions which sat for 1 h, and the sample spotted prior to division of the solution had the free and CF_3CO-tripeptide (chlorine-positive) in about a 1:1 ratio. Another sample of CF_3CO-Val-Phe-Gly (0.11 mg) was dissolved in 0.5 N NaOH (100 µl). By tlc (CM), 30 min at 24° was sufficient to remove the trifluoroacetyl group and give the free tripeptide, but after 24 h at 24°, there was almost complete hydrolysis resulting in the three amino acids.

Synthesis of Trp(Nps)-Ala-Phe Resin (I). Boc-Phe prepared by the modified Loffet method gave 0.52 mmol/g by the picric acid method. The subsequent incorporation of Boc-Ala and Boc-2-(2-nitrophenylsulfenyl)tryptophan [Boc-Trp(Nps)] (Yamashiro et al., 1976) on Boc-Phe resin (2.0 g) was performed by the same schedule as above with the exception that 2 equiv each of Boc-amino acid and DCCI were used. A final cycle of synthesis through step 8 of Table 7 gave the final deprotected and neutralized tripeptide resin (I), 2.511 g.

Preparation of CF_3CO-Trp(Nps)-Ala-Phe Resin. The tripeptide resin (501 mg) was washed with 10-ml portions of CH_2Cl_2 (6 times), treated with 10 ml of TFA anhydride-CH_2Cl_2 (1:99) for 5 min then diluted with 5 ml of CH_2Cl_2 and allowed to

react 10 min, washed with 10-ml portions of CH_2Cl_2 (6 times) and 10-ml portions of ethanol (3 times).

Isolation of Trp(Nps)-Ala-Phe (II) and CF_3CO-Trp(Nps)-Ala-Phe (III). A portion of I (252 mg) and all of the CF_3CO-Trp(Nps)-Ala-Phe resin (503 mg) were treated separately with 5 ml of HF at 0° for 15 min in the presence of anisole (0.5 ml). The HF was removed and the peptide-resin mixtures were worked up by the procedures described above for CF_3CO-Val-Phe-Gly. The peptides were isolated by lyophilization: 99 mg of II and 171 mg of III. II ran with R_f values of 0.68 and 0.55 on tlc in BAW and CM, respectively, and with R_{Lys} 0.31 on paper electrophoresis (pH 2.1, 2 kV, 1 h). III ran with R_f values of 0.82, 0.70 and 0.35 on tlc in BAW, CM, and CH, respectively, and stayed at the origin on paper electrophoresis (pH 2.1, 2 kV, 1 h). Amino acid analyses of acid hydrolysates gave for II, $Ala_{0.99}Phe_{1.0}$, and for III, $Ala_{0.94}Phe_{1.0}$. The Trp(Nps) content on a weight basis was obtained from absorbance at 365 nm and gave for II, $Trp(Nps)_{1.02}$, and for III, $Trp(Nps)_{1.08}$.

Synthesis of Lys-Trp(Nps)-Ala-Phe (IV). A portion of I (252 mg, 0.10 mmol theoretically) was stirred for 1 h with 1.0 ml of 0.20 M Boc-Lys(Z) in CH_2Cl_2 (2 equiv), 0.66 ml of CH_2Cl_2 and 0.95 ml of 0.2 M DCCI in CH_2Cl_2 and filtered. After washing with 20-ml portions of CH_2Cl_2, DIEA-CH_2Cl_2 (1:19), CH_2Cl_2 and ethanol, the dried material (280 mg) was

treated with HF in the presence of anisole and worked up as described above for II and III: 125 mg; tlc (BAW), R_f 0.45; paper electrophoresis (pH 2.1, 2 kV, 1 h), R_{Lys} 0.51. Amino acid analysis of an acid hydrolysate gave $Lys_{0.84}Ala_{0.95}Phe_{1.0}$. The Trp(Nps) content on a weight basis was obtained from absorbance at 365 nm and gave $Trp(Nps)_{0.85}$.

Separation of II, III and IV. Preliminary studies which were performed to quantitate the separation of mixtures of II, III and IV are briefly described below. Paper electrophoresis (pH 2.1, 2 kV, 30 min) of III and IV and the extraction of the cut-out yellow spots with acetic acid heated in boiling water for 5 min, resulted in incomplete recovery of III and IV but in the correct ratio by absorbance at 365 nm [Trp(Nps), ε_{365}=4,000]. The recovery of II, III and IV from tlc (BAW) by the extraction of the silica gel with acetic acid was unsatisfactory. Gel filtration of a mixture of II, III and IV on Sephadex G-10 (1.37 x 40.5 cm) in 0.05 N NH_4OH resulted in a single symmetrical peak (elution volume, V_e, of peak, 151 ml). Gel filtration of a mixture of II, III and IV on the same column in dioxane-acetic acid-water (1.5:1:3) resulted in a broad peak which analysed by the tlc (BAW) showed IV between V_e of 29 and 36 ml, II between V_e of 34 and 40 ml and III between V_e of 36 and 42 ml. Again, gel filtration of a mixture of II, III and IV

on the same Sephadex G-10 column in 20% aqueous acetic acid (Figure 11) resulted in a clean separation of IV (V_e of peak, 36 ml) and II (V_e of peak, 110 ml). However, elution of III was effected only after 222 ml of the 20% aqueous acetic acid and 58 ml of a 50% aqueous acetic acid solution had passed through the column. Overlapping peaks, which had V_e of peak for IV of 21 ml, for II of 21 ml and for III of 51 ml, were obtained from gel filtration of the same Sephadex G-10 column in 50% aqueous acetic acid. Partition chromatography on the same Sephadex G-10 column in ethyl acetate-0.05 N NH_4OH (1:1) resulted in III having an R_f value of 0.71, but II and IV were obtained in a single peak by a pyridine-0.5 M acetic acid (2:5) wash. On partition chromatography on Sephadex G-10 (0.96 x 21.5 cm) in 1-butanol-acetic acid-water (4:1:5), II and III eluted together in a peak with R_f 0.75 and IV eluted in a peak with R_f 0.43. After 40 transfers in a 4 ml/tube countercurrent distribution (CCD) apparatus (H.O. Post Scientific Instrument Co., Maspeth, N.Y.) in ethyl acetate-0.05 N NH_4OH, III had a K of 3.4, and II and IV had a K of 0.08. Separation of II, III and IV was achieved in a single chromatography on Sephadex G-10 (1.37 x 32.2 cm) in 33% aqueous acetic acid (Figure 12) with only a slight overlap of IV (V_e of peak, 23 ml) and II (V_e of peak, 46 ml), while III had a V_e of the peak of 164 ml. The amount of each peptide in the mixture was calculated from the total absorbance at 365 nm in each

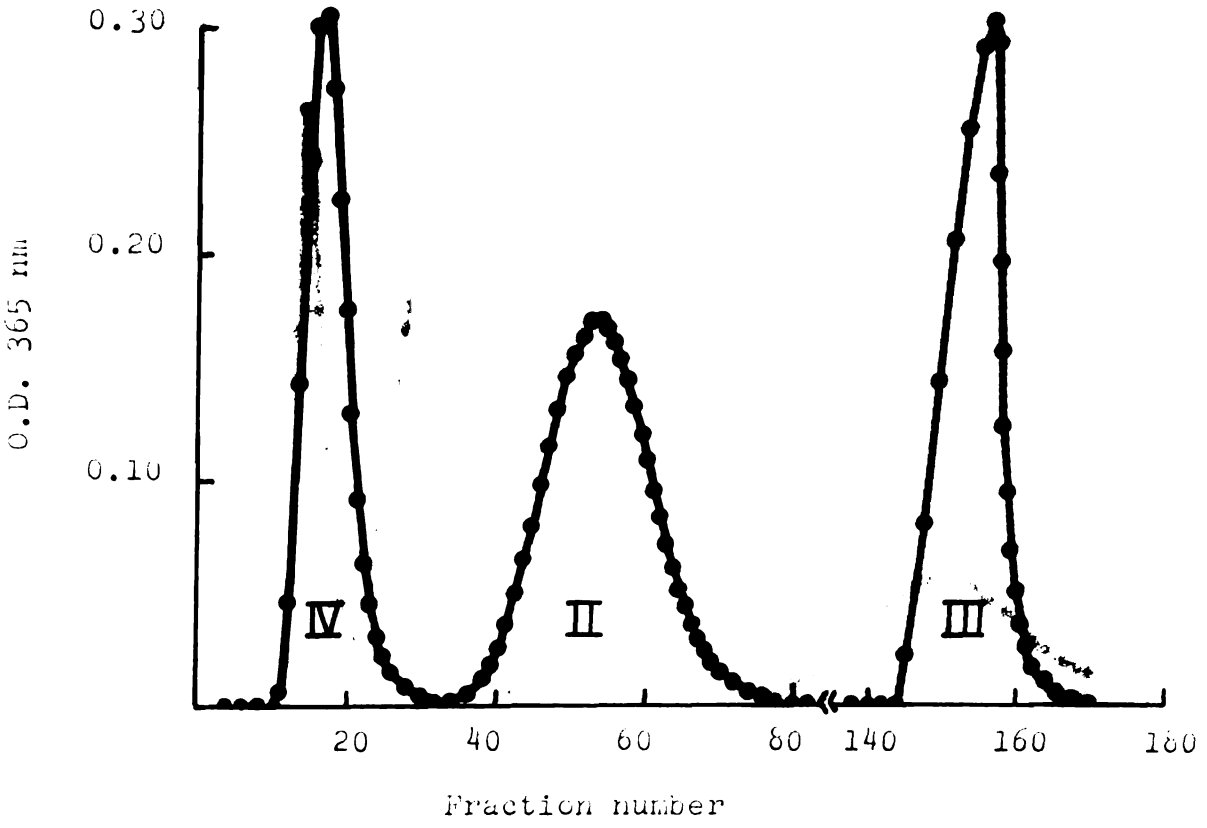
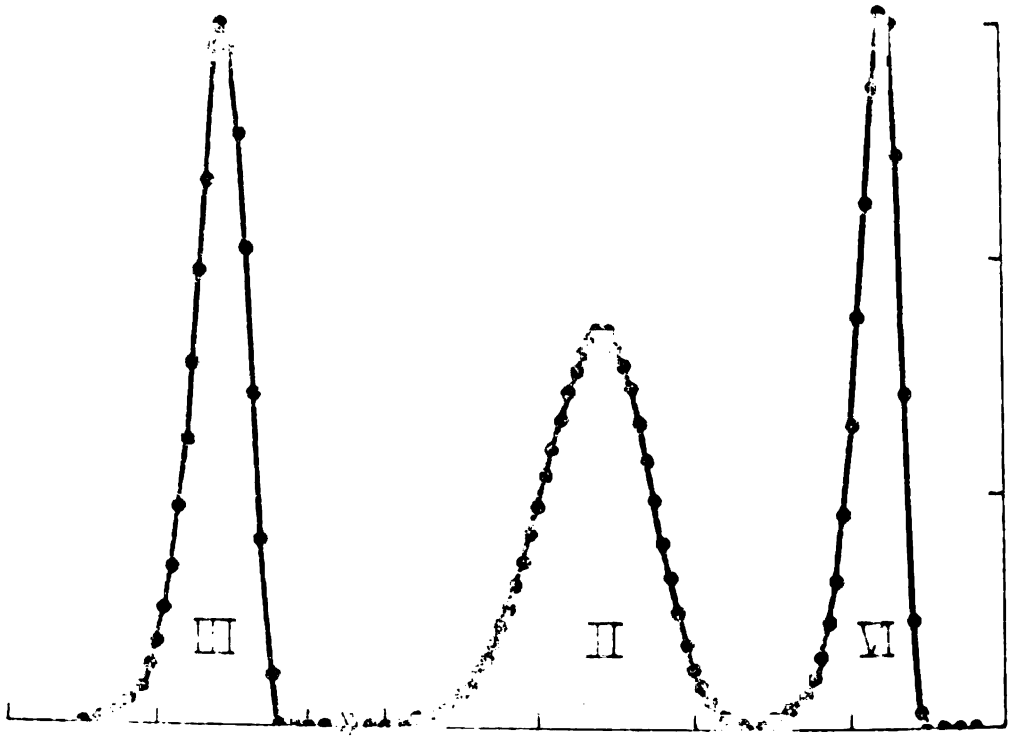


Figure 11. Chromatography on Sephadex G-10 (1.37 x 32.2 cm) of a mixture II, III and IV with 222 ml of 20% aqueous acetic acid (1.68 ml/fraction), then with 50% aqueous acetic acid (1.50 ml/fraction): elution volume, V_e, of 30 ml for IV, V_e of 96 ml for II and V_e of 272 ml for III.


III
II
VI

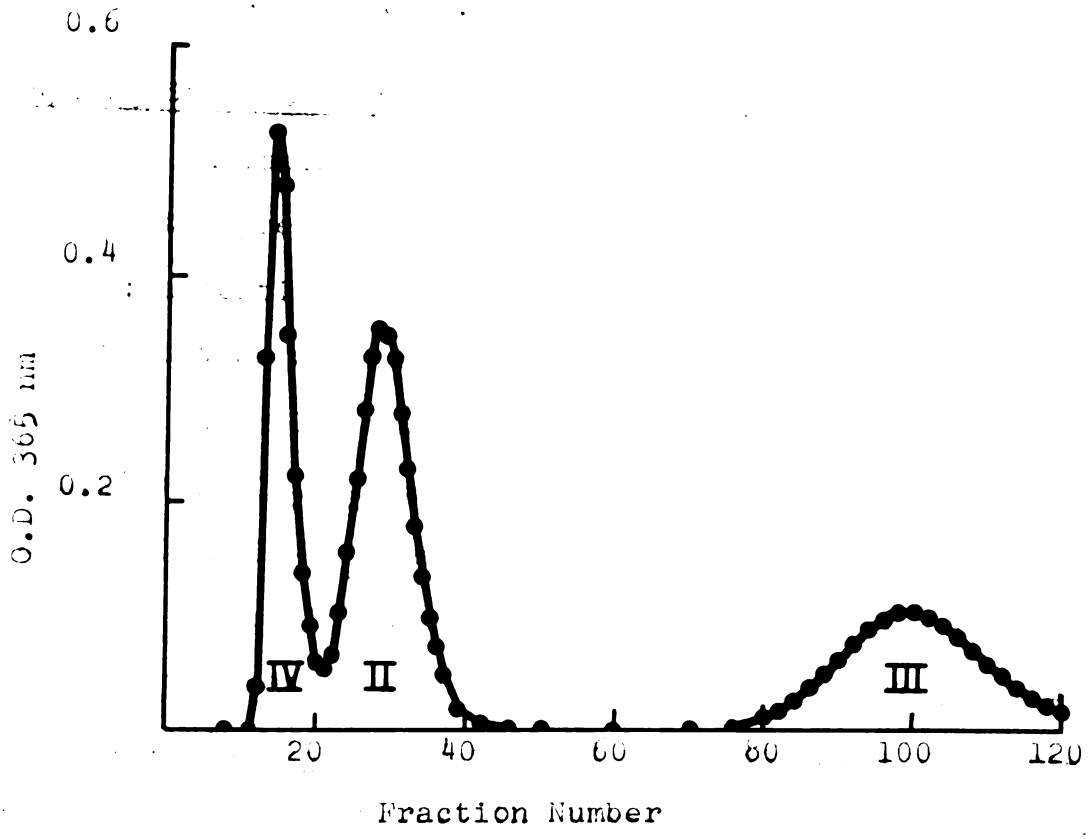
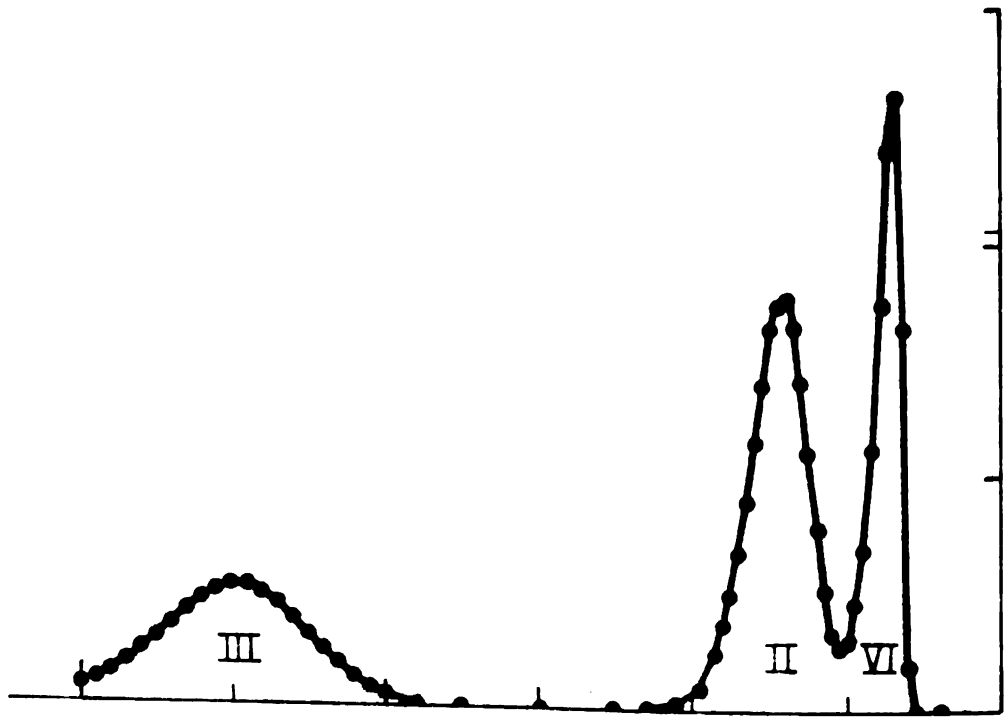


Figure 12. Chromatography on Sephadex G-10 (1.37 x 32.2 cm) of a mixture of II, III and IV with 33% aqueous acetic acid (1.64 ml/fraction): V_e of 23 ml for IV, V_e of 46 ml for II and V_e of 164 ml for III.


III
II
VI

peak and gave for IV a value of 28.0% of the total (31.2% theoretical),for II a value of 41.0% (36.6%), and for III a value of 31.0% (32.2%).

Coupling Studies in the Presence of TFA. The results are presented in Table 10. The in situ coupling (Merrifield, 1964) in the presence of TFA was typically performed as follows: a portion (65 mg, 27 μmol) of I was placed in a small test tube with a small magnetic stir bar, 54 μl of 0.5 M TFA in CH_2Cl_2 was added, then 0.538 ml of 0.2 M Boc-Lys(Z) in CH_2Cl_2 was added. After 5 min, 0.538 ml of 0.2 M DCCI in CH_2Cl_2 was added (95 mM coupling concentration) and the resin suspension stirred for 1 h and filtered. The resin was washed with 20-ml portions of CH_2Cl_2, DIEA-CH_2Cl_2 (1:19), CH_2Cl_2 and ethanol and then dried under reduced pressure over P_2O_5. The pre-mix coupling (Hagenmaier & Frank, 1972) was done in the same manner except that following the TFA addition an aliquot of the pre-mix solution was added and then stirred for 1 h. The pre-mix solution was prepared for a 52-mg (21.5 μmol) sample of I as follows: to 1.2 ml of 0.2 M Boc-Lys(Z) in CH_2Cl_2 in a test tube in an ice-bath and magnetically stirred was added 0.6 ml of 0.2 M DCCI in CH_2Cl_2; after 10 min, the solution was filtered through a glass wool plug in a Pasteur pipette; and a 0.645 ml (43 μmol of Boc-Lys(Z) symmetrical anhydride) aliquot taken and added to I.

All the peptide resins from the coupling studies were treated separately with 5 ml of HF for 15 min at 0° in the presence of anisole (0.5 ml). After removal of HF and drying, the peptide-resin mixture was stirred with 10 ml of TFA for 15 min and filtered. The filtrate was evaporated and twice more evaporated from acetic acid (5 ml).

The amounts of II, III and IV in each sample from the coupling study were determined by first the separation of II and IV from III by 40 transfers in a CCD machine in ethyl acetate-0.05 N NH_4OH (1:1). The contents from each tube (4 ml) were removed and made one phase by addition of 2 ml of acetic acid and the absorbance at 365 nm read. This gave the ratio of III to II plus IV. Then the fractions which contained II and IV were pooled and evaporated under reduced pressure, and the material subjected to chromatography on Sephadex G-10 in 20% aqueous acetic acid and the ratio of the total absorbance at 365 nm in the two peaks gave the ratio of II and IV in the sample. Thus, the ratios of II to III to IV in the original sample could be obtained from the CCD and G-10 derived ratios.

Removal of the Trifluoroacetyl Group from III. Two aliquots of a solution of III in acetic acid were taken so that 150 μg samples of III were obtained. These aliquots were dried overnight *in vacuo* over P_2O_5. To one sample, 100 μl of 1 N

aqueous piperidine (Goldberger & Anfinsen, 1962) was added and to the second sample 100 µl of 0.15 M K_2CO_3, pH 10.7 (Fanger & Harbury, 1965) was added. After 18 h at 24°, 10 µl of each was spotted for tlc (CH). Both showed complete loss of III (R_f 0.80) and appearance of II (R_f 0.68), and only the piperidine-treated sample showed another chlorine-positive spot, R_f 0.30-0.50, which was probably piperidine.

Preparation of N^{α}-CF_3CO-Lysine. N^{ϵ}-Benzyloxycarbonyllysine was obtained from Dr. J. Blake and a portion (2.8 g, 10 mmol) was dissolved in 25 ml of TFA and cooled to 4°. With stirring, 2.13 ml (15 mmol) of TFA-anhydride was added and the solution allowed to reach room temperature over 20 min. After removal of the solvent, the material was dissolved in ethyl acetate (30 ml) and washed with three 15-ml portions of 0.1 N HCl and then with ten 10-ml portions of water. After the ethyl acetate solution was dried over $MgSO_4$, the drying agent and solvent were removed to yield an oil (2.96 g). This oil was dissolved in ethyl acetate and 1.57 ml, 5.02 mmol (a mistake, should have been 2.46 ml, 7.88 mmol) of dicyclohexylamine (DCA) was added. The solution was evaporated to an oil which was dissolved in ether. From the ether, material crystallized and was collected by filtration: 2.12 g (40.8% yield), mp 129.5-131°.

The above material (1.12 g, 2.16 mmol) was suspended in 15 ml of ethyl acetate, cooled to 4° and stirred. After

addition of 2.0 ml of 1 N H_2SO_4, a solution resulted within 20 min at 4° with stirring. The organic phase was washed with two 10-ml portions of water, 10 ml of 0.1 N H_2SO_4, five 10-ml portions of water and dried over $MgSO_4$. Removal of the drying agent and solvent gave an oil (0.832 g). This oil was dissolved in 25 ml of methanol, purged with nitrogen, ca. 200 mg of freshly prepared pallidium catalyst (see above) was added. Then hydrogen was bubbled through the solution for 1 h followed by a nitrogen purge; tlc (BAW) R_f ca. 0.5, tlc (CH) origin. The catalyst and solvent were removed to yield an oil (497 mg) which crystallized at 24°. The material was collected with the aid of ether to yield of N^{α}-CF_3CO-lysine: 436 mg (99% yield from DCA salt), mp 213-214°.

Anal. Calcd for $C_8H_{13}N_2O_3F_3$(242.20): C, 39.7; H, 5.4; N, 11.6. Found: C, 39.8; H, 5.4; N, 11.5.

By quantitative amino acid analysis (Spackman et al., 1958), CF_3CO-lysine which appeared in the position of Leu was found to have a color constant of 17.7% of a methionine standard. With only the pH 3.25 buffer, Met appeared at 141 min and CF_3CO-lysine at 198 min. From the constant value above, the color constant of CF_3CO-lysine was then calculated to be 16.0% of that of Lys. Then, when submitted to the conditions of enzyme digestion [LAP buffer, pH 8.5 (Chapter 3), at 37° for 26 h] and amino acid analysis, the recovery of CF_3CO-lysine was 93.6% of theoretical. However, when the sample

after 26 h at 37° in the LAP buffer pH 8.5 was treated for 1 h at pH 12 by addition of NaOH to the buffer, and then submitted to amino acid analysis, no CF_3CO-lysine was detected.

Preparation of CF_3CO-Lys_5-Glu_3-Leu_2-Trp(Nps)-Phe. The [Lys-(2-BrZ)]$_5$-[Glu(OBzl)]$_3$-Leu_2-Trp(Nps)-Phe resin (Yamashiro et al., 1976) was synthesized by Mr. K. Hoey. A sample (274 mg) was stirred with a 2-ml solution of 2.5% trifluoroacetic-anhydride in CH_2Cl_2 (13.9-fold molar excess of the anhydride to the peptide-resin amine content) for 15 min and filtered with washes of CH_2Cl_2, DIEA -CH_2Cl_2 (1:19), CH_2Cl_2 and ethanol. After the peptide resin was dried, it was treated with HF (5 ml) for 1 h at 0° in the presence of anisole (0.3 ml). After the HF was removed by nitrogen, the peptide-resin mixture was dried and then stirred with 10 ml of ethyl acetate for 10 min, filtered, washed with ethyl acetate, and dried. The peptide was extracted with a total of 2.5 ml of 0.5 M acetic acid and subjected to gel filtration on Sephadex G-10 (2.16 x 26 cm) in 0.5 M acetic acid. The peptide material which was detected in the single peak (V_e, 32-48 ml) was isolated by lyophilization and gave 66.1 mg. A sample (31.6 mg) was submitted to chromatography on CMC (Figure 13) with a gradient employing the starting buffer, 0.10 M and 0.4 M ammonium acetate, pH 6.7 (see

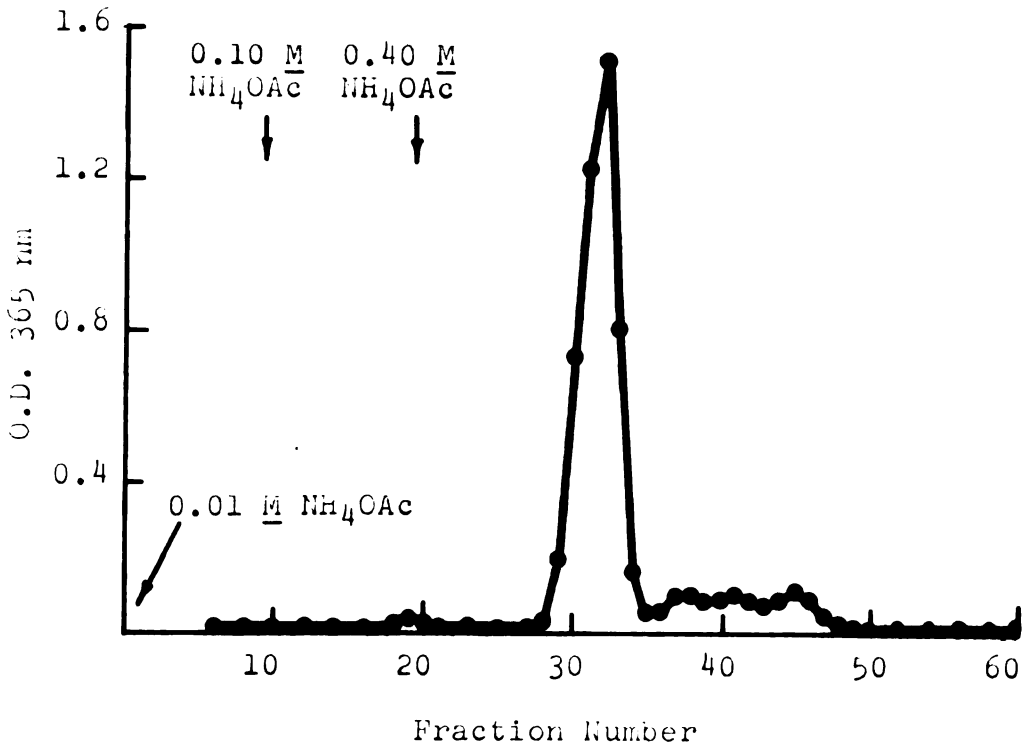
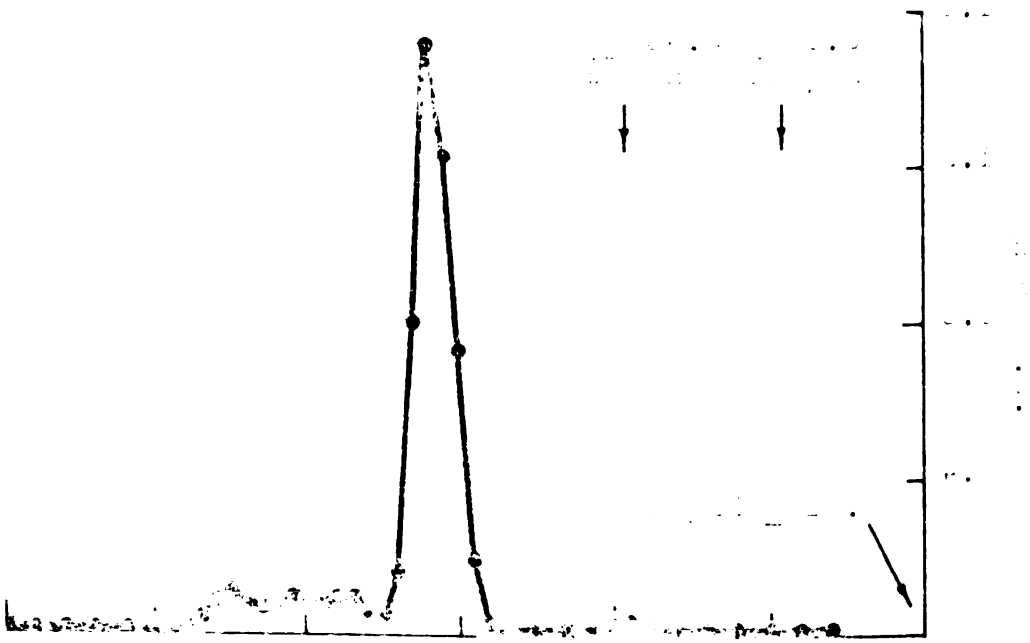


Figure 13. Carboxymethylcellulose chromatography (1.23 x 44.5 cm) by gradient elution (10 ml/fraction in 1-18, 5 ml/fraction in 19-60): 31.6 mg from gel filtration on Sephadex G-10, yield (fractions 29-34), 21.5 mg.



Chapter 3) and 10-ml fractions through fraction 18 and 5-ml fractions through fraction 60. The major peptide (V_e of peak, 245 ml) was detected spectrophotometrically at 365 nm and isolation by repeated lyophilizations gave 21.5 mg (50% overall yield based on starting Boc-Phe resin).

Tryptic digestion with an enzyme to peptide weight ratio of 1:25 of 1.60 mg samples for 21 and 54 h at 37° gave on amino acid analysis 58.5% and 79.1% recovery of CF_3CO-lysine, respectively. Tryptic digestion with a ratio of 1:1 for 26 h gave 87.2% recovery of CF_3CO-lysine. The control consisted of making one-half of the digestion solution pH 12 by addition of NaOH and after 1 h at 24° performing the amino acid analysis which showed the complete disappearance of CF_3CO-lysine.

Synthesis of Lys_6-HGH-(1-54). A sample (99.6 mg, 10.9 μmol theoretically) of protected HGH-(1-54) resin (prepared by the pre-mix coupling method and described in Chapter 7) was subjected to the schedule of synthesis shown in Table 11 for the six in situ couplings of Boc-Lys(2-BrZ). The steps used 1-ml volumes except step 11, which employed 0.87 ml of 0.05 M Boc-Lys(2-BrZ) in CH_2Cl_2 and 0.2 ml of CH_2Cl_2, and step 12, which used 0.6 ml of 0.072 M DCCI in CH_2Cl_2. The dried final protected peptide resin gave 121.2 mg.

The HF treatment and ethyl acetate washes were performed as described above for CF_3CO-Lys_5-Glu_3-Leu_2-Trp(Nps)-Phe. Extraction was similarly performed with

33% aqueous acetic acid. Gel filtration of the extract was performed on Sephadex G-10 (1.37 x 43 cm) in 33% aqueous acetic acid and resulted in a single peak (V_e, 19-27 ml). Isolation of the peptide material gave 60.5 mg.

A sample (50.8 mg) was submitted to digestion with trypsin at an enzyme to substrate ratio (w/w) of 1:81 for 24 h at 37° followed by addition of trypsin [1:81 (w/w)] and another 24 h at 37°. After lyophilization, the mixture was dissolved in 1.30 ml of 1 N HCl and 3.70 ml of 0.01 N HCl, and the solution divided into two 2.5-ml portions and lyophilized. One portion was submitted to amino acid analysis. The second portion was dissolved in 1 ml of 0.01 N NaOH and 0.20 ml of 2 N NaOH, allowed to sit 1 h at pH 12 and 24°, made pH 2 with addition of 3 N HCl, lyophilized and submitted to amino acid analyses. The two analyses were practically identical in that the high background masked the small differences expected for the presence of CF_3CO-lysine.

Results and Discussion

Trifluoroacetyl groups have been removed from N^{α} and N^{ϵ}- amino groups of peptides and proteins by treatment with aqueous solutions of 1 M piperidine (Goldberger & Anfinsen, 1962). This method also has been used as indirect evidence of trifluoroacetylated peptides. From just such evidence, it was concluded that the synthesis of acetyl-

Tyr-$(Pro)_{10}$-Tyr resulted in the formation of a series of CF_3CO-$(Pro)_n$-Tyr peptides to the extent of 11% (w/w) of acetyl-Tyr-$(Pro)_{10}$-Tyr (Bush et al., 1972). It has been suggested that, because of the higher basicity of the imino group of proline, the usual washings with triethylamine were not sufficient to remove all the trifluoroacetic acid (Ondetti et al., 1971). Trifluoroacetylation has been shown to occur in solution peptide synthesis if trifluoroacetic acid or the anion is present during the DCCI-mediated coupling (Fletcher et al., 1973). Thus it was of interest to determine if the above termination observed in solid-phase synthesis was unique to proline or if trifluoroacetic acid could compete during coupling in solid-phase synthesis under standard conditions.

First it had to be shown that a trifluoroacetylated peptide was stable to cleavage from the resin and could be isolated in a highly purified form. Synthesis of Val-Phe-Gly resin was performed by standard techniques. Following trifluoroacetylation, the trifluoroacetylated peptide resin was shown to be quite stable to attempts to remove the trifluoroacetyl group. The peptide was cleaved from the resin and the resulting material contained only a trace of ninhydrin-positive material. The final product, CF_3CO-Val-Phe-Gly, was isolated in highly purified form in good yield. The trifluoroacetyl group had the expected stability in solution

experiments on the CF_3CO-peptide. Therefore, peptides terminated by trifluoroacetylation during synthesis could be quantitated by isolation in solution by the techniques normally employed without concern for loss or conversion of the CF_3CO-peptides. For this reason, the coupling studies below could be undertaken.

The coupling studies were performed on the tripeptide resin, Trp(Nps)-Ala-Phe resin (I), and entailed the incorporation of Boc-Lys(Z) by various coupling conditions in the presence of an amount of trifluoroacetic acid equivalent to the amino group of the peptide resin. Thus, three peptides could be expected: Trp(Nps)-Ala-Phe (II); CF_3CO-Trp(Nps)-Ala-Phe (III); and Lys-Trp(Nps)-Ala-Phe (IV). The authentic samples of II, III and IV were prepared as described in the Experimental Section. As a convenient one-step separation of a mixture of the three peptides could not be found, countercurrent distribution was used to separate III from II and IV, and then II and IV were separated by gel filtration on Sephadex G-10. The Trp(Nps) moiety afforded an accurate measure of the amounts of II, III and IV.

As outlined above, the coupling study involved the coupling of Boc-Lys(Z) to I in the presence of an amount of trifluoroacetic acid equivalent to the amine content. The standard (<u>in situ</u>) couplings (Merrifield, 1964) were

performed by varying the ratio of Boc-Lys(Z) to DCCI used. The preformed symmetrical anhydride (pre-mix) coupling method (Hagenmaier & Frank, 1972) was carried out twice with different ratios of Boc-Lys(Z) to DCCI used. The results in terms of the amounts of the three peptides which were obtained in each coupling test are shown in Table 10.

The in situ coupling with 2 equivalents of both Boc-Lys(Z) and DCCI was assumed to be the minimum conditions which could probably drive the coupling reaction to completion. Obviously it was not sufficient, but none the less it resulted in trifluoroacetylation. The next in situ coupling investigated, 4 equivalents of Boc-amino acid and 2 equivalents of DCCI, was based on the "incremental DCC addition" (Sharp et al., 1973) coupling conditions which have been used to promote in situ symmetrical anhydride formation. Both significant trifluoroacetylation and incomplete coupling resulted and were similar to the amounts seen in the previous coupling condition. The most frequently used in situ coupling conditions, 4 equivalents each of Boc-amino acid and DCCI, would have indicated complete coupling by all the methods used to monitor the reaction. However, the result was actually a mixture of the desired product and a trifluoroacetylated peptide in a 4.5 to 1 ratio. The pre-mix coupling which employed 4 equivalents of Boc-Lys(Z) and 2-equivalents of DCCI, resulted in a 6-fold reduction

of the trifluoroacetylation obtained by the comparable *in situ* conditions and probably reflected the coupling of a mixed anhydride arising from the symmetrical anhydride and trifluoroacetic acid. The incompleteness of coupling was probably due to the slow coupling of the anhydrides to the protonated amino group. An experiment which probably should have been done was a *pre-mix* coupling in the presence of a trifluoroacetic acid salt (Fletcher et al., 1973). The lower amount of trifluoroacetylation in the *pre-mix* coupling with 5 equivalents of Boc-Lys(Z) and 2 equivalents of DCCI seemed to indicate the competition between the Boc-Lys(Z) present and trifluoroacetic acid for the symmetrical anhydride. Overall these preliminary studies demonstrated that extensive chain termination can occur during the standard coupling method in the presence of trifluoroacetic acid but not during the preformed symmetrical anhydride coupling method.

As a result of these experiments, it was decided to determine if chain termination by trifluoroacetylation can occur in synthesis under standard conditions where obviously trifluoroacetic acid is not added during the coupling reaction.

The synthesis of a nonadecapeptide fragment corresponding to residues 37-55 of ovine prolactin, Phe-Asn-Glu-Phe-Asp-Lys-Arg-Tyr-Ala-Gln-Gly-Lys-Gly-Phe-Ile-Thr-Met-Ala-Leu, was performed three separate times. The syntheses are discussed in detail in Chapter 6. All three syntheses were performed with

the use of the Boc group for N^{α} protection and TFA-CH_2Cl_2 (1:1) for deprotection. In the first synthesis, the triethylamine-salt procedure (Merrifield, 1964) was used for attachment to the resin and the couplings were carried out by the standard in situ method. As shown in Table 12, there was a dramatic loss of viable peptide chains (88% of theoretical) as indicated by amine determination. The other evidence of chain termination was the very low value for the diagnostic amino acid ratio of Asp to Leu determined by resin hydrolysis of the final peptide resin and amino acid analysis. The ratio of Asp, from the two residues in the amino-terminal portion, to Leu, the carboxyl-terminal residue, was only 0.61:1 instead of the theoretical 2:1. When the incorporation of quaternary ammonium sites on the resin (Rudinger & Gut, 1967; Beyerman et al., 1967) was avoided by the modified Loffet method of attachment, the synthesis with in situ couplings gave better values for the surviving amine content and the Asp to Leu ratio. The third synthesis employed the modified Loffet attachment and pre-mix couplings. There was a quite significant reduction of chain termination indicated by the surviving amine content and the Asp to Leu ratio. Since the terminated peptides were not characterized, it could be suggested that the results reflect only more complete coupling reactions. This is probably not so because of the differences in the surviving

amine content between the three syntheses. Therefore, chain termination was probably reduced because of the elimination of quaternary ammonium sites on the resin which can retain trifluoroacetic acid, and the use of pre-mix couplings which have been shown above to diminish the trifluoroacetylation that occurs in coupling in the presence of trifluoroacetic acid.

These experiments resulted in an examination of the products which were obtained from two syntheses of the same sequence, the fifty-four residue peptide fragment of HGH corresponding to residues 1-54, by the two DCCI-mediated coupling methods. The syntheses are described in detail in Chapter 7. The first indication of the extent of chain termination was the determination of the surviving amine content at the end of each synthesis by the Schiff base method (Esko et al., 1968). The synthesis which employed the in situ coupling method had a surviving amine content of 39% of theoretical, while the synthesis which utilized the pre-mix coupling method gave a value of 68% of theoretical amine content. After the protecting groups and resin were removed from the peptides of each synthesis, one of the purification steps was the separation of the lower molecular weight peptides from the desired molecular weight range peptides. This was accomplished by gel filtration on Sephadex G-25 in 0.5 M acetic acid. The weight ratios of the amounts of the lower to desired molecular weight peptides isolated from this gel

filtration were 1:2 for the in situ synthesis and 1:5.3 for the pre-mix synthesis. When the fluorine content of the low molecular weight peptides from each synthesis was determined by elemental analysis, it indicated that 80% of the shorter peptides from the in situ synthesis were trifluoroacetylated compared to 46% for the pre-mix synthesis. Following a treatment with 0.15 M K_2CO_3 which removes the trifluoroacetyl group (Fanger & Harbury, 1965), the appearance of new amino-terminal residues and an increase in the observed residues of controls were detected by the dansyl technique for the in situ short peptides and to a lesser extent for the pre-mix short peptides. Thus, for equal amounts of crude peptide material, the pre-mix coupling method evidently resulted in greater than a 5-fold reduction in the amount of trifluoroacetylated peptides.

Although the above studies on the HGH and prolactin fragments indirectly showed chain termination by trifluoroacetylation, isolation and identification of the authentic CF_3CO-peptides were lacking. It was decided to synthesize hexalysine on the above HGH peptide fragment resin. Since the coupling studies showed trifluoroacetylation can occur during the incorporation of a Lys residue, repeated incorporation of Lys residues could result in peptides with CF_3CO-Lys at the amino terminal. If tryptic digestion can cleave the CF_3CO-Lys residue, it could be quantitated by amino acid

analysis. Thus the scheme was as follows:

$$CF_3CO\text{-Lys-peptide} \xrightarrow{\text{Trypsin}} (CF_3CO\text{-Lys-OH} + \text{fragments})$$

$$\xrightarrow[\text{analysis}]{\text{amino acid}} CF_3CO\text{-Lys-OH}$$

To determine if this scheme was feasible, first CF_3CO-lysine was synthesized and characterized as described in the Experimental Section. Then, the model peptide, $CF_3CO\text{-Lys}_5\text{-Glu}_3\text{-Leu}_2\text{-Trp(Nps)-Phe}$, was obtained from $\text{Lys}_5\text{-Glu}_3\text{-Leu}_2\text{-Trp(Nps)-Phe}$ resin (Yamashiro et al., 1976) as described in the Experimental Section. A 54-h tryptic digestion of the model peptide with an enzyme to substrate ratio of 1:25 (w/w) gave a 79.1% recovery of CF_3CO-lysine by amino acid analysis. A 87.2% recovery of CF_3CO-lysine resulted from amino acid analysis of a 26-h tryptic digestion of the model peptide with a 1:1 (w/w) ratio of enzyme to substrate. This showed that an amino-terminal CF_3CO-Lys residue can be cleaved enzymatically and determined on an amino acid analyzer.

Therefore, to determine if chain termination by trifluoroacetylation can occur during the synthesis of a relatively large peptide, the fifty-four residue peptide resin synthesized by the pre-mix method (see Chapter 7) was subjected to six synthetic cycles for the incorporation of Boc-Lys(Z) by the in situ method . In this manner, the

average trifluoroacetylation in each coupling could be determined and would be represented by one-fifth of the amount of CF_3CO-lysine obtained. The resulting Lys_6-HGH-(1-54) resin was treated with liquid HF in the presence of anisole to remove the protecting groups and the resin. The peptide material was submitted to gel filtration on Sephadex G-10, and then to tryptic digestion. Unfortunately, when the total digestion mixture was submitted to amino acid analysis, the background was too great to permit an estimation of any CF_3CO-lysine that might have been present.

In summary the stability of the trifluoroacetyl group on a peptide resin and peptides in solution has been examined. From peptides in solution, trace amounts of the trifluoroacetyl group were removed by the conditions of the dansyl technique, but complete removal required either quite basic pH conditions or a sodium borohydride treatment. Coupling studies on the solid-phase in the presence of trifluoroacetic acid showed the standard *in situ* coupling method caused about a 6-fold greater chain termination by trifluoroacetylation than resulted from the preformed symmetrical anhydride coupling method. Indirect evidence showed that chain termination by trifluoroacetylation occurred during the syntheses of a nonadecapeptide and a 54-residue peptide even by the best extant techniques. When the *in situ* coupling method was replaced by the newly introduced preformed symmetrical

anhydride coupling method, chain termination by trifluoro-acetylation was significantly diminished, but not completely eliminated. A CF_3CO-model peptide was investigated to directly identify the trifluoroacetylated product; however, an attempt to apply this model to a large peptide resin resulted in inconclusive results.

Table 10

Coupling Studies in the Presence of TFA

Equivalents to 1 Equiv of amine-resin				% in Final Peptide Mixture[b]		
Boc-Lys(Z)	DCCI	TFA	Coupling(1 h[a])	IV	III	II
2	2	1	in situ	80[c]	9.4[d]	10[c]
4	2	1	in situ	75.7	11.1	13.2
4	4	1	in situ	81.7	18.3	0
4	2	1	pre-mix	76.7	1.7	21.6
5	2	1	pre-mix	60.0	<1.0	39.0

[a] Performed as described in text.

[b] Determined by separations described in text.

[c] Estimated by separation on tlc (BAW).

[d] Determined by CCD separation from II and IV.

Table 11

Schedule for Synthesis of

N^{α}-Boc-Lys(2-BrZ)$_6$-HGH-(1-54)-Resin

Step	Reagent and Operation	Time (min)
1	CH_2Cl_2 (2 times)	2
2	TFA-CH_2Cl_2, 1.08:1 (1 time)	1
3	TFA-CH_2Cl_2, 1.08:1 (1 time)	15
4	CH_2Cl_2 (3 times)	2
5	Dioxane-CH_2Cl_2, 1:2 (3 times)	2
6	CH_2Cl_2 (3 times)	2
7	DIEA-CH_2Cl_2, 1:19 (1 times)	5
8	CH_2Cl_2 (2 times)	2
9	DIEA-CH_2Cl_2, 1:19 (1 time)	5
10	CH_2Cl_2 (6 times)	2
11	Boc-Lys(2-BrZ) (4 equiv), (1 time)	5
12	DCCI (4 equiv), (1 time)	30
13	CH_2Cl_2 (3 times)	2
14	Ethanol-CH_2Cl_2, 1:2 (3 times)	2

Table 12

Properties of Nonadecapeptide Resins[a]

Order of Synthesis	Method of Attachment[b]	Coupling Method	Surviving Amine Content[c]	Asp:Leu of Final Peptide Resin[d]
First	TEA-salt	in situ	12%	0.61:1
Second	Modified Loffet	in situ	46%	1.2:1
Third	Modified Loffet	pre-mix	79%	1.8:1

[a] Nonadecapeptide corresponding to residues 37-55 of ovine prolactin.

[b] TEA-salt procedure, Merrifield, 1964; modified Loffet procedure, Yamashiro & Li, 1973b.

[c] Measured by the picric acid method.

[d] Determined by amino acid analysis of resin hydrolysate.

CHAPTER 6

DETECTION AND ISOLATION OF THE SULFONIUM FORM OF METHIONINE-CONTAINING PEPTIDES

Introduction

Ovine prolactin (LTH) is a protein consisting of 198 amino acids in a single polypeptide chain with three disulfide bridges, and its complete amino acid sequence has been elucidated (Li et al., 1970). A comparison of the primary structures of HGH and LTH indicated a total homology of about 60% between the two protein hormones (Bewley & Li, 1975). Immunochemical studies have shown that LTH is a potent antigen (Trenkle et al., 1963; Clarke & Li, 1974). During the course of investigating the immunoreactivity of various peptide fragments obtained from cyanogen bromide cleavage of LTH, it was found that a heptadecapeptide fragment corresponding to residues 37-53 possessed significant immunological activity in radioimmunoassay and complement-fixation experiments using antisera to LTH (Solis-Wallckermann, 1972). To substantiate this observation, synthesis of the fragment was undertaken using the solid-phase method. This chapter describes the synthesis and complement-fixation activity of the nonadecapeptide, Phe-Asn-Glu-Phe-Asp-Lys-Arg-Tyr-Ala-Gln-Gly-Lys-Gly-Phe-Ile-Thr-Met-Ala-Leu, corresponding to residues 37-55 of ovine LTH. The synthesis of the protected nonadecapeptide resin was performed three times, as briefly mentioned in the previous chapter, and the extent of chain

termination was shown to vary with the methods employed for the initial attachment and the subsequent couplings. In addition, the methionine residue which did not have the thioether group protected was found to have been modified during the course of synthesis.

In Chapter 2 it was pointed out that the incorporation of methionine in solid-phase peptide synthesis has generally been without protection of the thioether group. It has been assumed that subsequent side reactions could occur resulting in the alkylation of the sulfur and formation of the sulfonium ion. Evidence was presented in Chapter 3 that indicated the formation of a sulfonium ion side-product of the model heptapeptide, Leu-Gly-Arg-Leu-Gly-Met-Phe, during synthesis with use of N^{α}-Z(OMe) protection. The side-product was not obtained from a resynthesis of the model heptapeptide which used methionine sulfoxide for thioether group protection. In this chapter, the above heptapeptide was synthesized with the use of N^{α}-Boc protection, and with and without protection of the thioether group. Isolation of the highly purified sulfonium form of the model heptapeptide in high yield showed that alkylation of unprotected methionine occurred even in the presence of anisole.

Experimental Section

Methods and Materials. The methods and materials which have not been described in the previous chapters are below.

In this chapter, three buffer systems were used for paper electrophoresis: (A) 5% aqueous acetic acid, 400 V, 2 h; (B) collidine acetate buffer, pH 6.4, 2 kV, 1 h; and (C) formic acid-acetic acid buffer, pH 2.1, 2 kV, 1 h. Ninhydrin was used for detection.

Carboxymethylcellulose chromatography (Peterson & Sober, 1956) was performed as described in Chapter 3. In all cases, the initial buffer was 0.01 M NH_4OAc of pH 4.5.

Partition chromatography on Sephadex G-25 (1.89 x 23.3 cm) was performed by procedures previously described (Yamashiro, 1964) using the solvent system 1-butanol-0.5 M acetic acid (1:1). Partition chromatography on Sephadex G-50 (1.89 x 34.7 cm) was performed by procedures previously described (Yamashiro & Li, 1973b) using the solvent system 1-butanol-pyridine-0.1% aqueous acetic acid (5:3:11).

Protected Peptide Resins of Prolactin-(37-55).

A. First Synthesis. Boc-Leu resin prepared by the triethylamine-salt procedure (Merrifield, 1964) gave a substitution of 0.23 mmol/g as determined by the Schiff base amine test (Esko et al., 1968). The Boc-Leu resin (4.35 g, 1.0 mmol) was placed in a Beckman Model 990 peptide synthesizer and carried through the schedules in Table 9 with 40-ml washes and CH_2Cl_2 replacing $CHCl_3$. N^{α}-Boc protection was used throughout with the following side-chain protecting groups: Lys, Z(2-Br); Tyr, Bzl(2,6-Cl_2); Arg, tosyl; Asp, OBzl; Thr, Bzl; and Glu, OBzl(4-Br). Boc-Asn and Boc-Gln were coupled

as the p-nitrophenyl esters (Bodansky & du Vigneaud, 1959) (10 equiv, 10 mmol for 10 h). Boc-Arg(Tos)-OH was dissolved in 3 ml of DMF and diluted with 19 ml of CH_2Cl_2 before addition to the peptide resin. The finished peptide resin was dried under reduced pressure over P_2O_5 to yield 5.77 g.

Unpublished results of Dr. D. Yamashiro showed that by the Schiff base amine test the final deprotected peptide resin had an amine content (0.020 mmol/g) of 12% of theoretical. A sample (12.1 mg) of the final protected peptide resin was submitted to a 20-h resin hydrolysis (Scotchler et al., 1970). By amino acid analysis with a norleucine standard, the amount of leucine found was 78% (0.13 mmol/g) of theoretical and the amino acid composition was $Lys_{1.01}$-$Arg_{0.14}Asp_{0.61}Thr_{0.83}Glu_{0.88}Gly_{1.30}Ala_{1.34}Met_{0.66}Ile_{0.71}$-$Leu_{1.00}Tyr_{0.30}Phe_{1.25}$ (the low Arg value results from the incomplete removal of the tosyl group by the conditions of resin hydrolysis).

B. Second Synthesis. Boc-Leu resin prepared by the modified Loffet procedure (Yamashiro & Li, 1973b) gave a substitution of 0.20 mmol/g as determined by the Schiff base method. The Boc-Leu (1.53 g, 0.305 mmol) was treated by the same procedures as for the first synthesis, but obviously scaled down about 3-fold. After removal of a portion (150 mg) at the nonapeptide stage, the incorporation of the last 10 residues was performed by Dr. D. Yamashiro. The dried final protected peptide resin gave 2.45 g.

Unpublished results of Dr. D. Yamashiro showed that by the Schiff base method the final deprotected peptide resin had an amino content (0.083 mmol/g) of 46% of theoretical. By the picric acid method, the final deprotected and protected peptide resin gave amine contents of 0.0122 and 0.093 mmol/g, respectively. Thus, the picric acid method gave a positive test (14.7% of the amine content by the Schiff base method) even before deprotection of the amino group. The corrected total amine content (0.081 mmol/g) by the picric acid method was in agreement with the amine content by the Schiff base method. A sample (21.6 mg) of the final protected peptide resin was submitted to a 20-h hydrolysis. Amino acid analysis of the hydrolysate gave $Lys_{1.84}Asp_{1.15}Thr_{0.74}Glu_{1.39}$-$Gly_{2.60}Ala_{1.42}Met_{0.84}Ile_{0.71}Leu_{1.00}Tyr_{0.79}Phe_{1.90}$ (because of the incomplete removal of the tosyl group by this method of hydrolysis the Arg value was not calculated).

C. Third Synthesis. Boc-Leu resin prepared by the modified Loffet procedure gave a substitution of 0.385 mmol/g as determined by the picric acid method. The Boc-Leu resin (1.1 g, 0.426 mmol) was placed in a Beckman Model 990 peptide synthesizer and carried through the schedule in Table 13 for incorporating the remaining 18 amino acid residues, except for the incorporation of the Asn residue. Boc-Asn was coupled as the p-nitrophenyl ester (10 equiv, 4.26 mmol for 10 h) which required: step 13 in Table 13 to be repeated only 3 times; step 14, DMF (3 times) for 1 min; step 15, the coupling

reaction; step 16, DMF (3 times) for 1 min. The pre-mix reaction mixture used in all other couplings was prepared as previously reported (Yamashiro & Li, 1974) with 6.5 equiv (2.77 mmol) of tert-butyloxycarbonylamino acid and 3.0 equiv (1.28 mmol) of DCCI. Boc-Arg(Tos)-OH was dissolved in 1.2 ml of DMF and diluted with 3.6 ml of CH_2Cl_2 before the reaction with DCCI. For the reaction of Boc-Gln-OH with DCCI (Hemmasi & Bayer, 1974), Boc-Gln-OH was dissolved in 2.4 ml of DMF and diluted with 2.4 ml of CH_2Cl_2. The finished peptide resin was dried under reduced pressure over P_2O_5 for 3 h to yield 2.41 g, 96% of theoretical weight gain, and stored at -20°.

A sample (51 mg) of the final peptide resin was deprotected, neutralized, washed and dried under reduced pressure over P_2O_5 for 2 h at 65°. By the picric acid method, the amine content was 79% (0.1388 mmol/g) of theoretical. A sample of the protected final peptide resin gave 11% (0.0151 mmol/g) of the amine content of the deprotected sample by the picric acid method. Amino acid analysis of a resin hydrolysate gave $Lys_{1.37}Arg_{0.22}Asp_{1.76}Thr_{0.81}Glu_{1.86}Gly_{1.74}Ala_{1.91}Met_{0.83}Ile_{0.77}Leu_{1.00}Tyr_{0.82}Phe_{2.59}$.

Isolation of Prolactin-(37-55). Protected peptide resin (1.00 g) was treated with TFA-CH_2Cl_2, 1:1 (10 ml), for 15 min at 25° and filtered. The resin was washed with 25 ml each of CH_2Cl_2, DIEA-CH_2Cl_2 (1:19), CH_2Cl_2, and absolute ethanol. After drying for 80 min under reduced pressure over P_2O_5, the

partially protected peptide resin (971 mg) was treated with liquid HF (10 ml) in the presence of anisole (1.0 ml) for 30 min at -30 to -20° and for 30 min at 0° (Baba et al., 1973). After removal of the HF at 0° by a stream of nitrogen for 10 min, the peptide-resin mixture was dried under reduced pressure over NaOH for 30 min and then stirred with TFA (20 ml) for 20 min and filtered. The filtrate was evaporated to give an oil which was twice reevaporated from acetic acid (5 ml). The oily residue was then dissolved in 10% aqueous acetic acid (50 ml) and washed with two 20-ml portions of ether. The aqueous solution was lyophilized and the resulting material (359 mg) was subjected to gel filtration on Sephadex G-10 (2.2 x 28.5 cm) in 0.5 M acetic acid. The peptide material (V_e of peak, 45 ml) was isolated by lyophilization to give 305 mg, 77% overall yield based on starting Boc-Leu resin. An aliquot (50.0 mg) was submitted to chromatography on CMC, Figure 14A, and essentially three peaks were detected. Isolation by lyophilization yielded fraction A, 36 mg, and fraction B, 2.95 mg. Another aliquot (180 mg) chromatographed on CMC yielded 130 mg corresponding to the material in fraction A. Upon partition chromatography on Sephadex G-50 (1.89 x 35.4 cm), Figure 15A, a 60-mg aliquot of fraction A resulted in a major peak with R_f 0.47 and a minor peak with R_f 0.28. Isolation of material represented by the major peak (R_f 0.47) gave 50.5 mg (47% overall yield) of highly purified prolactin-(37-55): tlc (BPAW), R_f 0.48;

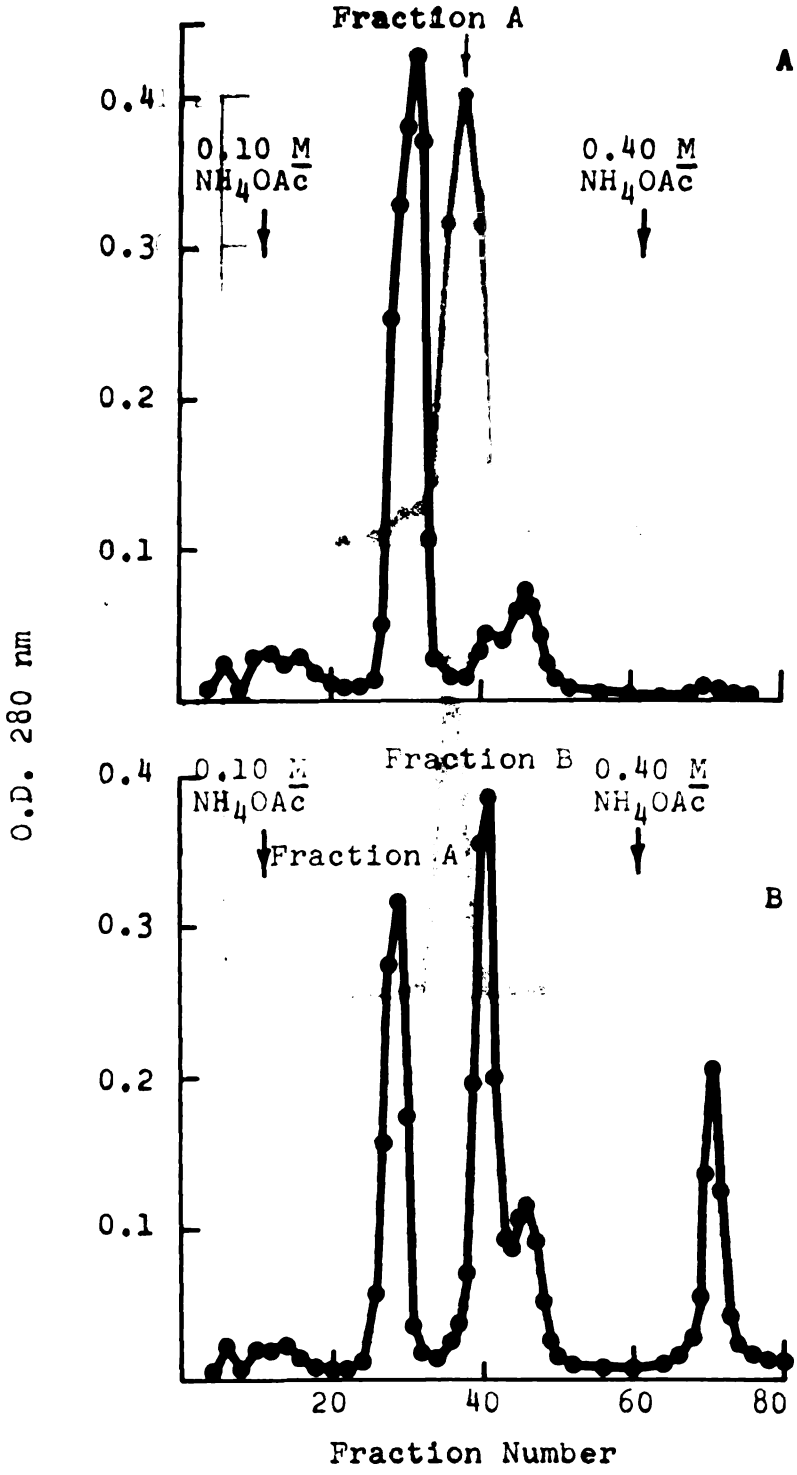


Figure 14. Carboxymethylcellulose chromatography of partially purified prolactin-(37-55) obtained by HF treatment of (A) TFA-deblocked peptide resin and (B) Boc-peptide resin. Collected 10.3 ml per fraction.

Figure 14. Carboxymethylcellulose chromatography of proteolytic peptides produced by treatment of (A) TIA-deficient pituitary resin and (B) reserpine-treated rat pituitary resin. Collected 10.3 ml per fraction.

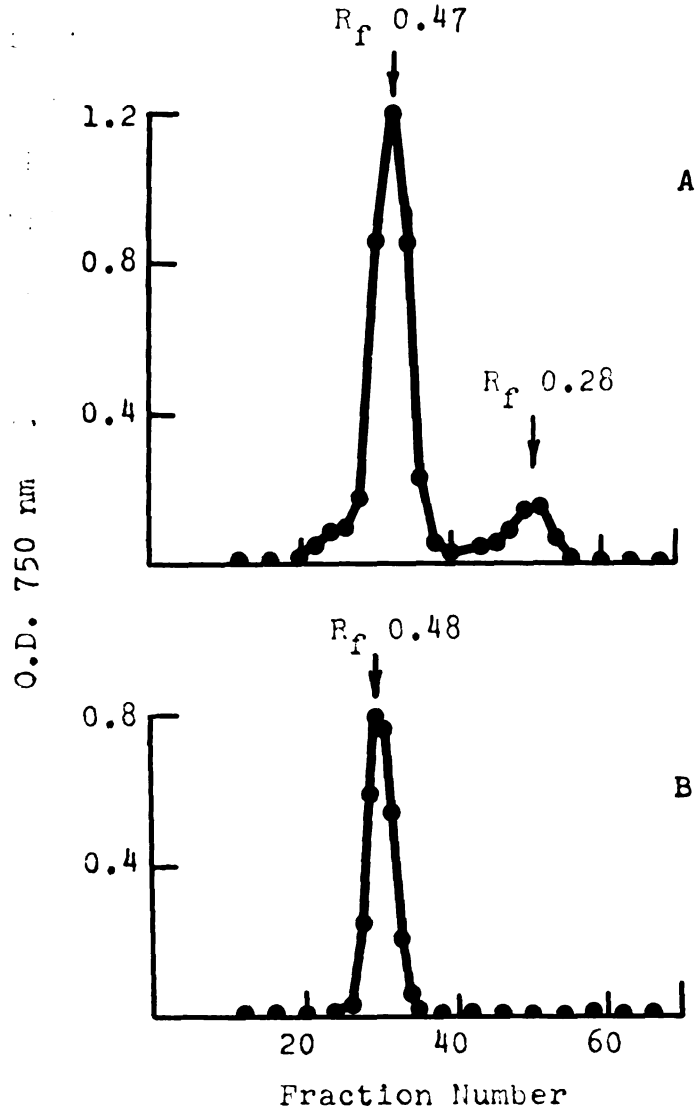
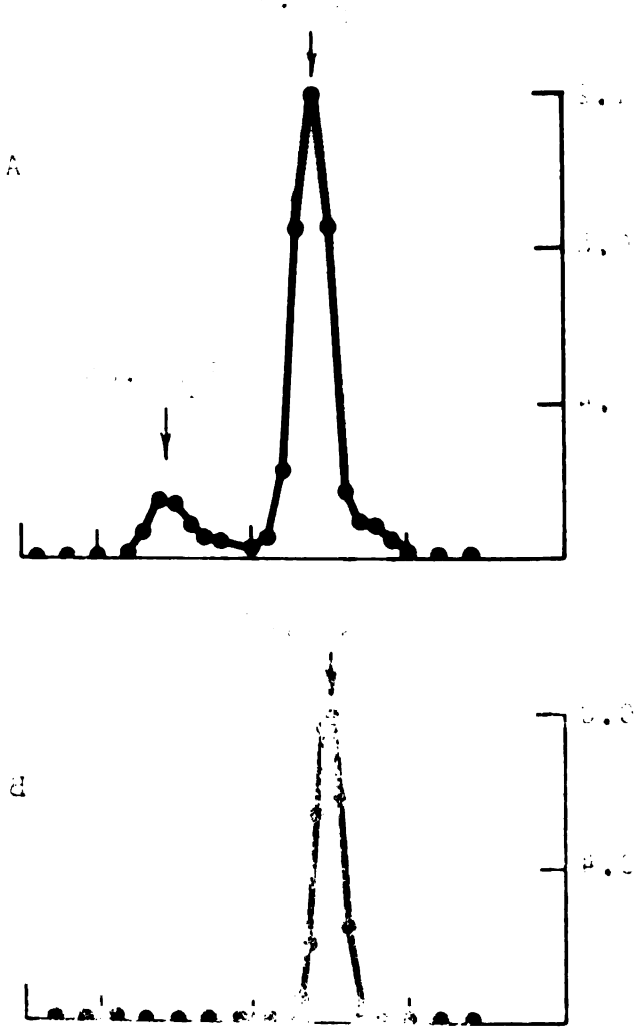


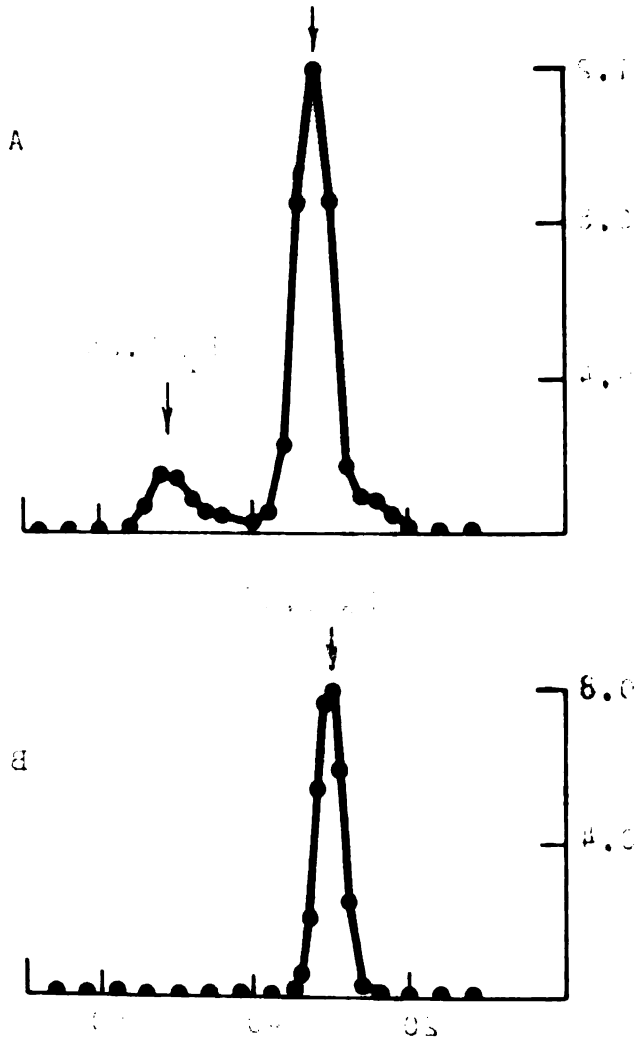
Figure 15. Partition chromatography on Sephadex G-50 (1.9 ml per fraction): (A) 60 mg of fraction A, yield (fractions 28-37), 50.5 mg; (b) 20.5 mg of highly purified prolactin-(37-55), yield (fractions 28-33), 19.0 mg.



tlc (BAW), R_f 0.12; $[\alpha]_D^{24}$ -50.03° (*c* 0.985, 1.0 *M* acetic acid).

A sample (20.5 mg) of the R_f 0.47 material was again subjected to partition chromatography on Sephadex G-50 and a single symmetrical peak with R_f 0.48 was obtained as shown in Figure 15B. Paper electrophoresis in buffer systems (see above) A, B, and C each gave a single spot with R_{Lys} values of 0.62, 0.31, and 0.26, respectively. A 24-h chymotryptic digestion with a 1:50 (w/w) ratio of enzyme to substrate gave the four expected ninhydrin-positive spots when submitted to paper electrophoresis in buffer system B; R_{Lys} 0.54 (also Pauly-positive), R_{Lys} 0.50, R_{Lys} 0.14, and R_{Glu} 0.25. Amino acid analyses of acid and enzyme hydrolysates are shown in Table 14.

When Boc-protected peptide resin (494 mg) was not treated with TFA but taken directly to HF treatment and worked up as described above, 153 mg (78% overall yield based on starting Boc-Leu resin) of peptide was isolated after the Sephadex G-10 step. An aliquot (50 mg) subjected to chromatography on CMC gave the pattern shown in Figure 14B; yields, fraction A, 18 mg (28% overall yield), and fraction B, 23 mg. On paper electrophoresis in buffer system A, fraction A gave R_{Lys} 0.61 and fraction B gave two spots with R_{Lys} 0.61 and 0.76. At this stage of purity the overall yield of prolactin-(37-55) was 28%. Another aliquot of crude peptide (50 mg) was heated at 50-55° for 20 h and then subjected to chromatography on CMC. A pattern similar to that shown in


A
B

tlc (BAW), R_f 0.12; $[\alpha]_D^{24}$ -50.03° (c 0.985, 1.0 M acetic acid).

A sample (20.5 mg) of the R_f 0.47 material was again subjected to partition chromatography on Sephadex G-50 and a single symmetrical peak with R_f 0.48 was obtained as shown in Figure 15B. Paper electrophoresis in buffer systems (see above) A, B, and C each gave a single spot with R_{Lys} values of 0.62, 0.31, and 0.26, respectively. A 24-h chymotryptic digestion with a 1:50 (w/w) ratio of enzyme to substrate gave the four expected ninhydrin-positive spots when submitted to paper electrophoresis in buffer system B; R_{Lys} 0.54 (also Pauly-positive), R_{Lys} 0.50, R_{Lys} 0.14, and R_{Glu} 0.25. Amino acid analyses of acid and enzyme hydrolysates are shown in Table 14.

When Boc-protected peptide resin (494 mg) was not treated with TFA but taken directly to HF treatment and worked up as described above, 153 mg (78% overall yield based on starting Boc-Leu resin) of peptide was isolated after the Sephadex G-10 step. An aliquot (50 mg) subjected to chromatography on CMC gave the pattern shown in Figure 14B; yields, fraction A, 18 mg (28% overall yield), and fraction B, 23 mg. On paper electrophoresis in buffer system A, fraction A gave R_{Lys} 0.61 and fraction B gave two spots with R_{Lys} 0.61 and 0.76. At this stage of purity the overall yield of prolactin-(37-55) was 28%. Another aliquot of crude peptide (50 mg) was heated at 50-55° for 20 h and then subjected to chromatography on CMC. A pattern similar to that shown in

Figure 14A was obtained, and the material isolated from the major peak (29 mg) was further purified (20-mg sample) by partition chromatography on Sephadex G-50 to give 16 mg of highly purified prolactin-(37-55) (36% overall yield).

Synthesis and Isolation of Leu-Gly-Arg-Leu-Gly-Met-Phe (I). Boc-Phe resin was prepared by the method used for the Boc-Leu resin as above. Boc-Phe resin (1.0 g, 0.483 mmol) was placed in a Beckman Model 990 peptide synthesizer and essentially carried through the same procedures employed for the nonadecapeptide. About 10% of the resin was removed for analytical purposes during the synthesis (see below). The finished peptide resin was dried under reduced pressure over P_2O_5 for 3 h at 25° to yield 1.25 g, 97% of the theoretical weight gain, and stored at -20°.

Aliquots of the protected peptide resin had been taken after the following residues were incorporated: Boc-Met, the first Boc-Gly, Boc-Arg(Tos), and the final Boc-Leu. The picric acid method was used to detect a charged group that could retain the picric acid. The aliquot taken after the incorporation of Boc-Met did not retain picric acid. However, the aliquots taken after the incorporation of Boc-Gly, Boc-Arg(Tos) and Boc-Leu retained picric acid to the following values of the theoretical amine content, respectively, 5.3%, 8.7% and 7.0%. When the 'Boc-Gly' aliquot was heated at 140° for just 5 min the amount of picric acid retained dropped to 0.86% of the theoretical amine content.

Protected peptide resin (300 mg) was treated with liquid HF (10 ml) for 30 min at 0° in the presence of anisole (0.30 ml). The cleaved material was worked up and subjected to gel filtration as described for the nonadecapeptide. Isolation by lyophilization gave 91.2 mg. This material was heated at 50-55° for 24 h and then submitted to chromatography on CMC. The column (1.23 x 47 cm) was eluted with 200 ml of the initial buffer, 0.01 M NH_4OAc of pH 4.5. The desired product emerged between elution volumes of 130 and 200 ml, and isolation by lyophilization gave 66.5 mg, 80% overall yield based on starting Boc-Phe resin. Then, the column gradient was first raised with the introduction of 160 ml of 0.10 M NH_4OAc, pH 6.7 through a 500-ml mixing chamber, and next with 0.40 M NH_4OAc, pH 6.7. A peak emerged between 88 and 124 ml after the start of the 0.40 M buffer, and isolation by lyophilization gave 3.5 mg of a peptide side product. The desired product (64 mg) was further purified by partition chromatography on Sephadex G-25 (R_f 0.26) to give 55 mg of peptide I (68% overall yield): tlc (BPAW), R_f 0.68; $[\alpha]_D^{24}$-22.07° (c 1.22, 1.0 M acetic acid). Paper electrophoresis in buffer systems A and B each gave a single spot with R_{Lys} values of 0.58 and 0.45, respectively. Amino acid analysis of an acid hydrolysate gave $Arg_{1.0}Gly_{1.8}Met_{1.0}Leu_{2.1}Phe_{1.0}$.

Another portion of the protected peptide resin (323 mg) was treated with HF and worked up in the same manner. The material after gel filtration was not heated but taken directly

to CMC chromatography. The yield of peptide I was 18.8 mg, 21% of overall yield, while the yield of the slow-moving side product was 60 mg. The preparation of peptide I obtained in this run was identical with an authentic sample of peptide I by tlc, paper electrophoresis, and amino acid composition.

Another portion of the protected peptide resin (122 mg) was treated with TFA-CH_2Cl_2 1:1 and neutralized as described above for the nonadecapeptide resin. One-half of the peptide resin was treated with HF and worked up as described above to give an oily residue. The other half of the peptide resin was heated at 80-85° for 4 h and processed as the first half to give an oily residue. On paper electrophoresis in system A, peptide I traveled with R_{Lys} 0.58 and the side product with R_{Lys} 0.81. By these criteria the material obtained from the unheated resin showed a peptide I to side product ratio of 4:1. The material from the heated resin showed a 20:1 ratio.

Isolation of Peptide II, Sulfonium Salt of I. Protected peptide resin (102 mg) was treated with 5 ml of liquid HF for 30 min at 0° in the presence of 0.10 ml of anisole. The cleaved material was worked up and subjected to gel filtration as described for the nonadecapeptide. The crude peptide material was isolated by lyophilization in which the lyophilizing flask was immersed in an ice-water bath. Subjecting this material to chromatography on CMC gave a slow-moving major peak

in the same position as the side product (see previous section). Isolation by lyophilization as described above yielded 47.4 mg of the sulfonium salt of I, 66% overall yield based on $\varepsilon_{257\ nm}$ of Phe: tlc (BPAW), R_f 0.45, with a trace of I with R_f 0.68; paper electrophoresis in system A gave R_{Lys} 0.81, with a trace of I with R_{Lys} 0.58. Amino acid analysis of an acid hydrolysate gave $Arg_{1.0}Gly_{1.9}Met_{1.0}Leu_{2.1}Phe_{1.0}$. Amino acid analysis of a performic acid oxidized sample gave $Hsl_{0.1}Arg_{0.9}Met(O)_{0.1}Met(O_2)_{0.2}Hse_{0.1}Gly_{1.8}Met_{0.1}Leu_{2.0}$, while I under the same conditions gave $Arg_{1.0}Met(O_2)_{1.0}Gly_{1.7}Leu_{2.0}$. Under the conditions described here phenylalanine was altered by chlorination (Vithayathil & Richards, 1960).

NMR spectra were taken with a Varian T-60 with 2,2-dimethyl-2-isopentane-5-sulfonate (DSS) as internal reference. The sulfonium salt II (47 mg) and the authentic peptide I (54 mg) were dissolved in D_2O, 0.25 and 0.35 ml, respectively.

The sample of peptide II in D_2O used in the NMR study was recovered by lyophilization at 0°. The solid (20 mg) was stored at room temperature for 3 months and then a sample (18.9 mg) was subjected to chromatography on CMC. Peptide I was recovered in good yield (17.1 mg) in highly purified form identical with an authentic sample of I by tlc (BAW and BPAW) and paper electrophoresis in system A.

When peptide II was obtained from CMC chromatography by normal lyophilization, stored for 3 days at room temperature, and then resubmitted to CMC chromatography, there was a

40% recovery of peptide material in the position of peptide I (Figure 16).

Peptide I could be converted to the sulfonium salt by the following procedure. Peptide I (9.7 mg, 12 μmol) was dissolved in 0.1 ml of TFA-CH_2Cl_2 1:1 and treated with tert-butyl bromide (125 μmol) for 60 min at room temperature. After evaporation, the product was subjected to CMC chromatography in the usual manner. Peaks corresponding to the positions of peptide I and the sulfonium salt II were detected and isolation by lyophilization gave 2.8 and 5.2 mg, respectively.

Synthesis and Isolation of Leu-Gly-Arg-Leu-Gly-Met-d-(O)-Phe (III). The synthesis of the peptide resin was performed on the same scale and in the same manner as in the synthesis of I, except Boc-Met was replaced by Boc-Met-d-(O). The Boc-Met-d-(O) prepared by Mr. W. F. Hain was dissolved in 1 ml of DMF and diluted with 7 ml of CH_2Cl_2 before reaction with DCCI. The weight of the final protected peptide resin was 1.31 g.

A portion (324 mg) of the peptide resin was treated with HF in the presence of anisole and worked up in the same manner as described for I. All of the cleaved material was also subjected to gel filtration and chromatography on CMC as described for I. The chromatography on CMC is shown in Figure 17. Isolation by lyophilization of the peptide material in the major peak gave III, 72 mg (81% overall yield). The material was identical to the same peptide synthesized with

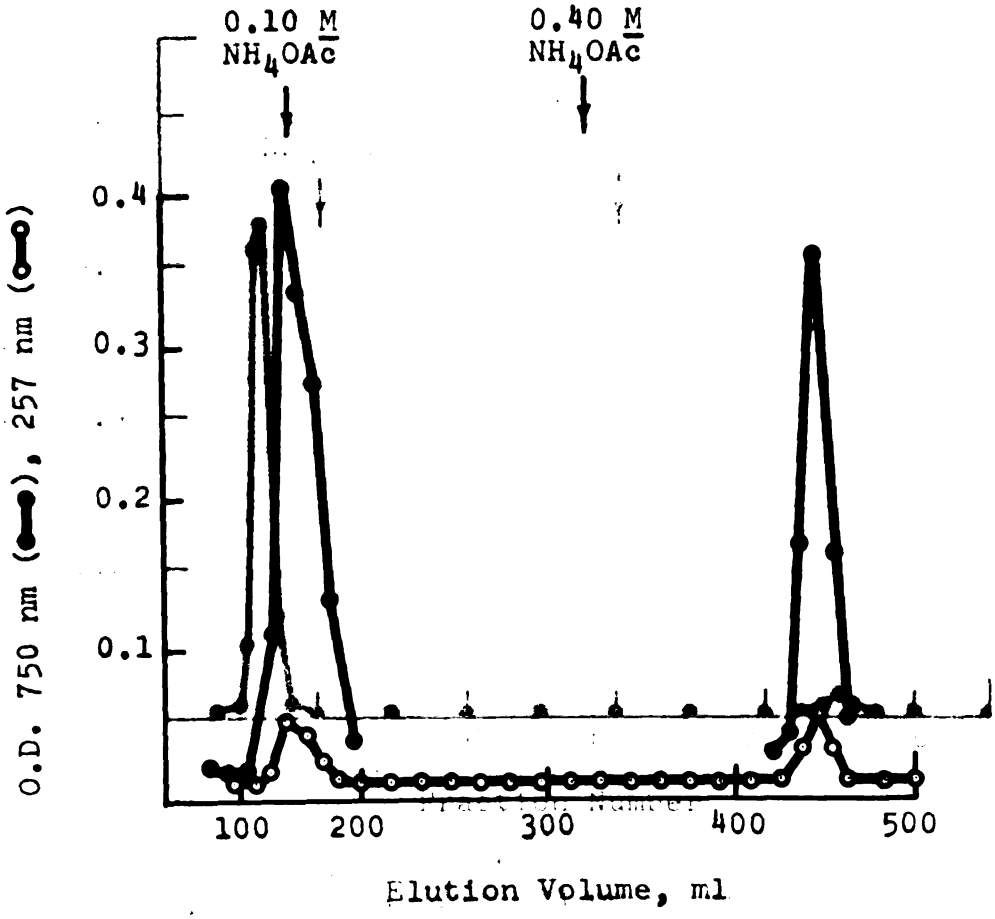


Figure 16. Rechromatography on CMC of the side-product peptide II obtained by normal lyophilization: 8.7 mg on a 1.23 x 47-cm column, yield (V_e, 140-184 ml), 3.5 mg; (V_e, 424-452 ml), 5.0 mg.

0.4
0.3
0.2
0.1
200
250

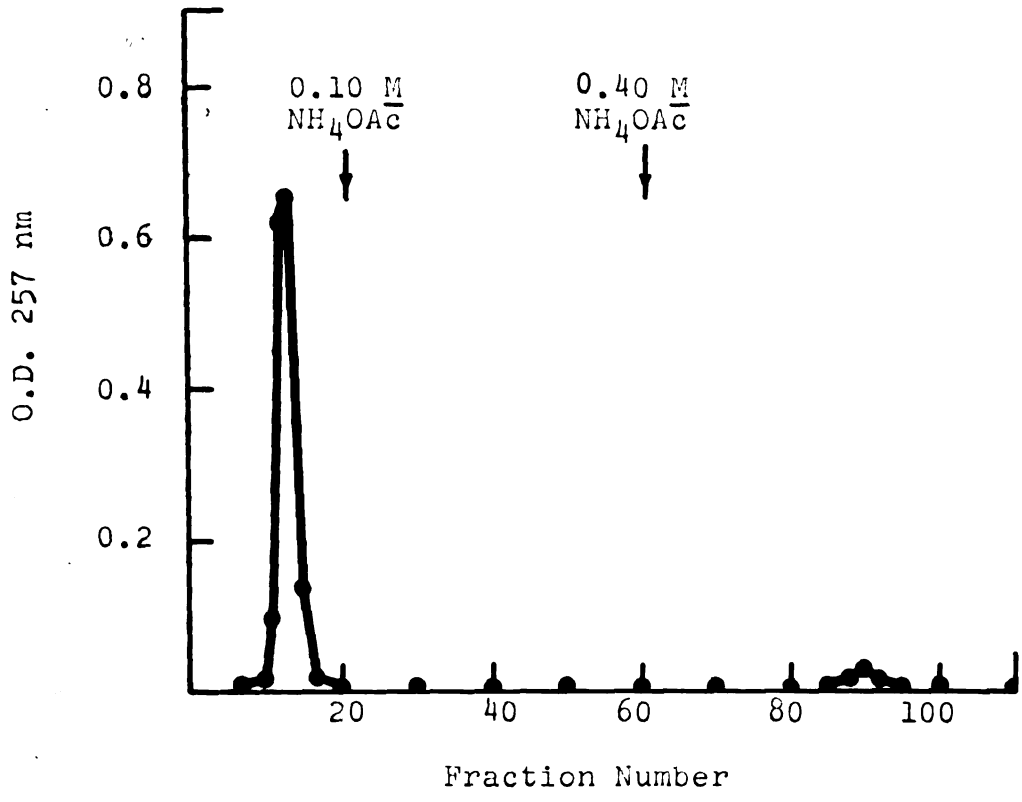


Figure 17. Chromatography on CMC of the Met(O)-heptapeptide, III: 73 mg of crude peptide material, yield (fractions 11-16), 72 mg. Collected 10 ml/fraction for fractions 1-20 and 4 ml/fraction from fraction 21 on.

Z(OMe) protection in Chapter 3 on tlc (BPAW) and paper electrophoreses in buffer systems B and C.

Attempted Reduction of III. Two samples (5 mg each) of III were dissolved separately in 0.20 ml of 0.1 M acetic acid. To one solution, 4 equiv of dithiothreitol (DTT) were added, while to the other solution, 4 equiv of β-mercaptoethanol (β-ME) were added. Two more samples (5 mg each) of III were dissolved separately in 0.20 ml of 0.01 M NH_4OAc, pH 8.4 and the same amounts of the above reducing agents were added. The four solutions were heated in a 50° bath and the reduction followed by tlc (BPAW) of 1 μl aliquots. After 20 h, reduction of III to I was 25% complete in the DTT-0.1 M acetic acid sample, 50% complete in both the β-ME-0.1 M acetic acid and DTT-0.01 M buffer samples, and 75% complete in the β-ME-0.01 M buffer sample; and by 48 h, reduction was 40% complete for the first 3 samples above and still 75% complete for the β-ME-0.01 M buffer sample.

Immunological Studies. Guinea pig antiserum to ovine prolactin was obtained as previously described for the porcine hormone (Clarke & Li, 1974). Specific antibodies were prepared by affinity chromatography using a prolactin-Sepharose immunoadsorbent according to the procedure of Sairam et al. (1974). The antibodies were eluted with 2 M sodium trichloroacetate. Microcomplement fixation was carried out by Dr. W. C. Clarke as described by Wasserman and Levine (1961).

Results and Discussion

Previous studies (Solis-Wallckermann, 1972) showed that the heptadecapeptide fragment corresponding to residues (37-53) of ovine LTH obtained by cyanogen bromide cleavage of LTH had significant immunoreactivity to antisera to native LTH. Since the results could be obtained by a 1% contamination on a molar basis of the native protein, synthesis of this fragment would allow unambiguous determination of the fragment's immunoreactivity. For synthetic convenience, it was decided to synthesize the nonadecapeptide fragment corresponding to residues 37-55.

The first synthesis was carried out on Boc-Leu resin prepared by the triethylamine-salt procedure and employed the standard *in situ* coupling method. The final peptide resin had only 12% of the theoretical amine content by the Schiff base method. This loss of amine was not due to acidolytic cleavage of peptide chains by the deblocking conditions during the synthesis, because 78% of the theoretical carboxyl-terminal leucine was found by quantitative amino acid analysis of a resin hydrolysate. This amino acid analysis gave a 0.61:1 ratio for the diagnostic amino acid ratio of Asp to Leu, the two Asp residues arising from Asn_{38} and Asp_{41}. Thus, chain termination must have proceeded at a totally unacceptable rate during the synthesis.

The second synthesis was identical to the first, except for the method of attachment. That the quaternary

ammonium sites (Rudinger & Gut, 1967; Beyerman et al., 1967) generated in the resin during the above attachment method could have possibly retained trifluoroacetic acid and led to chain termination was discussed in the previous chapter. Thus, the modified Loffet method was the attachment procedure used for the second synthesis. The couplings were again performed by the standard *in situ* method. When compared to the first synthesis, the final peptide resin had a greatly improved amine content even though it was only 46% of theoretical, and the diagnostic amino acid ratio of Asp to Leu just about doubled. Examination of the protected final peptide resin by the picric acid method, nevertheless, showed the presence of a charged group which was about 15% of the above amine content, but probably not a quaternary ammonium group. The nature of this charged group was studied in more detail in the model peptide below. The overall conclusion was that chain termination had been significantly but not completely eliminated.

The third synthesis employed, in addition to the attachment by the modified Loffet procedure, the preformed symmetrical anhydride (*pre-mix*) coupling method. There was a vast improvement over the second synthesis in both the amine content (79% of theoretical) of the final resin and the diagnostic amino acid ratio of Asp to Leu (1.76:1). Again, the final protected peptide resin had a positively charged group that retained picric acid (11% of the final amine content).

The final protected peptide resin from the third synthesis was treated in TFA-CH_2Cl_2 (1:1) to remove the Boc group. Removal of the solid support as well as the side-chain protecting groups was accomplished in HF in the presence of anisole. Gel filtration on Sephadex G-10, in which only one peak was detected, was followed by chromatography on CMC (Figure 14A). Further purification of fraction A by partition chromatography on Sephadex G-50 (Figure 15A) gave ovine LTH-(37-55) in highly purified form with 47% overall yield based on the starting Boc-Leu resin. Partition chromatography of this material on Sephadex G-50 gave a single symmetrical peak with R_f 0.48 (Figure 15B). The product was homogeneous on paper electrophoresis in three buffer systems and on tlc in two solvent systems. Amino acid analyses of acid and enzyme hydrolysates were in agreement with expected values (Table 14).

The immunological activity of the synthetic LTH-(37-55) was estimated by microcomplement fixation using purified guinea pig antibodies to ovine LTH. As shown in Figure 18, the natural LTH-(37-53) reached equivalence at 50 ng to fix 32% complement whereas the synthetic LTH-(37-55) at the same equivalent dose fixed 26%.

In the above procedure to obtain the final product, the Boc group was removed from the protected peptide resin with TFA before proceeding to the HF step. When the Boc group was not removed in this manner but was removed along

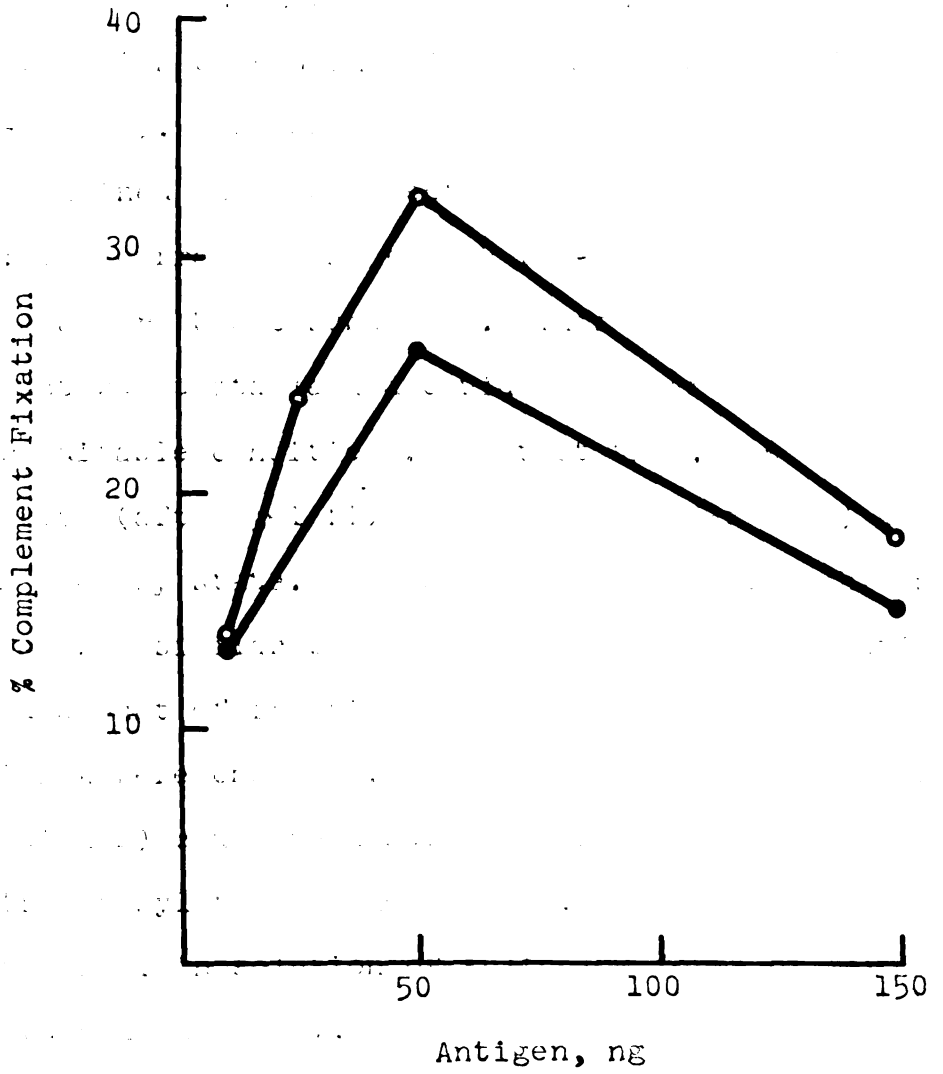


Figure 18. Microcomplement fixation curve obtained with CNBr derived fragment (37-53) from native prolactin (o—o) and synthetic prolactin-(37-55) (●—●) using purified guinea pig antibodies to ovine prolactin.

with all the other protecting groups with HF, the crude peptide material from gel filtration gave a pattern on CMC chromatography shown in Figure 14B. Ovine LTH-(37-55) (fraction A) was obtained in diminished yield. The major side product (fraction B) had an amino acid composition identical with LTH-(37-55). Two spots were observed when this material was examined on paper electrophoresis in 5% aqueous acetic acid: one corresponding to LTH-(37-55) and the other traveling like a more basic substance. This suggested that the side product was unstable and could be converted to LTH-(37-55) under suitable conditions. To test this, the crude peptide material (after gel filtration on Sephadex G-10) was heated in the dry state. The resulting chromatographic pattern on CMC was similar to that of Figure 14A. A greatly diminished amount of fraction B and a corresponding increase in fraction A were observed. It appeared that the side product (fraction B) could be a sulfonium form of LTH-(37-55) resulting from alkylation of methionine. The possibility of partial reversal in solution of sulfonium forms of methionine is known (Gundlach et al., 1959), but the reversal observed here appears to occur in the solid state.

To support the suggestion that fraction B (Figure 14B) was the sulfonium form of the nonadecapeptide, a methionine-containing model heptapeptide, Leu-Gly-Arg-Leu-Gly-Met-Phe (I), was synthesized with an attempt to isolate its sulfonium form (II). This seemed feasible because when this heptapep-

tide was synthesized with use of N^{α}-Z(OMe) protection (Chapter 3), a more basic side product was obtained from CMC chromatography and could be converted to I. Thus, like the nonadecapeptide, the heptapeptide was synthesized with N^{α}-Boc protection. In addition, like the second and third syntheses of the nonadecapeptide resin, the final protected heptapeptide resin had a charged group that retained picric acid. This group was not present on the Boc-Met-Phe-resin, but was present to the extent of 5.3% of the theoretical amine content on the Boc-Gly-Met-Phe-resin. However, the charged group was eliminated from this protected tripeptide resin by a short heat treatment. A similar group was detected in the final heptapeptide resin synthesized with Z(OMe) protection and represented 41% of the final amine content of the resin. Since it was not known if the more basic side product (possibly the charged group retaining picric acid on the solid support) was generated by the TFA-deprotection step during the synthesis or by the HF treatment for cleavage and side-chain deprotection, various routes were devised for the work up as shown in Figure 19.

For isolation of peptide I, route 1 in Figure 19 was selected. Final purification was effected by partition chromatography on Sephadex G-25, and isolation by lyophilization gave I in 68% overall yield based on starting Boc-Phe-resin.

The yield of I was drastically diminished (to 21%) when the heat treatment was omitted (route 2, Figure 19). In the chromatography on CMC, a major side product was detected

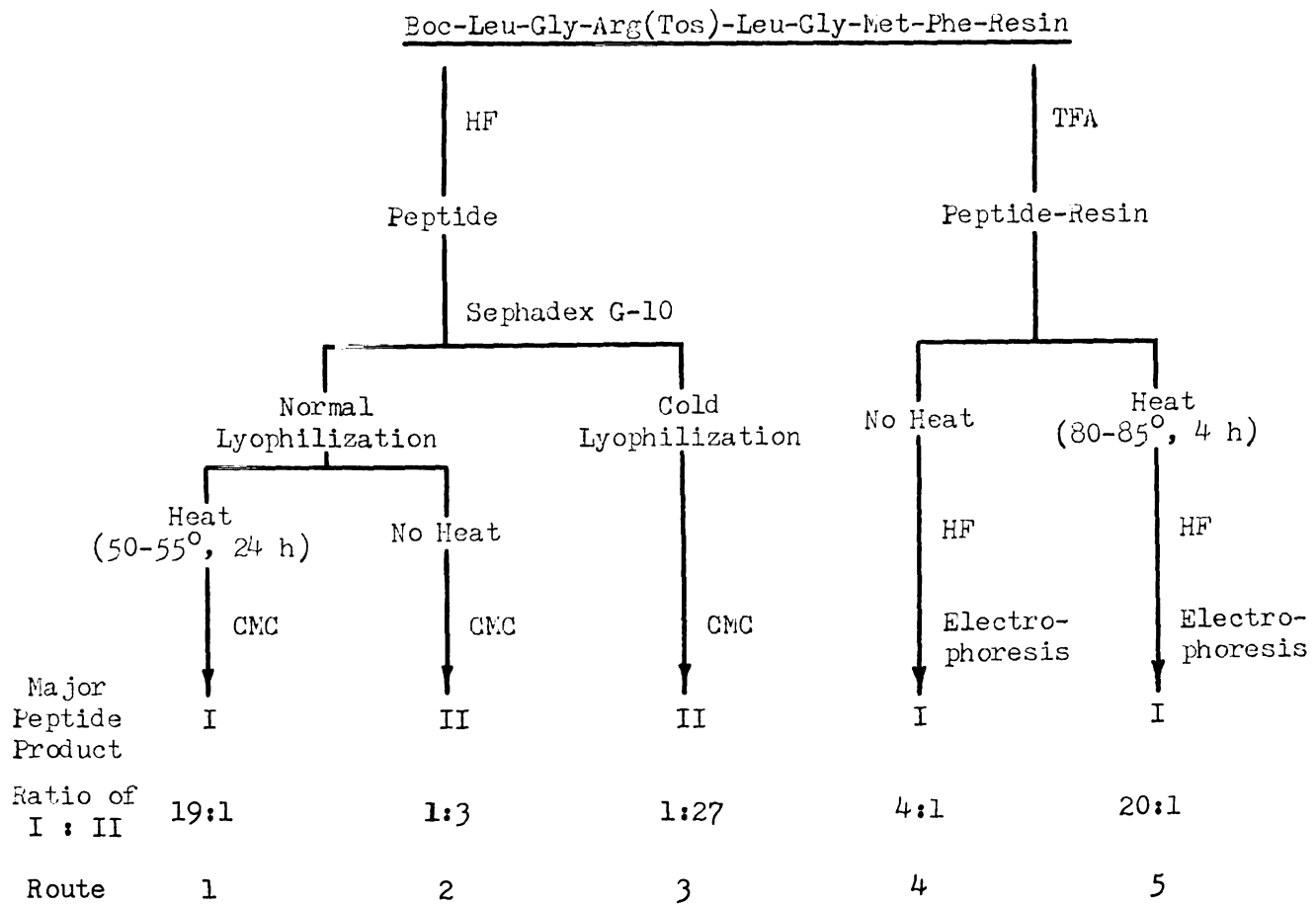


Figure 19. Outline of the various routes investigated (see text) which resulted in different proportions of the desired methionyl peptide I and a side-product peptide II.

which was eluted as a symmetrical peak much later than I, suggesting that it was a more positively charged substance. The amino acid composition of an acid hydrolysate was identical with I. Experiments indicated that the side product was relatively stable while in solution but was partially converted to I when the solvent in which it was dissolved was removed as during spotting for thin-layer chromatography or paper electrophoresis, and even during lyophilization or storage of the solid at room temperature. Thus, when the side product was isolated by lyophilization, stored for 3 days at room temperature, and rechromatographed on CMC, the pattern shown in Figure 16 was obtained. The first peak corresponds to the position of I and the second peak to the position of the side product. By paper electrophoresis, the peptide material in the first peak is identical with peptide I.

For isolation of an analytical sample of the side product, route 3 in Figure 19 was devised. The "cold lyophilization" was accomplished in a vessel immersed in an ice-water bath at all times. Chromatography on CMC gave a major peak corresponding to the previously observed position of the side product (as in Figure 16), and cold lyophilization gave peptide II in 66% overall yield based on starting Boc-Phe resin. The peptide showed homogeneity on thin-layer chromatography and paper electrophoresis only after careful spotting and immediate development. To confirm that II was

the S-tert-butylsulfonium form of peptide I, proton NMR spectroscopy was carried out. The methyl peak of methionine located at δ 2.00 downfield from DSS for peptide I was shifted downfield to δ 2.75 for peptide II. Peptide II also gave a nine-proton tert-butyl peak at δ 1.52.

Alternatively, peptide II was synthesized by the reaction of peptide I with tert-butyl bromide in TFA-CH_2Cl_2 (1:1).

The amino acid composition of an acid hydrolysate of II was identical with peptide I. When I was first treated with performic acid and then acid hydrolysed, all the methionine was converted to methionine sulfone. However, when peptide II was treated in like manner (Gundlach et al., 1959), the methioninesulfonium residue was almost completely destroyed to give a complex mixture of products. Thus, the tert-butylsulfonium form of methionine was affected by performic acid in a different manner than was methionine but was converted to methionine in acid hydrolysis.

Peptide II was converted to peptide I during storage of II in the solid form at room temperature, a fact in agreement with preliminary observations on this conversion. Although the exact nature of this conversion needs further investigation, the results in Figure 19 delineate routes for maximizing yields of either I or II. It is evident that the methionine residue in the crude peptide after HF treatment of the Boc-protected peptide resin is largely of the tert-butyl-

sulfonium form. If the Boc group is first removed with TFA before the HF treatment, then the crude peptide consists largely of peptide I (routes 4 and 5 in Figure 19). In Chapter 3 this was done for the work up of I from the synthesis with Z(OMe) protection but a greater amount of the side product was detected on CMC chromatography. Thus, besides indicating stability differences between different sulfonium forms, it supports the fact that sulfonium formation occurs even during the synthesis in the deprotection step and generates a positively charged group on the resin.

To avoid this side reaction, I was synthesized with the protection of the thioether group by use of the methionine sulfoxide derivative and obtained after reduction of the Met(O)-containing peptide. However, complete reduction was not achieved indicating that protection of methionine still requires a final separation of peptides differing only in the nature of the methionyl residue. Further studies would be required to show if the side reaction during synthesis which generates a positive charge has to be eliminated to diminish chain termination as was shown for the quaternary ammonium sites obtained from the triethylamine salt method of attachment.

In summary, trace amounts of the S-tert-butylsulfonium ion form of human calcitonin M resulting from the final deprotection of the protected peptide synthesized in solution by fragment condensation have been reported but not charac-

terized (Riniker et al., 1972). The studies in this chapter showed that indeed the S-tert-butylsulfonium form of methionine-containing peptides resulted from deprotection of tert-butyl groups. Routes have been described which allow a minimal amount of the sulfonium form in the crude peptide material obtained from solid-phase synthesis.

Table 13

Schedule of Synthesis for LTH-(37-55)

Step	Reagent and Operation	Time (min)
1	CH_2Cl_2 (4 times)	1
2	TFA-CH_2Cl_2, 1.22:1 (1 time)	1
3	TFA-CH_2Cl_2, 1.22:1 (1 time)	12
4	Dioxane-CH_2Cl_2, 1:3 (3 times)	1
5	CH_2Cl_2 (3 times)	1
6	DIEA-CH_2Cl_2, 1:39 (1 time)	2.5
7	CH_2Cl_2 (2 times)	1
8,10,12	Repeat step 6	
9,11	Repeat step 7	
13	CH_2Cl_2 (6 times)	1
14	Pre-mix reaction mixture (1 time)	30
15	3.3 ml of 0.4 M DIEA/CH_2Cl_2	15
16	CH_2Cl_2 (3 times)	1
17	Ethanol-CH_2Cl_2 (3 times)	1

Table 14

Amino Acid Analyses of Synthetic Prolactin-(37-55)

Amino Acid	Theoretical	Acid Hydrolysate	Enzyme Digest[a]
Lys	2	2.07	2.03
Arg	1	1.00	1.01
Asp	1	2.02	1.06
Asn	1		
Thr	1	0.95	3.16
Gln	1		
Glu	1	2.05	0.98
Gly	2	1.89	1.86
Ala	2	1.98	2.06
Met	1	0.97	0.95
Ile	1	0.90	0.94
Leu	1	1.01	1.00
Tyr	1	1.06	1.02
Phe	3	3.00	3.00

[a] Digestion with chymotrypsin for 24 h at 37°, followed by 24-h digestion with leucine aminopeptidase.

CHAPTER 7

SYNTHESIS OF THE AMINO-TERMINAL FIFTY-FOUR RESIDUE PEPTIDE FRAGMENT OF HUMAN GROWTH HORMONE AND ITS IMMUNOLOGICAL AND BIOLOGICAL ACTIVITIES

Introduction

In Chapter 1 it was noted that about 60 peptide fragments of HGH have been synthesized. Of these syntheses, fifteen were fragments synthesized to determine if an "active core" for the growth-promoting activity resided in the middle third of the molecule. These studies stemmed from the isolation of a fragment from bovine growth hormone that was reported to have growth-promoting activity (Yamasaki et al., 1970), but a definitive study to answer the "active core" idea is still lacking. It has been suggested that HGH-(1-134) has the full growth-promoting potential of HGH (Kostyo et al., 1976). The chemistry and biology of this fragment have been actively studied by Li and colleagues as noted in Chapter 1 (also see Li et al., 1976). Most of the fifteen synthetic fragments above correspond to regions in the carboxyl-terminal portion of the HGH-(1-134) fragment. However, much less attention has been given to the amino-terminal region of HGH. The insulin-potentiating activity of synthetic peptides containing residues 6-13 has been reported (Bornstein, 1976), while a lipolytically active fragment, residues 31-44, has been both isolated from HGH and synthesized (Keda et al., 1973; Yudaev et al., 1976). Thus, it was decided to synthesize an amino-terminal fragment of large enough size so that it

would be possible to study the two activities above and eventually obtain a fragment that could form the first disulfide bridge linking residues 53 and 165. This chapter describes the synthesis of the amino-terminal 54 residue fragment of HGH with the sulfhydryl group permanently blocked, [Cys(Cam)^{53}]-HGH-(1-54). The differences between two syntheses of the fragment which differ mainly in the coupling method used will be described, along with the immunoreactivity and lipolytic activity of the final product.

Experimental Section

Methods and Materials. The methods and materials which have not been described in the previous chapters are below.

In this chapter, the tlc (BPAW) system means 1-butanol-pyridine-acetic acid-water (5:5:1:4). Paper chromatography was performed using the upper phase of 1-butanol-acetic acid-water (4:1:5). Partition chromatography on Sephadex G-50 was performed by procedures previously described (Yamashiro & Li, 1973b). Chromatograms were delineated by either absorbance at 280 nm, Folin-Lowry analysis (Lowry et al., 1951), and/or an automatic fluorescence monitor (Böhlen et al., 1975).

Isoelectric focusing on polyacrylamide gel (IFPA; Catsimpoolas, 1970) was carried out at 20° in gels of 2% Ampholine pI 5-8 (LKB, Bromma, Sweden), 8 M urea, 6% T, 15% C_{DATD} (DATD, N,N'-diallyltartardiamide), where %T = (g acrylamide + g cross-linker)/100 ml and %C = cross-linker (100/%T), 0.06% (w/v) ammonium persulfate, 2×10^{-3}% (w/v) riboflavin

and 5×10^{-4}% TEMED (TEMED, N,N,N',N'-tetramethylethylenediamine). The acrylamide, ammonium persulfate, riboflavin, DATD, and TEMED were obtained from Bio-Rad Laboratories. The upper and lower buffers were 0.1 N KOH and 0.1 N H_2SO_4, respectively. The preparative gels (1.45 x 13 cm) were run in itially at a constant current of 1 mamp/cm^2 gel for 30 min, then at a constant voltage of 100 V for 16 h. The analytical gels (0.6 x 13 cm) were run in the same manner and the bands detected by precipitation with 12% (w/v) aqueous trichloroacetic acid for 20 min, removal of the excess trichloroacetic acid by three 30-min water washes, and staining with Coomassie Brilliant Blue G-250 (Serva, Heidelberg, West Germany) in a perchloric acid solution (Reisner et al., 1975).

Fluorine analysis was performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

The lipolytic assay using isolated rabbit fat cells was performed as previously reported (Ramachandran et al., 1972) by Dr. A. J. Rao.

Antiserum to [Cys(Cam)^{53}]-HGH-(1-54) was prepared by the immunization of rabbits with 0.20 mg antigen per week over a period of 7 weeks according to previously reported procedures (Vaitukaitis et al., 1971). Antisera to HGH and [Cys(Cam)^{53}]-HGH-(1-134) were prepared by the immunization of rabbits as previously described (Clarke et al.,1974) and were obtained from Dr. C. H. Li, along with samples of HGH,

[Cys(Cam)^{53}]-HGH-(1-134) and [Cys(Cam)^{53}]-HGH-(15-125). Microcomplement fixation was carried out as previously reported (Wasserman & Levine, 1961). The fixation of complement was conducted in a volume of 1 ml for 14 h at 4°. The volume was increased to 7 ml by the addition of activated sheep erythrocytes and the tubes were incubated for 1 h at 37°. After cooling to 0° and centrifugation to remove the unlysed cells, the absorbance of the supernatant solution was measured at 413 nm.

Attempted Synthesis of [Cys(Cam)^{53}]-HGH-(1-54) by Standard Coupling Method. Boc-Phe resin prepared by the modified Loffet method (Yamashiro & Li, 1973b) gave a substitution of 0.430 mmol/g as estimated by the method of Gisin (1972). The Boc-Phe resin (1.30 g, 0.56 mmol) was placed in a Beckman Model 990 peptide synthesizer and subjected to 53 cycles of synthesis by the schedule in Table 15. The volumes used were 25 ml, except: step 9 was a 15-ml solution of Boc-amino acid in CH_2Cl_2; step 10, a 4-ml solution of 0.56 M DCCI in CH_2Cl_2; and step 11, 3.3 ml of 0.68 M DIEA in CH_2Cl_2 (Yamashiro & Li, 1974). N^{α}-Boc protection was used throughout with the following side-chain protecting groups: Lys, Z(2-Br); Tyr, Z(2-Br); Arg, Tos; His, Boc; Asp, OBzl; Glu; OBzl; Ser, Bzl; Thr, Bzl; and Cys, Cam. Boc-Asn and Boc-Gln were coupled as the p-nitrophenyl ester (10 equiv, 5.6 mmol for 10 h) in

DMF in step 10, which was preceeded and followed by replacement of steps 9 and 12 by 1-min DMF washes (3 times) and elimination of step 11. Boc-Arg(Tos)-OH was first dissolved in 1.5 ml of DMF and then diluted to 15 ml with CH_2Cl_2. The weight of the finished protected peptide resin was 4.00 g, 54% yield based on theoretical weight gain. The amine content of a deprotected and neutralized sample gave 39% of theoretical by the Schiff base method. Resin hydrolysis (Scotchler et al., 1970) of a sample (20.6 mg) followed by amino acid analysis gave an amount of S-carboxymethylcysteine equal to 86% of the theoretical Cys(Cam) content of the peptide resin.

A portion (1.00 g) of the final protected peptide resin was treated with liquid HF (10 ml) in the presence of anisole (1.0 ml) for 1 h at 0°. After removal of the HF by a stream of nitrogen, the peptide-resin mixture was stirred with 15 ml of ethyl acetate for 15 min, filtered and dried under reduced pressure. Then the peptide-resin mixture was stirred with 15 ml of TFA for 20 min, filtered and the filtrate evaporated to an oil. After dissolving the oil in 5 ml of acetic acid and reevaporation, the peptide material was submitted to gel filtration on Sephadex G-10 (2.16 x 26 cm) in 20% aqueous acetic acid. A single symmetrical peak (V_e of peak, 30 ml) was detected and the peptide material isolated by lyophilization gave 384 mg. A sample (51 mg) was subjected to chromatography on Sephadex G-25 in 0.5 M acetic acid (Figure 20) and resulted

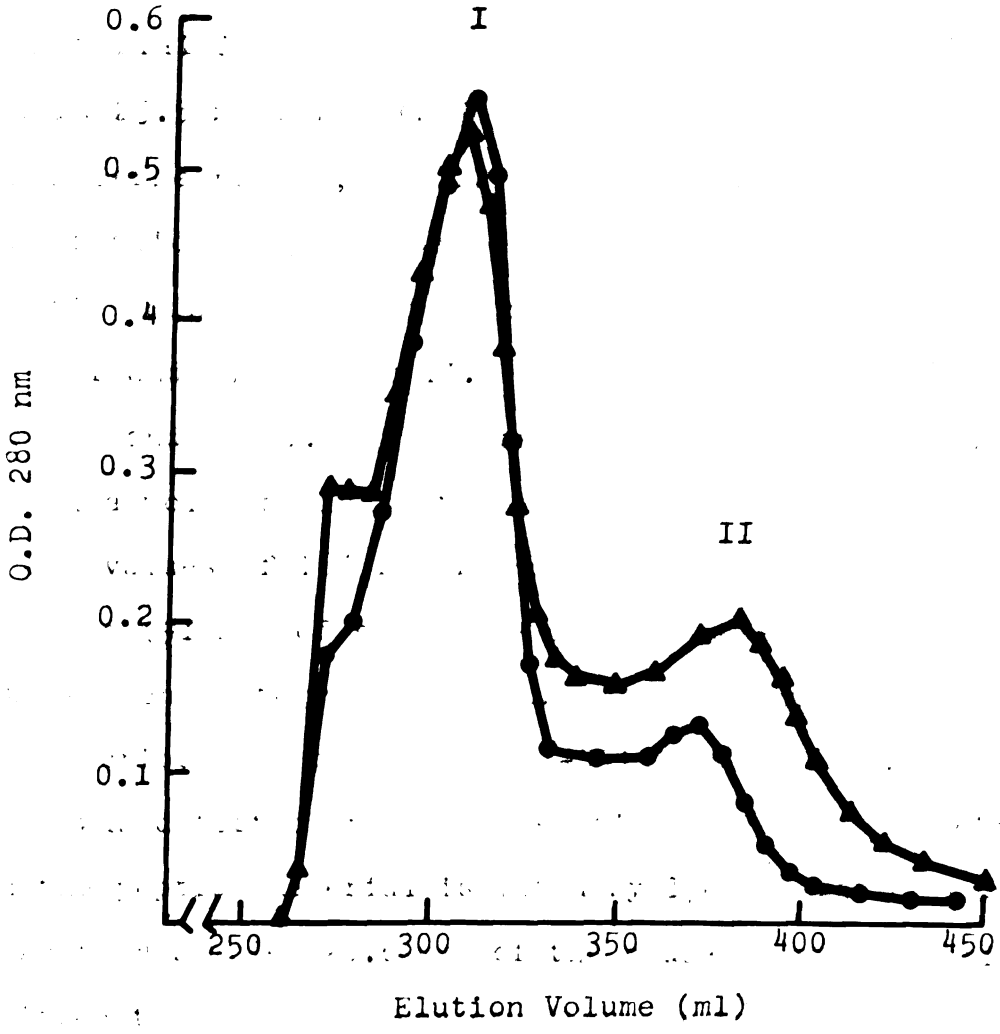


Figure 20. Gel filtration on Sephadex G-25 (2.51 x 140 cm) in 0.5 M acetic acid of crude peptide material from the standard coupling synthesis (▲—▲) and the pre-mix coupling synthesis (●—●) of [Cys(Cam)53]-HGH-(1-54).

I
II

in two peaks (I, V_e, 283-320 ml; II, V_e, 321-435 ml). The peptide material in each peak isolated by lyophilization resulted in 23.5 mg for I (22% overall yield based on starting Boc-Phe resin), and 12.0 mg for II. Amino acid analyses of acid hydryolysates of I and II (Table 16) showed a diagnostic amino acid ratio for Met^{14} to $Cys(Cam)^{53}$ of 0.95:1 for I and 0.17:1 for II. When the fluorine content of I (0.13%) and II (1.00%) was calculated as CF_3CO- and compared on a molar basis to the amount of Cys(Cam) in each sample; the values of 16% for I and 80% for II were obtained.

A sample (91 mg) of the crude peptide material from Sephadex G-10 chromatography was submitted to CMC chromatography as described for ovine LTH-(37-55) in Chapter 6. A major peak was detected only after elution with 2.0 M acetic acid and the peptide material isolated by lyophilization was 13 mg. Another sample (20.6 mg) of the crude peptide material was submitted to chromatography on CMC under the conditions of Katsoyannis et al.(1967) employing 8 M urea in the buffers. A single unretarded peak was detected, which after dialysis and lyophilization gave 8.3 mg. When a sample (0.350 mg) of I (from the above chromatography on Sephadex G-25) was submitted to analytical isoelectric focusing on polyacrylamide gels (IFPA) in 8 M urea and stained as described above, the pattern of three major bands (pI 6.93, 6.38, and 5.83) in Figure 21 was obtained.

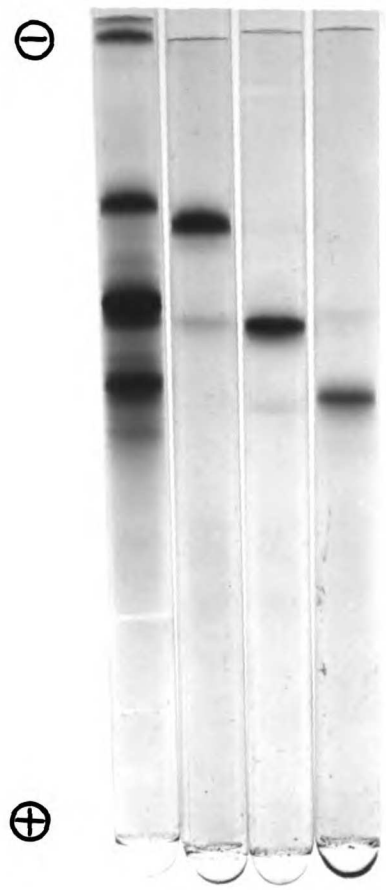


Figure 21. Isoelectric focusing on polyacryl amide gel (IFPA) in 8 M urea and 2% Ampholine, pI 5-8, of (A) 0.35 mg of I resulting for the 'in situ' synthesis of [Cys(Cam)^{53}]-HGH-(1-54) and chromatography on Sephadex G-25 and (B,C,D) 0.10 mg samples of the three major peptide materials (pI 6.93, 6.38, and 5.83) isolated from preparative IFPA.

Peptide material corresponding to I was combined from several chromatographic runs on Sephadex G-25 and subjected to preparative IFPA in 8 M urea: 54 mg on 9 separate gels in the same focusing run. At the end of the focusing, one gel was treated with 12% (w/v) aqueous trichloroacetic acid to locate the three major bands. The regions corresponding to these bands were cut out of the remaining 8 gels and combined according to region. Then separately the regions were minced with a razor blade, stirred in 60 ml of 20% aqueous acetic acid for 24 h, and filtered. The filtrates were dialysed against water and lyophilized. Each of the three resulting peptide materials were chromatographed first on Sephadex G-25 (1.89 x 20.5 cm) in 0.5 M acetic acid (isolated by lyophilization), and then on Sephadex G-100 (1.89 x 64.5 cm) in 0.5 M acetic-1 mM NH_4OAc, and isolated by repeated lyophilization. Samples (0.10 mg) of each of the three peptide materials were submitted to analytical IFPA (Figure 21). The three peptide materials by amino acid analyses of acid and enzyme (24 h with chymotrypsin and trypsin, followed by 48 h with leucine aminopeptidase) hydrolysates showed no significant differences, and the values were quite acceptable for those expected of [Cys(Cam)^{53}]-HGH-(1-54). Based on the amount of Leu present, the total yield of the three bands from IFPA was 11% of theoretical; and the materials with pI of 6.93, 6.38, and 5.83 were,

respectively, 32%, 48%, and 20% of the total isolated material. When a 1.15-mg sample of the pI 6.38 peptide material (from the middle band) was submitted to partition chromatography on Sephadex G-50 (0.96 x 19.5 cm) in 1-butanol-acetic acid-water (4:1:5), a very unsymmetrical peak with R_f 0.53 was detected.

Synthesis of [Cys(Cam)^{53}]-HGH-(1-54) by Pre-Mix Method. The above Boc-Phe resin (1.16 g, 0.50 mmol) was placed in a Beckman Model 990 peptide synthesizer and subjected to 53 cycles of synthesis by the schedule in Table 17. The volumes used were 27 ml, except for step 9, the pre-mix reaction mixture, and step 10, addition of 0.5 ml of 3.0 M DIEA in CH_2Cl_2 (Yamashiro & Li, 1974). The protecting groups employed were the same as described in the above synthesis. While Boc-Gln was coupled after reaction with DCCI (Hemmasi & Bayer, 1974), Boc-Asn was coupled as the p-nitrophenyl ester (10 equiv, 5.0 mmol for 10 h) in DMF in step 16, which was preceeded and followed by replacement of CH_2Cl_2 by DMF in steps 15 and 18, and elimination of step 17. The pre-mix reaction (Hagenmaier & Frank, 1972) mixture was prepared as follows: 3.0 mmol (6 equiv) of Boc-amino acid in 9 ml of CH_2Cl_2 were cooled to 0° and mixed with 2.0 ml of 0.75 M DCCI in CH_2Cl_2. After the mixture was stirred for 15 min at 0°, the precipitate was filtered at 25° and washed with 1 ml of CH_2Cl_2. The filtrate was added immediately to the resin manually followed

by a rinse of 2 ml of CH_2Cl_2. The only exceptions to this procedure were for the incorporation of the following residues: Cys(Cam), Gln, Arg(Tos), Leu and Ile. Respectively, the derivatives of these residues were first dissolved in DMF and then diluted to 9 ml with CH_2Cl_2 to give a final DMF/CH_2Cl_2 solution of 50%, 50%, 22%, 5.5% and 5.5% prior to reaction with DCCI. For these solutions the reaction mixture was stirred for 20 min at 0°. The finished peptide resin was dried under reduced pressure over P_2O_5 to give 4.57 g, 76% yield based on theoretical weight gain. The amine content of a deprotected and neutralized sample gave 68% of theoretical by the Schiff base method. The final peptide resin had an amount of S-carboxymethylcysteine [Cys(Cam)] equal to 95% of the theoretical Cys(Cam) content when analysed by the procedure described for the above synthesis.

Isolation of [Cys(Cam)^{53}]-HGH-(1-54). The protected peptide resin (1.01 g) was treated as follows to avoid the sulfonium ion formation discussed in Chapter 6 and Noble et al. (1976): a 15-min treatment with 10 ml of TFA-CH_2Cl_2 (1:1); filtration with 25-ml washes of CH_2Cl_2, DIEA-CH_2Cl_2(1:19), CH_2Cl_2, and ethanol; and dried over P_2O_5 under reduced pressure for 90 min. Then, the partially protected peptide resin (980 mg) was treated with HF (10 ml) in the presence of anisole (1.0 ml) for 30 min at -30 to -20° and for 30 min at 0° (Baba et al.,

1973). After removal of the HF at 0° by a stream of nitrogen for 10 min, cold ethyl acetate (25 ml) was added. The peptide-resin mixture was stirred for 20 min at 25°, filtered, dried under reduced pressure over P_2O_5 for 20 min, stirred with 50% aqueous acetic acid (25 ml) for 20 min, and filtered. The filtrate was evaporated to 4 ml and subjected to gel filtration on Sephadex G-10 (1.37 x 44 cm) in 20% aqueous acetic acid. The peptide material (V_e of peak, 33 ml) was isolated by lyophilization to give 514 mg. A sample (50 mg) was subjected to chromatography on Sephadex G-25 in 0.5 M acetic acid (Figure 20) and resulted in two peaks (I, V_e, 283-320 ml; II, V_e, 321-435 ml). The peptide material in each peak isolated by lyophilization resulted in 24.5 mg for I (40% overall yield), and 4.6 mg for II. Amino acid analyses of acid hydrolysates of I and II (Table 16) showed a diagnostic amino acid ratio for Met^{14} to $Cys(Cam)^{53}$ of 0.96:1 for I and 0.13:1 for II. When the fluorine content of I (0.072%) and II (0.60%) was calculated as CF_3CO- and compared on a molar basis to the amount of Cys(Cam) in each sample, the values of 8% for I and 46% for II were obtained. A total of 464 mg of crude peptide material from Sephadex G-10 chromatography when submitted to 8 separate (ca. 55 mg each) chromatographic runs on Sephadex G-25 (2.4 x 137 cm) in 0.5 M acetic acid gave a combined yield of 267 mg of peptide material corresponding

to I in Figure 20. Samples of I, 150 and 50 μg, were submitted to analytical IFPA. As shown in Figure 22, the same pattern of three major bands with pI 6.93, 6.38 and 5.83 as in the first synthesis was obtained. A sample (49.7 mg) was subjected to preparative IFPA on 10 separate gels as described above. At the end of the focusing period, the gels were removed from the tubes and placed for 2 min in water, which resulted in removal of enough urea from the gel that the peptide material precipitated and allowed detection of the main band, pI 6.38. The combined gel regions of the main band were minced with a razor blade, stirred with 150 ml of 5 M acetic acid for 12 h at 25°, and filtered. The filtrate was dialysed against 2 one-liter portions of water for 6 and 3 h, respectively. The non-diffusible material which still contains urea and Ampholine was isolated by lyophilization and then subjected to gel filtration on Sephadex G-100 as described for the above synthesis. The peptide material (V_e of peak, 155 ml) was isolated by lyophilization and resubmitted to the Sephadex G-100 chromatography (Figure 23). The main peak (V_e, 140-171 ml) was isolated by repeated lyophilization to yield 22.8 mg, 19% overall yield based on starting Boc-Phe resin. A sample, 21.0 mg, of this peptide material was submitted to partition chromatography on Sephadex G-50 (1.89 x 31.6 cm) in 1-butanol-acetic acid water (BAW, 4:1:5) (Figure 24). A major peak

Figure 22. Isoelectric focusing on polyacrylamide gel in 8 M urea. (A) 0.15 mg of I resulting from the 'pre-mix' synthesis of [Cys(Cam)^{53}]-HGH-(1-54) and chromatography on Sephadex G-25 using 2% Ampholine, pI 5-8. (B) 0.05 mg of I from the same sample and under the same conditions used in (A) except the buffers were reversed and 2% Ampholine, pI 3.5-10, was used.

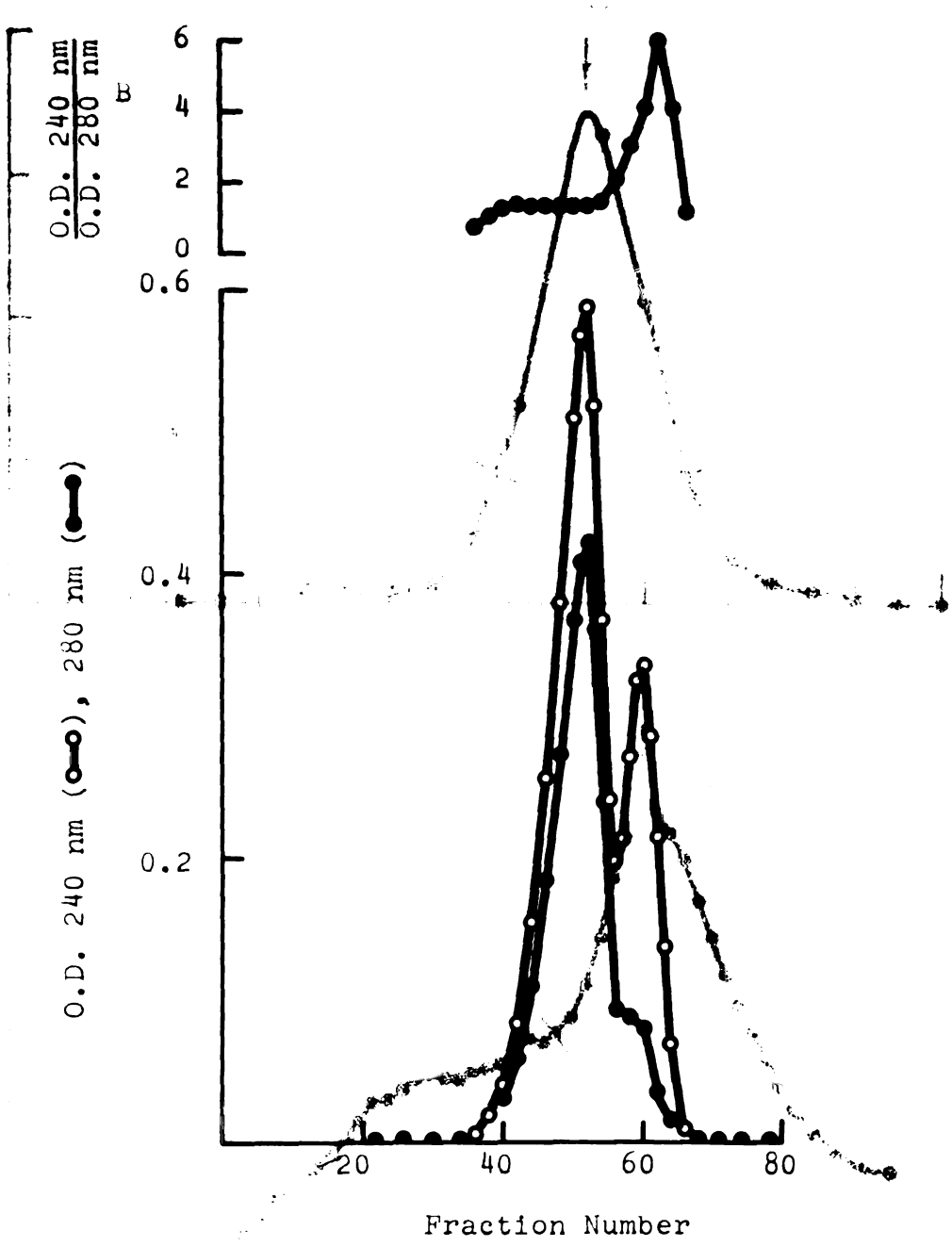


Figure 23. Rechromatography on ~~Sephadex G-100 (see text for conditions)~~ of peptide material with pI 6.38 from preparative IFPA: yield (fractions 45-55, 3.1 ml/fraction), 22.8 mg.

[illegible]
[illegible] (280 tons) [illegible]
ditions) of peptide [illegible]
ml 6.30 from [illegible] and yield
(fractions 45-52, 3.1 [illegible]),
52.0 mg.

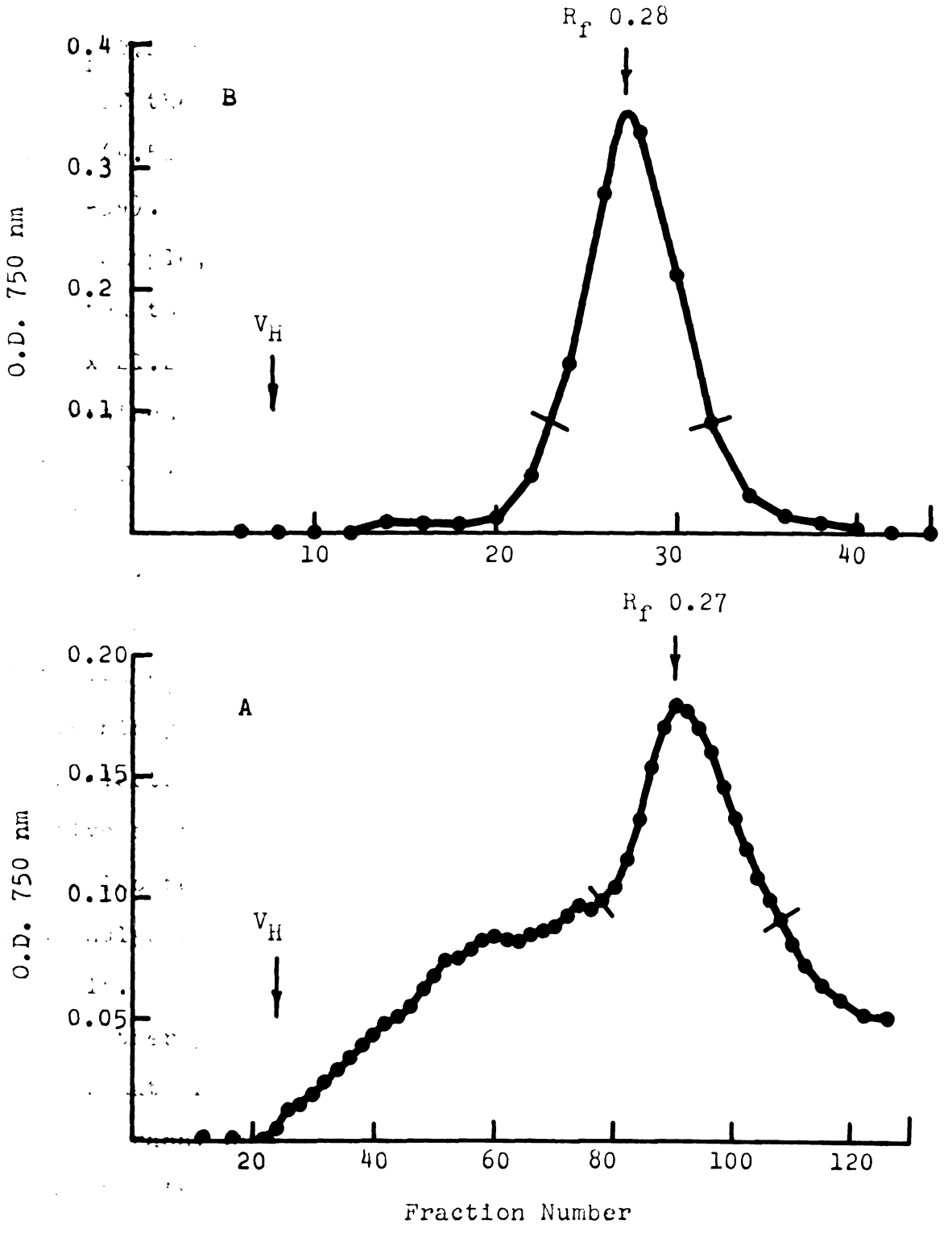


Figure 24. (A) Lower: partition chromatography of 21.0 mg of peptide material with pI 6.38 on Sephadex G-50 (1.89 x 31.6 cm), yield (fractions 78-108, 0.93 ml/fraction), 5.1 mg. (B) Upper: partition chromatography of 1.85 mg of highly purified [Cys(Cam)^{53}]-HGH-(1-54) on Sephadex G-50 (0.96 x 21.2 cm), yield (fractions 23-32, 0.46 ml/fraction), 1.70 mg.

B
A

with R_f 0.27 with tailing on both sides was detected. Isolation of the peptide material between R_f 0.31 and 0.22 gave 5.1 mg (4.5% overall yield) of highly purified [Cys(Cam)^{53}]-HGH-(1-54).

A sample, 1.85 mg of the R_f 0.27 material, was again subjected to partition chromatography on Sephadex G-50 (0.96 x 21.2 cm) and a single symmetrical peak with R_f 0.28 was obtained as shown in Figure 24. Samples, 50 and 100 µg, of the R_f 0.27 material subjected to analytical IFPA as described above gave single bands with pI 6.38 as shown in Figure 25.

Paper chromatography gave a single ninhydrin-positive spot with R_f 0.24. Thin-layer chromatography (BPAW) gave a single ninhydrin-positive, chlorine-positive spot, R_f 0.56. Paper electrophoresis (pH 2.1, 2 kV, 1 h) gave one ninhydrin-positive spot, R_{Glu} 0.74. Phenylalanine was shown to be the only amino terminal amino acid by the dansyl technique. Amino acid analyses of acid and enzyme hydrolysates are shown in Table 18.

Another portion of the peptide material I (254 mg) from the chromatography on Sephadex G-25 was subjected to partition chromatography on Sephadex G-50 (1.89 x 32 cm) in the above BAW system in four separate batches (ca. 60 mg each). The peptide material isolated from the peaks with R_f 0.27 were combined to give 98.6 mg, 16% overall yield. A sample of this peptide material (48.7 mg) was subjected to preparative IFPA and the main band (pI 6.38) isolated by extraction and gel

Figure 25. Isoelectric focusing on polyacrylamide gel (IFPA) in 8 M urea and 2% Ampholine, pI 5-8, of samples, (A) 0.05 mg and (B) 0.10 mg, of highly purified [Cys-(Cam)^{53}]-HGH-(1-54) obtained from preparative IFPA (pI 6.38) and subsequent partition chromatography on Sephadex G-50 (see text for details).

filtration as described above to yield 23.7 mg. All of this peptide material was again subjected to partition chromatography on Sephadex G-50 as above and isolation of the major peak with R_f 0.24 gave 6.8 mg, 2.2% overall yield, of highly purified [Cys(Cam)^{53}]-HGH-(1-54).

Treatment of [Cys(Cam)^{53}]-HGH-(1-54) with HF. A sample (1.31 mg, 200 nmol) of the highly purified [Cys(Cam)^{53}]-HGH-(1-54) was treated for 30 min at 0° with HF (5 ml). The HF was removed and the peptide sample (III) dried *in vacuo* over NaOH. Another 1.13-mg sample was treated with HF (5 ml) for 30 min at 0° in the presence of anisole (0.5 ml) and 8 mg of N^{α}-Ac-Ser(Bzl)-NH_2 [34 μmol, a 10-fold excess of Bzl groups over the amount of Bzl and Z(2-Br) groups on the final protected peptide resin]. After the HF was removed, the peptide material was dissolved in 5 ml of 1.0 *M* acetic acid, washed with three 2-ml portions of ethyl acetate, and recovered by lyophilization to give IV. Both III and IV (*ca.* 260 μg) were subjected to analytical IFPA along with an untreated sample (100 μg) of the synthetic HGH fragment. After the focusing period, the peptide materials were precipitated with 12% (w/v) aqueous trichloroacetic acid (Figure 26A) and then stained with Coomassie Brilliant Blue G-250 (Figure 26B). Both of the treated samples had two major bands in about a 1:1 ratio. One band corresponded to the starting material,

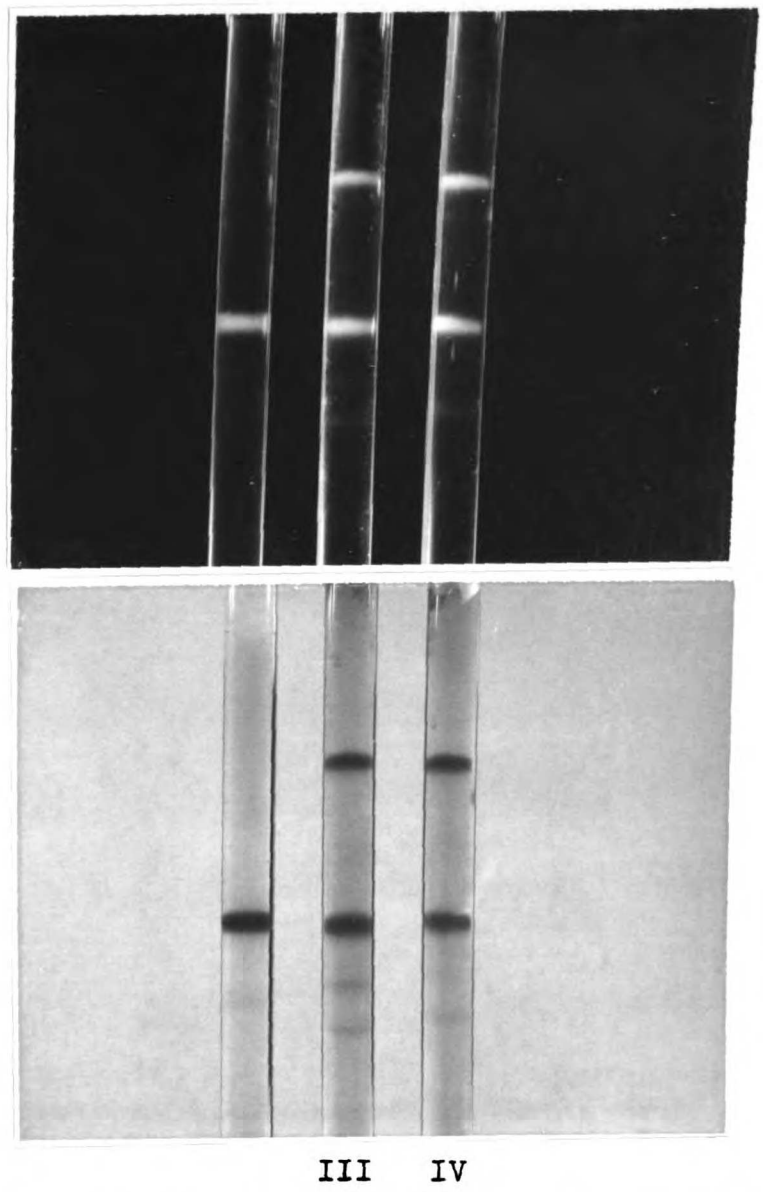


Figure 26. Isoelectric focusing on polyacrylamide gel in 8 M urea and 2% Ampholine, pI 5-8., of highly purified [Cys(Cam)^{53}]-HGH-(1-54) before (0.10 mg sample on left) and after (0.26 mg each of III and IV) treatment with HF. See text for difference between III and IV. (A) Upper: After trichloroacetic acid precipitation. (B) Lower: Same gels as above after staining.

while the other band ran with pI 6.93 as seen in the sample of I (Figure 22).

Attempted CNBr Cleavage of [Cys(Cam)^{53}]-HGH-(1-54). For reaction with CNBr (Gross & Witkop, 1962), a sample (2.2 mg) of the R_f 0.27 peptide material from the above Sephadex G-50 partition chromatography was dissolved in 1.0 ml of 70% formic acid (Steers et al., 1965). To this solution, 0.100 ml of a solution of 23.20 mg of CNBr in 1.52 ml of 70% formic acid was added. After 16 h at 25° in the dark, water (10 ml) was added and the solution lyophilized. The peptide material was subjected to chromatography on Sephadex G-50 (1.15 x 42 cm) in 20% aqueous acetic acid. As shown in Figure 27, two peptide-containing peaks (V_e of peaks, 36 and 49 ml) were detected and isolated by lyophilization to give V (V_e of peak, 36 ml) and VI (V_e of peak, 49 ml). Amino acid analyses of acid hydrolysates of V and VI are shown in Table 19.

Results and Discussion

The best extant techniques were used in the first attempt to synthesize [Cys(Cam)^{53}]-HGH-(1-54). These techniques as described in the Experimental Section included: an attachment method that did not introduce quaternary ammonium sites on the resin; an efficient deprotection step (Reid, 1976); a more sterically hindered agent for neutralization than triethylamine which can form quaternary ammonium sites on the resin; and

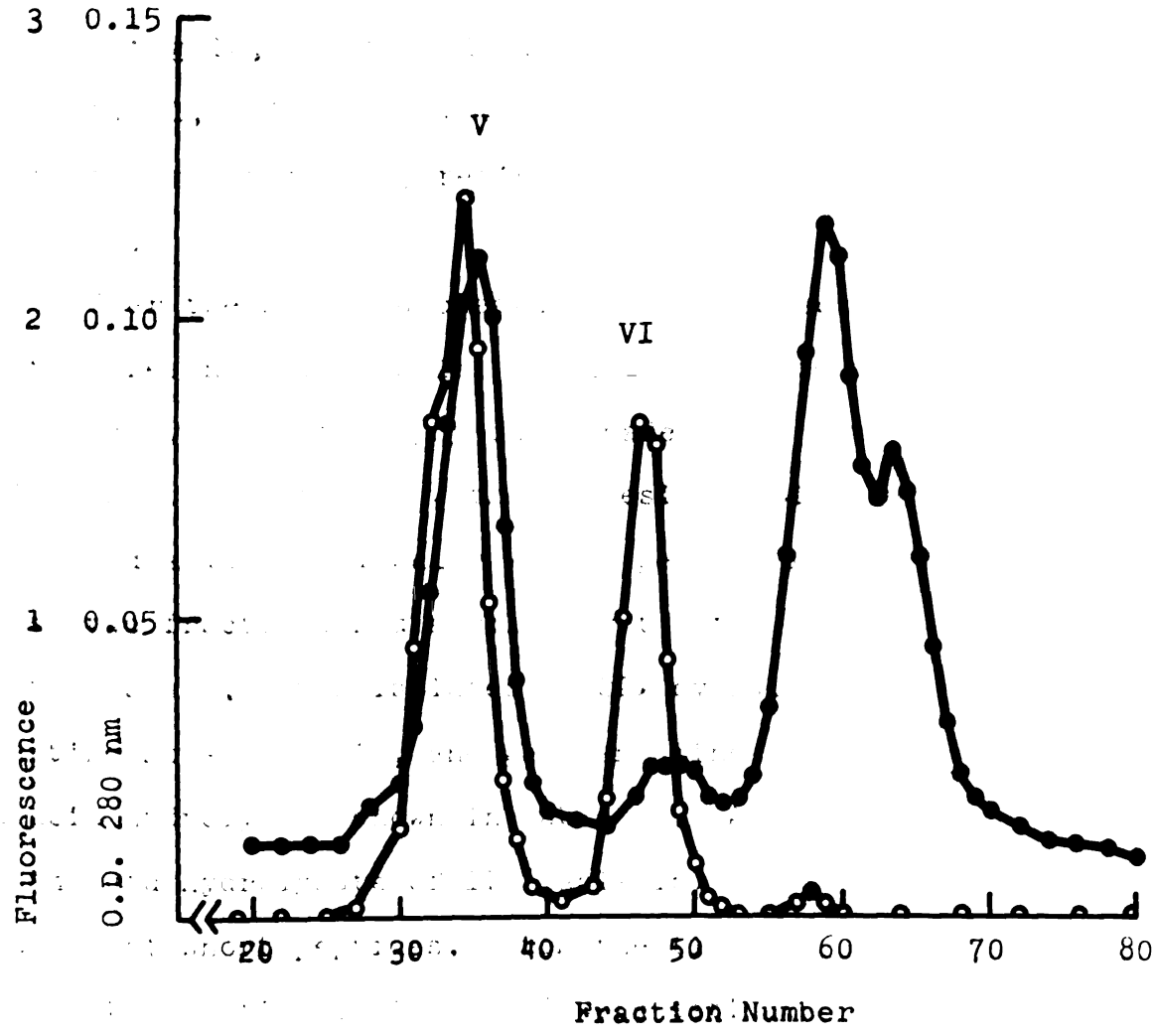
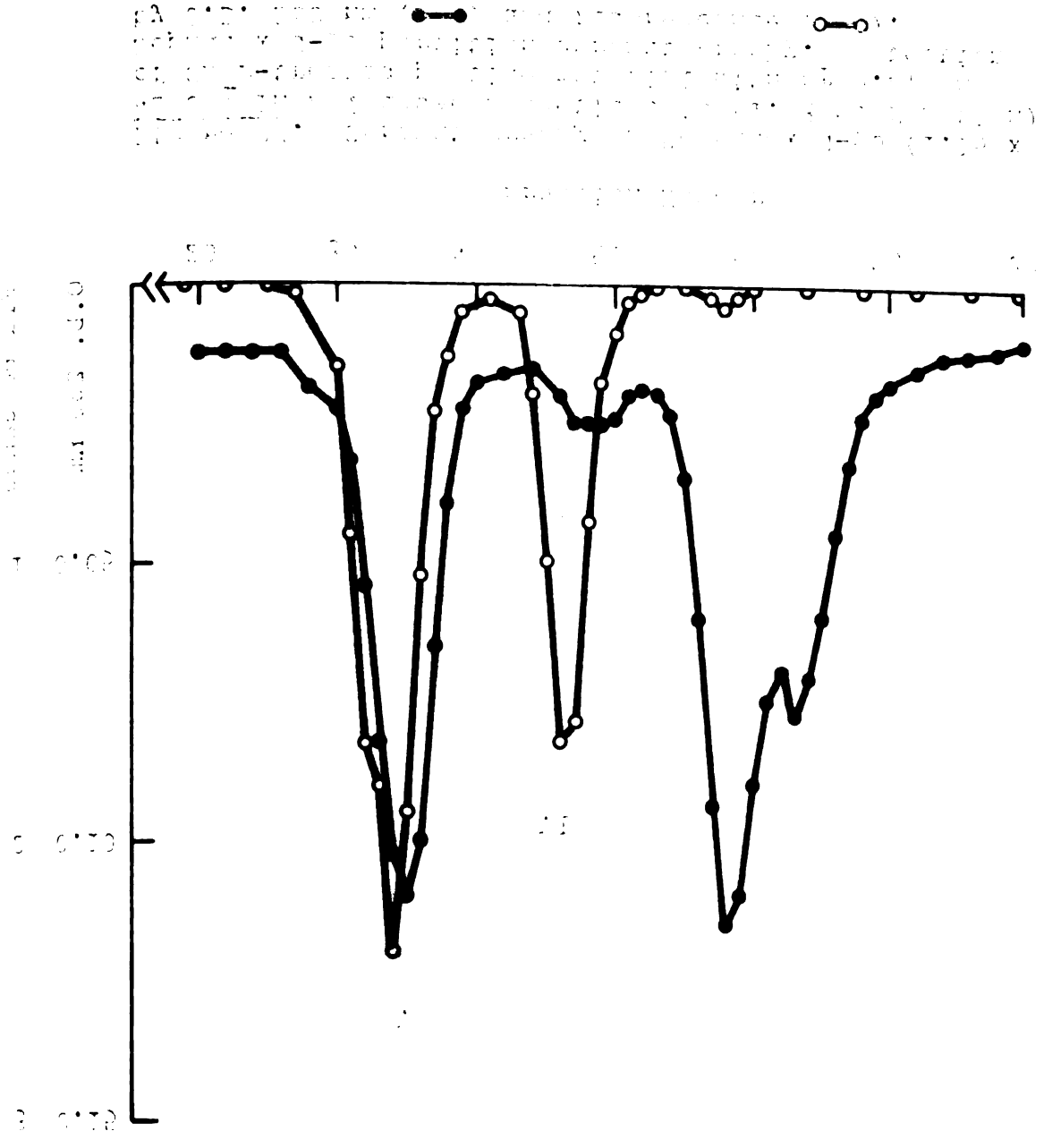


Figure 27. Chromatography on Sephadex G-50 (1.15 x 42 cm) in 20% aqueous acetic acid (1.03 ml/fraction) of CNBr-treated peptide material with R_f 0.27 on Sephadex G-50 partition chromatography. Detection by O.D. 280 nm (●—●) and fluorescence (○—○).



the standard coupling method employing 4 equivalents each of Boc-amino acid and DCCI. Nevertheless, the yield of the final protected peptide resin was 54% of theoretical based on weight gain, and the final amine content was 39% of theoretical, while 86% of the theoretical Cys(Cam) residue content was obtained by resin hydrolysis and amino acid analysis.

After the standard cleavage and deprotection with HF in the presence of anisole, the crude peptide material was resolved by chromatography on Sephadex G-25 into a fraction (I) of peptide material with the desired molecular weight range and a lower molecular weight peptide fraction (II). These two fractions had a 2:1 weight ratio, respectively. At this stage, I was isolated in 22% overall yield based on starting Boc-Phe resin, and gave an amino acid analysis of an acid hydrolysate shown in Table 16. Amino acid analysis of an acid hydrolysate of II (Table 16) indicated II was a mixture of short peptides. From the fluorine content of II, 80% of the peptide chains of II were estimated to be terminated by a trifluoroacetyl group. This led to a resynthesis by an alternative coupling technique which had been used with success in the synthesis of much smaller peptides.

The second synthesis was carried out essentially in the same manner as the first, except that DCCI-mediated couplings were performed by the preformed symmetrical anhydride (pre-mix)

method (Hagenmaier & Frank, 1972). The yield of the final protected peptide resin was 76% of theoretical based on weight gain. The final amine and Cys(Cam) contents were 68% and 95% of theoretical, respectively. The deprotection and cleavage step incorporated the initial lower temperatures introduced by Baba et al. (1973). As a result of the above improvements, the weight ratio of I to II from chromatography on Sephadex G-25 was 6:1, and I was isolated in 40% overall yield at this stage. From the fluorine content of II, 46% of the peptide chains of II were estimated to be trifluoroacetylated. The amino acid analyses of acid hydrolysates of I and II are given in Table 16.

Although I was insoluble in aqueous buffers normally employed in ion exchange chromatography, it was soluble in 8 M urea solutions. Attempted purification on CMC chromatography in 8 M - 0.04 M sodium acetate, pH 4.0 (Katsoyannis et al., 1967) resulted in a single unretarded peak. When I from the first synthesis was submitted to analytical isoelectric focusing on polyacrylamide gel (IFPA) in 8 M urea, a separation of three main peptide species was detected (Figure 21). Then, it was shown that from preparative IFPA of I from the first synthesis, the three peptide materials could be isolated on the basis of charge in fairly homogeneous states (Figure 21). Therefore, I from the pre-mix synthesis (which showed the pattern in Figure 22 on analytical IFPA) was purified on the

basis of charge by preparative IFPA in 8 M urea, 2% Ampholine pI 5-8. Thus, I was separated into a main band (ca. 75% of the total) with pI 6.38 (uncorrected for presence of urea; Ui, 1973), two minor bands with pI 6.93 and 5.82 (ca. 20% and 5% of the total, respectively) and trace bands between pI 6.93 and 5.82. After extraction, dialysis and gel filtration (to remove the Ampholine; Bauman & Chrambach, 1975) on Sephadex G-100, the peptide material with pI 6.38 was further purified by partition chromatography on Sephadex G-50 (Figure 24A) to give the final product in 4.5% overall yield. Whether I was or was not submitted to partition chromatography on Sephadex G-50 prior to IFPA, the band pattern and relative intensities were the same, indicating the impurities could not be eliminated solely by either partition chromatography or IFPA.

The highly purified [Cys(Cam)^{53}]-HGH-(1-54) gave a single symmetrical peak on partition chromatography on Sephadex G-50 (Figure 24B) and a single band with pI 6.38 on analytical IFPA (Figure 25). Amino acid analysis of an acid hydrolysate gave values shown in Table 18, in agreement with expected values. Amino acid analysis of an enzyme digestion with acid protease, trypsin and chymotrypsin, followed by leucine aminopeptidase gave the values also shown in Table 18, again in good agreement with expected values. The synthetic HGH fragment was homogeneous by thin-layer and paper chromatography, and paper electrophoresis. Phenylalanine was the only amino terminal amino acid detected by the dansyl technique.

When the highly purified [Cys(Cam)^{53}]-HGH-(1-54) is retreated with neat HF or with HF under conditions similar to those during deprotection and cleavage from the resin, analytical IFPA (Figure 26) of the resulting peptide materials (respectively, III and IV) showed an approximately 50% conversion to the pI 6.93 species observed in the earlier stages of purification.

Preliminary studies on CNBr cleavage of the synthetic HGH fragment resulted in the isolation of two peptide fractions (V and VI) from chromatography on Sephadex G-50. The amino acid analysis of acid hydrolysates of V and VI are shown in Table 19. The values for VI are in agreement with expected values of HGH-(1-14) isolated from CNBr (loss of methionine, with appearance of homoserine and its lactone). The values for V indicated incomplete separation of [Cys(Cam)^{53}]-HGH-(15-54) from the starting synthetic HGH fragment.

The HGH fragment, HGH-(31-44), obtained from native HGH or syntheses, has been reported to have *in vitro* lipolytic activity over a range of 0.57 to 11.4 μM in rat, rabbit and human adipose tissue (Keda et al., 1973; Yudaev et al., 1976; Pankov et al., 1976). When assayed for lipolytic activity in isolated rabbit fat cells (Ramachandran et al., 1972), synthetic [Cys(Cam)^{53}]-HGH-(1-54) showed no lipolytic activity over a range of 0.18 to 15.3 μM measured by glycerol production over controls (Table 20).

The immunoreactivity of synthetic [Cys(Cam)^{53}]-HGH-(1-54) was assessed by the microcomplement fixation technique. Interaction of 3.06 pmol of the synthetic HGH fragment with rabbit antiserum to HGH and rabbit antiserum to [Cys(Cam)^{53}]-HGH-(1-134) resulted in the equivalent amount of complement fixation, 77% and 80% respectively, as shown in Figure 28. However, as the concentration of the synthetic HGH fragment was increased, the two antisera clearly differed in their immunoresponse to the synthetic fragment. This could reflect the possibility that the conformation of the amino-terminal portion of the 134 residue fragment is more like that of the synthetic HGH fragment ('exposed more') than is the corresponding region in Native HGH. Support for this was that when rabbit antiserum to the synthetic HGH fragment was assayed against the synthetic HGH fragment, [Cys(Cam)^{53}]-HGH-(1-134), [Cys(Cam)^{53}]-HGH-(15-125), and HGH as shown in Figure 29, there was greater cross-reaction between the antiserum and the 134 residue fragment than with HGH. Interestingly, the loss of the first 14 residues from the 134 residue fragment apparently causes a diminution of the cross-reaction; however, the conditions of CNBr treatment employed to obtain [Cys(Cam)^{53}]-HGH-(15-125) may also be responsible for the observed immunoreactivity.

In summary, the amino-terminal 54-residue fragment of HGH, [Cys(Cam)^{53}]-HGH-(1-54), was synthesized twice by the

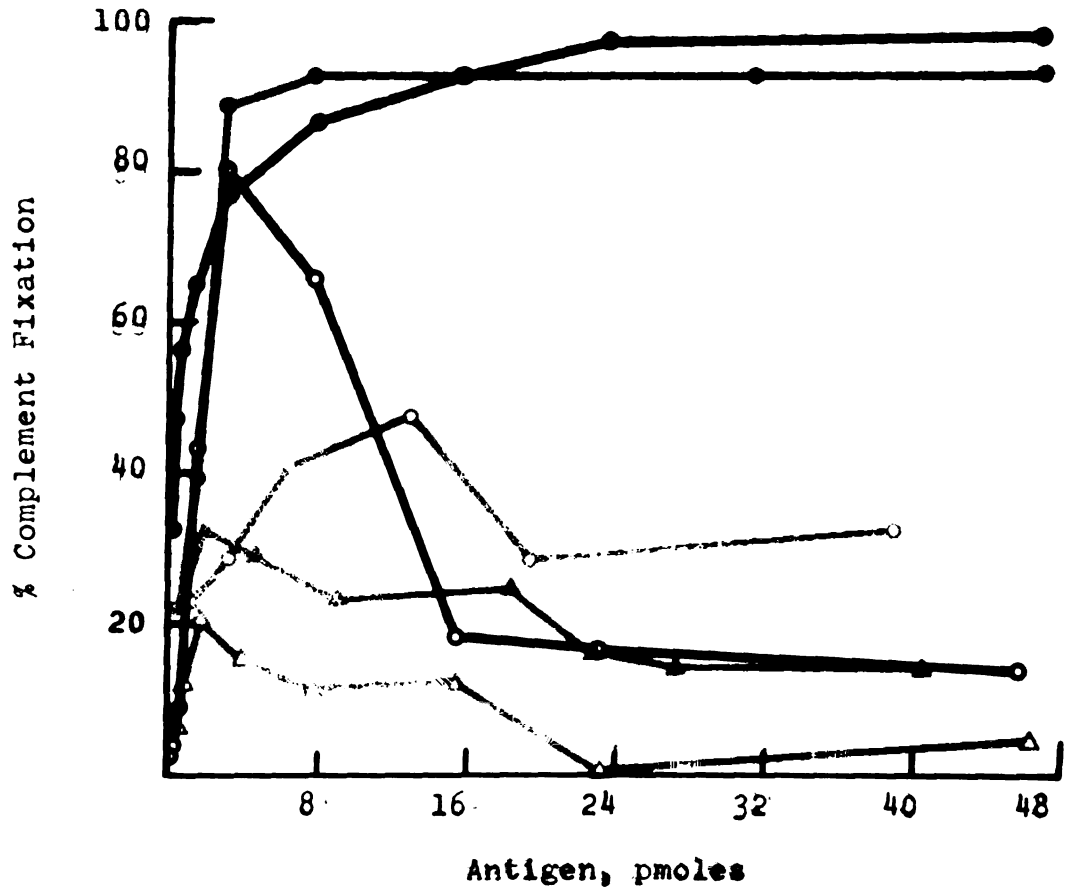
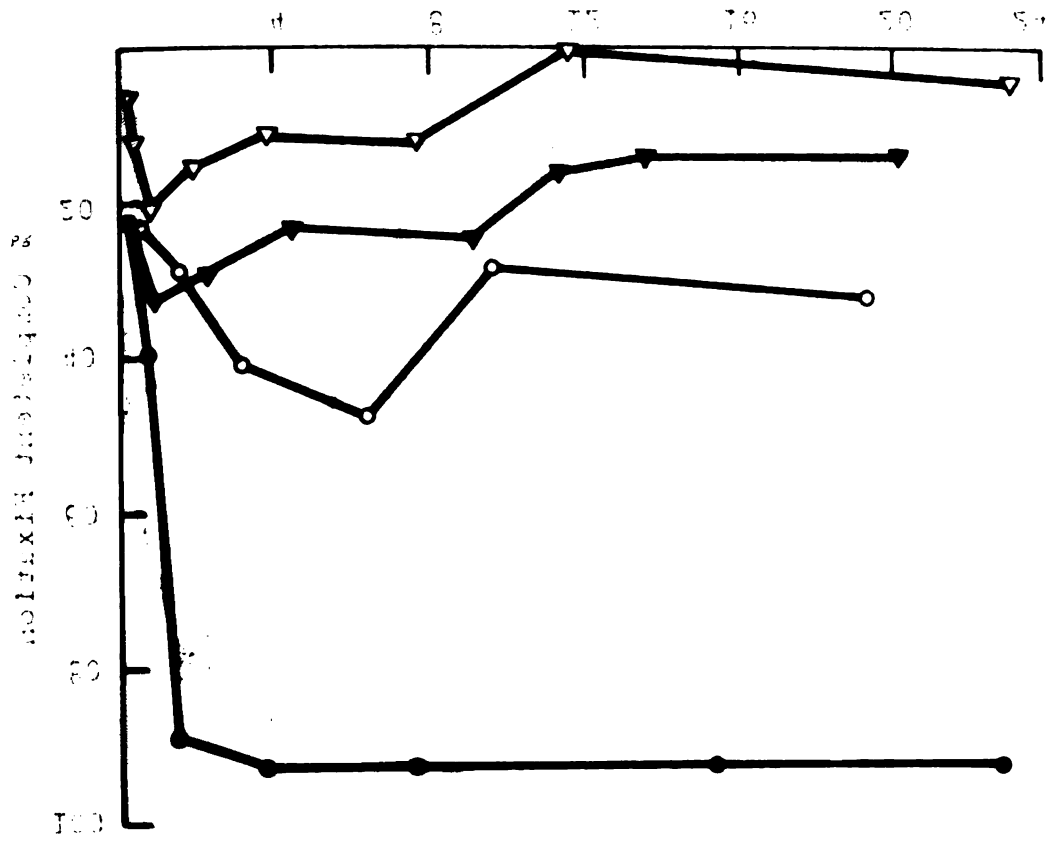


Figure 28. Microcomplement fixation curve obtained with synthetic [Cys(Cam)^{53}]-HGH-(1-54) using rabbit antisera to HGH (●—●) and [Cys(Cam)^{53}]-HGH-(1-134) (○—○). In both cases, the 1 ml reaction mixture contained 100 µl of a 1:20 dilution of antiserum (final dilution, 1:200).



solid-phase method. The first synthesis employed the standard *in situ* coupling method and resulted in chain termination as evidenced by examination of the final peptide resin and of peptide materials obtained during the attempted isolation of the final product. There was no separation of peptide material by partition chromatography on Sephadex G-50 which yielded a major product. However, by IFPA the crude peptide material gave only 3 major bands. A synthesis of HGH-(125-156) (Chillemi et al., 1972), carried out by the standard *in situ* coupling method, resulted in a product with 22 bands on IFPA (Kopwillem et al., 1974).

The second synthesis of [Cys(Cam)^{53}]-HGH-(1-54) employed the preformed symmetrical anhydride (*pre-mix*) coupling method. In contrast to the first synthesis, partition chromatography on Sephadex G-50 of the crude peptide material yielded a major peptide product; however, the route employed to obtain the final desired product involved preparative IFPA prior to the partition chromatography. As in the first synthesis, the crude peptide material gave 3 major bands by IFPA. Preparative IFPA in 8 *M* urea was utilized to obtain separation of the crude peptide material based on charge differences. After preparative IFPA, the final desired product was obtained from partition chromatography on Sephadex G-50.

Although inactive as a lipolytic agent, the final highly purified product showed immunochemical activity to both

antisera to native HGH and [Cys(Cam)^{53}]-HGH-(1-134) in the microcomplement fixation assay. Rabbit antiserum raised against the synthetic 54-residue fragment showed immunoreactivity by the above assay in the homologous system and also to native HGH and fragments derived from native HGH.

Table 15

Schedule of Synthesis for [Cys(Cam)^{53}]-HGH-(1-54)

Using the Standard Coupling Method

Step	Reagent and Operation	Time(min)
1	CH_2Cl_2 (4 times)	1
2	TFA-CH_2Cl_2, 1.22:1 (1 time)	1
3	TFA-CH_2Cl_2, 1.22:1 (1 time)	15
4	CH_2Cl_2 (3 times)	1
5	Dioxane-CH_2Cl_2, 1:2 (3 times)	1
6	CH_2Cl_2 (3 times)	1
7	DIEA-CH_2Cl_2, 1:19 (1 time)	5
8	CH_2Cl_2 (6 times)	1
9	Boc-Amino acid (4 equiv), (1 time)	5
10	DCCI (4 equiv), (1 time)	15
11	0.68 M DIEA in CH_2Cl_2 (3.3 ml, 4 equiv),(1 time)	15
12	CH_2Cl_2 (3 times)	1
13	Ethanol (3 times)	1

Table 16

Amino Acid Analyses of Peptide Material Isolated from Chromatography on Sephadex G-25

Amino Acid	Theoretical	Syntheses			
		Standard		Pre-Mix	
		I	II	I	II
Lys	2	2.64	2.86	2.01	1.41
His	2	2.27	0.46	1.79	0.30
Arg	3	3.65	0.86	2.84	0.35
Cys(Cam)[a]	1	1.00	1.00	1.00	1.00
Asp	4	3.94	1.80	4.33	1.71
Thr	3	3.00	1.68	2.84	1.44
Ser	3	3.06	2.16	2.77	1.97
Glu	9	9.49	6.75	9.76	5.97
Pro	4	4.60	3.85	3.81	2.38
Ala	4	4.32	1.20	3.80	1.53
Met	1	0.95	0.17	0.96	0.13
Ile	2	1.77	0.96	2.02	0.94
Leu	7	7.57	3.56	6.99	2.97
Tyr	3	3.29	2.64	3.31	2.73
Phe	6	6.00	4.23	5.70	3.47
Total	54	57.55	34.18	54.19	28.30

[a] Based on Cys(Cm).

Table 17

Schedule of Synthesis for [Cys(Cam)^{53}]-hGH-(1-54)

Using the Pre-Mix Coupling Method

Step	Reagent and Operation	Time(min)
1	CH_2Cl_2 (4 times)	1
2	TFA-CH_2Cl_2, 1.22:1 (1 time)	1
3	TFA-CH_2Cl_2, 1.22:1 (1 time)	15
4	CH_2Cl_2 (3 times)	1
5	Dioxane-CH_2Cl_2, 1:2 (3 times)	1
6	CH_2Cl_2 (3 times)	1
7	DIEA-CH_2Cl_2, 1:19 (1 time)	2
8	CH_2Cl_2 (3 times)	1
9	Repeat step 7	2
10	Repeat step 8	1
11	Repeat step 7	2
12	Repeat step 8	1
13	Repeat step 7	2
14	CH_2Cl_2 (3 times)	1
15	CH_2Cl_2 (3 times)	1
16	Pre-mix reaction mixture (1 time)	20
17	3.0 M DIEA in CH_2Cl_2 (0.5 ml, 3 equiv), (1 time)	10
18	CH_2Cl_2 (3 times)	1
19	Ethanol-CH_2Cl_2, 1:2 (3 times)	1

Table 18

Amino Acid Analyses of Synthetic [Cys(Cam)^{53}]-HGH-(1-54)

Amino Acid	Theoretical	Acid hydrolysate	Enzyme digest[a]
Lys	2	2.12	2.21
His	2	2.17	1.71
Arg	3	2.69	2.82
Cys(Cam)	1	0.94[b]	0.27[c]
Asp	2	4.26	2.02
Asn	2		
Thr	3	2.74	13.12
Ser	3	2.69	
Gln	5		
Glu	4	9.38	3.87
Pro	4	4.35	2.89
Ala	4	4.06	4.32
Met	1	0.96	0.98
Ile	2	1.82	2.05
Leu	7	7.12	7.66
Tyr	3	2.93	3.38
Phe	6	5.76	5.90

[a] Digestion with acid protease for 24 h at 37°, followed by 24 h digestion with trypsin and chymotrypsin, followed by 48 h digestion with leucine aminopeptidase gave 96% recovery.

[b] Determined as Cys(Cm).

[c] At least 60% of an authentic sample of Cys(Cam) is lost under the conditions of the digestion as judged by amino acid analysis (Yamashiro et al., 1973).

Table 19

Amino Acid Analyses of Products Obtained by CNBr Treatment[a]

Amino Acid	HGH-(15-54) Theoretical	V	HGH-(1-14) Theoretical	VI
Lys	2	1.35		0.07
His	2	1.21		0.03
Hsl[b]		0.03		0.32
Arg	2	1.66	1	0.92
Cys(Cm)	1	0.45		0.09
Asp	2	2.61	2	2.22
Thr	2	1.94	1	0.98
Ser	2	1.91	1	1.06
Hse[b]				0.49
Glu	9	8.13		
Pro	2	2.58	2	2.04
Ala	3	3.03	1	1.30
Met		0.20	1	
Ile	1	1.22	1	1.13
Leu	5	5.36	2	2.37
Tyr	3	2.25		
Phe	4	4.00	2	2.00

[a] See text for details. Values based on recovered Phe.

[b] Hsl, homoserine lactone; Hse, homoserine.

Table 20

Stimulation of Lipolysis in Rabbit Fat Cells by Synthetic [Cys(Cam)^{53}]-HGH-(1-54)

Concentration (μM)	Glycerol Production[a,b]
Control, 0 min	1.27 ± 0.04[c]
Control, 60 min	1.29 ± 0.04
0.18	1.27 ± 0.04
0.57	1.27 ± 0.04
1.68	1.27 ± 0.04
5.04	1.27 ± 0.04
15.25	1.23 ± 0

[a] μmoles glycerol/g cells/h.

[b] β_h-LPH has shown a 3.6-fold increase in glycerol production when assayed at 12 to 37 nM (Li & Rao, unpublished results).

[c] Mean ± S.E.

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