A NOVEL USE OF ENZYMES AS REAGENTS IN PEPTIDE SYNTHESIS: ENZYMATIC REMOVAL OF AMINE PROTECTING GROUPS

by

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Abstract

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By

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A system is described for the enzymatic deprotection of suitably masked amino groups during stepwise peptide synthesis. Nitrophenyl esters of amino acids, N-protected with trypsin-labile carbobenzoxyarginyl groups, were prepared as crystalline, analytically pure picrate salts in a standardized procedure. These intermediates reacted with amino components to yield carbobenzoxyarginyl peptides. Carbobenzoxyarginine was specifically released from such peptide intermediates by the action of trypsin at pH 8.0 in aqueous solution. In this way a series of model peptides were synthesized to probe the stereoselectivity of the enzymatic deprotection step and to provide synthetic precursors from which the potent oxytocin analog, deamino oxytocin, was prepared in good yield and high potency. In the course of these studies the rate of tryptic hydrolysis of arginyl bonds was found to be strongly influenced by the stereochemistry of the amino acid residue donating its amino group to the arginyl bond. This stereoselectivity in the enzymatic deprotection step was useful for rectification of partial racemization in a newly added

residue during stepwise peptide synthesis.

In addition to the mild conditions of the deprotection reaction in aqueous media and the stereoselectivity achieved, the foregoing system of stepwise peptide synthesis offers several technical advantages for purification and characterization of synthetic intermediates. Ion exchange methods were developed in this context which provided convenient standardized methods for the separation of protected and deprotected intermediates. The course and degree of the deprotection reaction is conveniently monitored by titration of liberated carboxyl groups and by the content of arginine in the deprotected product.

The results indicate that C-activated amino acid derivatives, N-protected with trypsin-labile groups, are readily prepared in convenient form and that the peptide derivatives prepared from these intermediates are readily freed of their amino-protecting groups under mild aqueous conditions with a potentially useful degree of stereospecificity. Theoretical implications of this first enzyme-catalyzed step in the repetitive cycle of stepwise peptide elaboration are discussed in conjunction with existing reagents and techniques. Immediate applications of the method are proposed and an ultimate system of peptide synthesis functioning under essentially physiologic conditions is envisioned.

Acknowledgement

I am immensely grateful to Dr. John Glass whose inspired guidance made the successful completion of this work possible. By having had the rare opportunity of working in close personal contact with a man as dedicated, imaginative, patient and friendly as Dr. Glass, I have grown on both a professional and personal level.

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I wish to thank my sister Wendy for generously donating her time to expertly type the draft of this manuscript.

To my wife, Chris, I can only inadequately express my appreciation for her patience, understanding and perseverance throughout the program.

I should like to dedicate this work to my recently departed father whose example of the highest standards and warm personality will continue to guide my efforts.

Publications resulting from this experimental work:

1. Glass, J.D., Meyers, Chester, Schwartz, I.L., and Walter, Roderich, An Approach Towards Bidirectional Solid-Phase Peptide Synthesis in Aqueous Media. Proceedings of the 13th European Peptide Symposium, in Peptides 1974, Y. Wolman, ed. p. 143.
2. Meyers, Chester and Glass, J.D., Enzymes as Reagents for Peptide Synthesis: Enzymatic N-deprotection. Proc. Nat'l Acad. Sci., in press.
3. Glass, J.D. and Meyers, Chester, Thin-Layer Chromatographic Analysis of Carbobenzoxy-Arginyl Amino Acid Nitrophenyl Esters, manuscript in preparation.
4. Meyers, Chester and Glass, J.D., Enzymes as Reagents in Peptide Synthesis: A Model Synthesis of Deamino Oxytocin Demonstrating the Use of Trypsin-labile N-Protection, manuscript in preparation.
5. Meyers, Chester and Glass, J.D., Enzymes as Reagents in Peptide Synthesis, to be presented at the 4th Amer. Peptide Symp., New York, N.Y., June, 1975.

Abbreviations follow the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature, J. Biol. Chem. 242, 555 (1967), and Biochem. J. 126, 773 (1972). All optically active amino acids are of the L-configuration unless otherwise noted. Additional Abbreviations: DMF, N,N Dimethylformamide; HOAc, Glacial Acetic Acid; DCCI, N,N' Dicyclohexylcarbodiimide; AcM, Acetamidomethyl; EtOAc, Ethyl Acetate; MP, 3-mercaptopropionyl.

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INTRODUCTION

A. Definition and Nomenclature of Peptides and Proteins:

Peptides are linear condensation products (polyamides) of α-amino-carboxylic acids (amino acids) whose basic structural features are presented in Fig. 1.

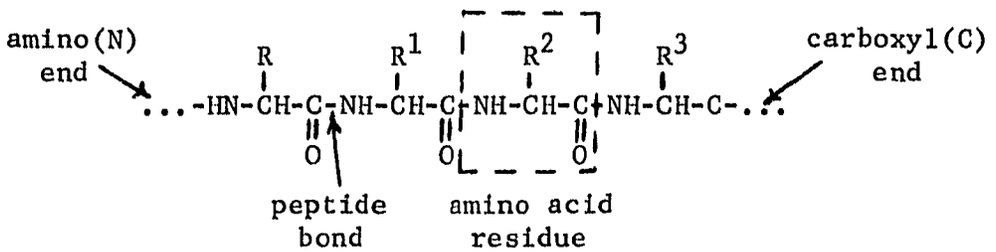


Figure 1: Major Structural features of a linear peptide.

The diagram shows that the peptide is comprised of a repetitive unit which is termed an amino acid residue. Conventionally, the residues are numbered consecutively from left to right (N- to C-terminal), the N-terminal residue being number one. Cyclic peptides have direction but no N- or C-terminus. The backbone of the peptide chain, which is the portion of the molecule exclusive of the "R" group, is completely defined by its length (number of residues) and by its direction. The amino acids are characterized by a particular side-chain moiety ("R" group) attached to their respective α-carbon atoms. These functional groups encompass a wide range of chemical classes. For example, they may be aliphatic or aromatic, polar or non-polar, acidic, neutral, basic, phenolic, or alcoholic. It is these varied side chains which make the individual peptides so unique and distinguishable despite the repetitive backbone.

The structural foundation for the biological function of a given physiologically active peptide is provided principally by a precise sequence of specific amino acids. Structural variants of a peptide may

result either from amino acid substitutions or from recombination of the same amino acids in different sequential arrangements. The number of possibilities for different peptides from the more than 20 amino acids available to biological systems is therefore virtually limitless by this consideration alone, even for only relatively small chains. In addition, secondary and tertiary structures arise through covalent, electrostatic and hydrophobic interaction among the various side chains in a peptide. This brings about particular conformations which may bring distant amino acids in a peptide chain into proximity. Longer chain, high molecular weight compounds with complex three dimensional configurations of this nature are referred to as proteins. Functional proteins, such as enzymes, are frequently composed of aggregated subunits which may consist of several similar or dissimilar individual chains. This quaternary type of structure imparts even more information and versatility to the protein class of molecules.

B. Importance of Peptides and Historical Development of their Synthesis:

It can be readily seen that the peptides and proteins incorporate a vast body of information which make them well suited for a wide variety of critically important biological functions. Many hormones which are vital to the maintenance of regulatory control systems are peptides. For example, ion transport and metabolism, control of blood sugar levels, water transport, and the release of other regulatory and metabolic agents are some of the processes under the control of peptide hormones. The enzymes which specifically catalyze the countless biochemical reactions of living systems are proteins, as are many structural components of all organisms.

The importance of the peptides and proteins was recognized early and attempts to synthesize them naturally followed. The development of agents which activated the carboxyl moiety sufficiently to facilitate peptide bond formation with another amino acid met with great initial success. Yet, if two unaltered amino acids were condensed in this fashion, each activated specie could react with the amino group of either amino acid resulting in an uncontrolled polymerization. Emil Fischer and T. Curtius investigated possibilities for circumventing random polycondensation in order to synthesize peptides of designed sequence. Approaching the problem through classical organic chemistry, they developed several basic concepts which persisted as fundamental elements of modern peptide chemistry. By acylating the amino function prior to carboxyl activation, a controlled, directed coupling to amino acid or peptide esters could be accomplished*. Initially, no acyl group could be found which was selectively removable after the peptide bond was formed, but this need was satisfied in 1932 when Bergmann introduced the carbobenzoxy group which could be removed by catalytic hydrogenolysis. Thus, stepwise elaboration of unambiguous peptide chains was made possible through the reversible blocking of functional groups not meant to participate in the coupling reaction. As additional masking groups and activating agents were developed along with increasingly sophisticated purification techniques, the task of synthesizing biologically active peptide sequences became feasible.

Of the numerous categories of peptides and proteins, the peptide hormones offer several features which make them the best suited as

*Preactivated intermediates such as N-protected amino acid chlorides or azides could be coupled directly to free amino acids or peptides.

synthetic models: 1) Many possess well defined, conveniently assayed biological properties. 2) Some are also low molecular weight, short chain molecules which greatly simplifies their preparation. 3) Often they can be crystallized and their physical and chemical analysis can be routinely performed. 4) They frequently produce multiple physiological or pharmacological effects which provides an opportunity formore rigorous testing of synthetic products. 5) They are good models with which to study the interaction of peptides and proteins with macromolecules such as enzymes and receptors in order to determine their nature and mechanisms of action. 6) The peptide hormones and their synthetic analogs often have profound medical importance.

It is for the above reasons that the peptide hormones have been extensively investigated throughout the development of peptide chemistry. The neurohypophyseal nonapeptide hormone oxytocin was the first naturally occurring biologically active peptide to be synthesized (1,2). Hundreds of analogs of the neurohypophyseal hormones have since been prepared and characterized, making them by far the best studied group of peptides to date. They therefore provide an excellent testing ground for the development of new methods of synthesis and for a comparison of these techniques with already established procedures.

C. Deficiencies in Existing Methodology and Rationale for their Rectification:

Neurohypophyseal hormones also serve to illustrate the need for improved synthetic methods since the knowledge gained from numerous studies with conveniently prepared analogs has created an interest in the behavior of new, more delicate analogs which cannot be synthesized by the existing methods. In addition to this specific limitation, the qualities of many

synthetic peptide and protein products and intermediates are frequently unsatisfactory. Certain large sequences or those which contain particularly fragile residues, as in the example just cited for neurohypophyseal hormone analogs, are not yet accessible by the available synthetic procedures. Thus, serious disadvantages severely limit the scope and applicability of the modern synthetic methods. These shortcomings arose as a direct result of the historic derivation of peptide synthesis from classical organic chemistry. As outlined earlier, the basic approach to the control of the synthetic direction of peptidyl intermediates had been the deliberate masking of chemically reactive groups. In addition to their protective function, these masking groups provided the amino acids with better solubility in the organic solvents required as the medium for the majority of the coupling reactions known at the time. The intentional selection of protecting groups which would enhance the organic solubility of the protected intermediates naturally accompanied that development. The unfortunate outcome of this approach was that the chemically functional groups which were masked, and the aqueous solubilities which were suppressed were precisely those properties which formed the bases of the most powerful techniques presently available for isolation, purification, and analysis of polypeptides. Hence, the most highly developed synthetic procedures and the most sophisticated purification methods conflict in their basic requirements and as a result are performed in different milieu. The consequences of this dichotomy - primarily the loss of solubility of the protected intermediates and the denaturation of the unprotected products-become especially obvious as synthetic goals become more ambitious. For example, in order to advance the technique of semisynthesis (3-5) - which utilizes naturally occurring subfragment sequences as synthetic intermediates - to the total synthesis of large

polypeptides, problems intrinsic to commuting between aqueous and non-aqueous media must be eliminated or at least minimized.

Usually the techniques and materials chosen tend to be more compatible with either the synthetic approach or the purification routine. As much of the biology of naturally occurring peptides and proteins is firmly entrenched in aqueous media, and as many proteins are irreversibly inactivated upon leaving the aqueous environment, it seems necessary that the existing chemical methods of peptide synthesis be altered to accommodate the polypeptide within its native milieu. Thus, one major consideration in the design of the work to be presented for the improvement of the existing synthetic methods was that the system must function in aqueous media or be, in principle, adaptable to use in aqueous media.

In addition to the above problem, other major factors contribute to the existing limitations in peptide synthetic efforts; primarily the treatments with strong acids and reducing agents. The use of such harsh conditions developed from the need for several types of blocking groups during a stepwise synthesis and from the limited array of mechanisms for removal of protecting groups. For example, the protecting group on an amino acid side chain might be required to endure many cycles of the synthesis, whereas the N-protection would need to be removed at each cycle. Thus, the side chain group chosen would have to be stable to the conditions of repetitive N-deprotection and would therefore require more severe conditions for its own removal if both groups were cleaved by a common mechanisms (eg acidolysis), as is commonly the case.

The degree of severity separating the selective deblocking procedures spans several orders of magnitude since the N-deprotection treatment is repetitive. Although the problem may be avoided by employing groups

which are removable by differing mechanisms, the availability of such options are extremely limited. Moreover, many excellent side chain groups currently available, which are removed smoothly by mild treatments, are often excluded from use because they are also unstable to the conditions of N-deprotection. Recent developments in peptide synthesis have increasingly utilized the selective deprotection of the C-terminal blocking group from the protected polypeptide intermediate. This requires that the protecting groups which are intended for removal last be even more stable than was necessary for those in the previous example with only two masking groups. Even when a combination of substituents which meet these requirements can be found, the conditions which displace the most stable groups are so severe that they are plainly deleterious to many chemical groups which might be desired as constituents of the peptide chain. Again, this may be minimized by seeking new protecting linkages which are cleaved by dissimilar reaction mechanisms.

It seemed unlikely that a search for alternative groups which are subject to the most severe cleavage methods would overcome the problem because the heirarchy which exists was created and is sustained by the relatively more mild, repetitive amino deblocking conditions. Hence, if this procedure could be accomplished in an environment physiologic to the extent that it operates in aqueous solution at room temperature within the pH range of 6-8, other blocking groups might be chosen which could be removed by correspondingly milder treatments. In addition, many of the existing mildly removable groups unstable to current N-deprotection conditions would then become more widely applicable. Thus, it became clear that the best approach to the ultimate development of a system of peptide synthesis which can function under physiologic conditions was through a

modified N-terminal protecting group. The particular requirements being sought for such a group - especially that it be suited to removal in physiologic environments - prompted an investigation into the development of an enzyme-labile amino blocking group. Enzymes are well appreciated as subtle and efficient reagents in many areas of peptide and protein chemistry, including synthetic applications peripheral to the basic cycle of stepwise peptide elongation (6-9). The unique properties of enzymes as selective catalysts have not generally been brought to bear on the repetitive reactions of controlled, stepwise peptide synthesis, despite the fact that the chemical precision, mild reaction conditions, and stereospecificities so characteristic of enzymatic reactions are at a premium in this repetitive process. Therefore, a model system was devised within that framework and it was subsequently applied, in conjunction with several technical improvements, to the synthesis of a highly potent hormone analog. These developments, along with the experimental data, results obtained, and a discussion of the advantages, disadvantages, and potentials of the novel method are detailed in later sections of this manuscript. However, it should be noted here that the scheme fulfills all of the initial criteria sought for the repetitive amine deprotection system and it offers several additional unique advantages. For example, the protecting group chosen confers aqueous solubility on the protected intermediates and provides the basis for the development of ion exchange methods for the convenient, standarized isolation and purification of protected and deprotected intermediates. Further, the stereoselective capabilities of the enzyme, in conjunction with the ion exchange procedures, were successfully exploited for the rectification of partial racemization in an amino acid residue most recently incorporated during stepwise peptide synthesis.

D. The Model System:

The model system, involving enzymatic deprotection of amino functions, is based upon three general principles: 1) The substrate specificity of the proteolytic enzyme, trypsin (10,11), 2) The selective acylation of amino groups in the presence of protonated guanido functions (12,13), and 3) The "backing off" method for preparing activated esters of peptide derivatives (14). Carbobenzoxyarginine was selected as the amino blocking group removable by trypsin. Thus, Cbz-Arg-OH·HCl, through the action of DCCI, coupled with several amino acid nitrophenyl esters to yield the respective nitrophenyl Cbz-Arg-aminoacylates (Cbz-Arg-X-ONp's) which were isolated as crystalline, analytically pure picrate salts (Fig 2). These activated dipeptide derivatives reacted with amino compounds to yield the corresponding Cbz-Arg-aminoacylamine derivatives. For example, in order to assess their suitability to form peptide bonds in a semi-quantitative system, they were allowed to react with an excess of glycine esterified to chloromethylpolystyrene - 2% divinylbenzene resin (glycine-resin ester) (15). Based upon the observed extent of coupling to the glycine, this system indicated that all of the Cbz-Arg-X-ONp derivatives examined would be generally adaptable to the routine formation of peptide bonds.

Cbz-Arg-L-Gln-ONp and Cbz-Arg-D-Gln-ONp picrates were also coupled to $(H\text{-Asn-Cys-Pro-Leu-GlyNH}_2)_2$ (16) in solution to yield $(Cbz\text{-Arg-L-Gln-Asn-Cys-Pro-Leu-GlyNH}_2)_2$ ("All-L-peptide") and $(Cbz\text{-Arg-D-Gln-Asn-Cys-Pro-Leu-GlyNH}_2)_2$ ("2-D-peptide"), respectively. These products, after the exchange of picrate for alternate counter-ions, were incubated with trypsin. Cbz-Arginine was specifically released from both the "All-L-peptide" and the "2-D-peptide", but the former compound was hydrolyzed much more rapidly than its diasteriomer.

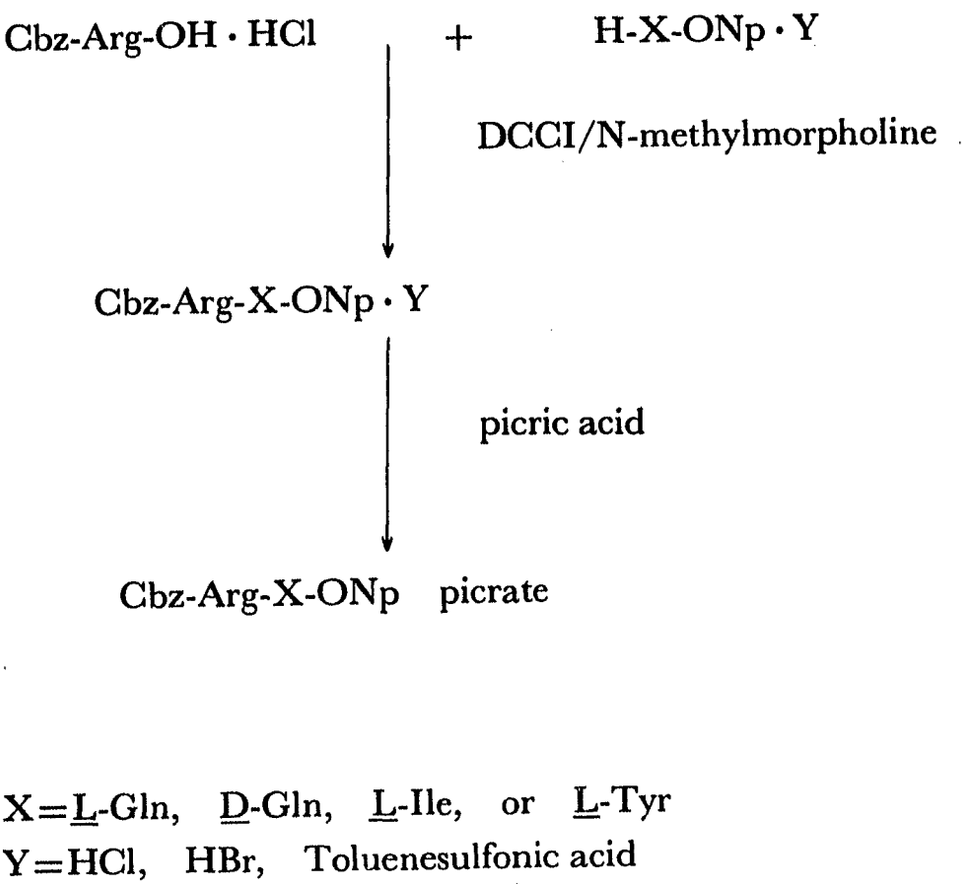


Figure 2: Scheme for synthesis of Cbz-Arg-X-ONp picrates.

E. Model Synthesis of Deamino Oxytocin:

Reasons were given earlier for the choice of neurohypophyseal hormone analogs as models to test experimental synthetic innovations. Deamino oxytocin (Fig. 3), in which the N-terminal cysteine residue of oxytocin (Fig. 4) is replaced by 3-Mercaptopropionic acid, is an attractive choice since it can be crystallized, has been well characterized chemically and pharmacologically, and also possesses higher biological potency than the parent hormone (17-19). This analog was prepared stepwise, using all of the Cbz-Arg-L-amino acid-ONp picrates described for the model system, from the single chain H-Asn-Cys(AcM)-Pro-Leu-GlyNH_2 (20). Several improvements in the methodology were developed at each cycle. Thus, the incorporation of glutamine into the peptide chain and the exchange of picrate were accomplished by the methods developed for the model system, but purification was affected on a carboxylic acid ion exchanger which discriminated between the strongly basic guanidinium ion and the weaker ammonium ion of the uncoupled starting material.

The incorporation of isoleucine into the growing chain was consistently accompanied by significant racemization of this residue*. The protected DL isomeric chains provided a challenging opportunity to demonstrate the stereoselective capabilities of the enzymic deprotection reaction. It was necessary to develop more convenient and reliable ion exchange procedures along with this investigation, and it was found that a sulfonic acid ion exchange resin which permitted the use of buffers at a lower pH was an excellent alternative. Tryptic hydrolysis of the

*The presence of salts in the coupling reaction mixture was unavoidable at this step owing to certain properties of free N-terminal glutaminyl residues. The problems associated with this isolated case are detailed in the discussion section.

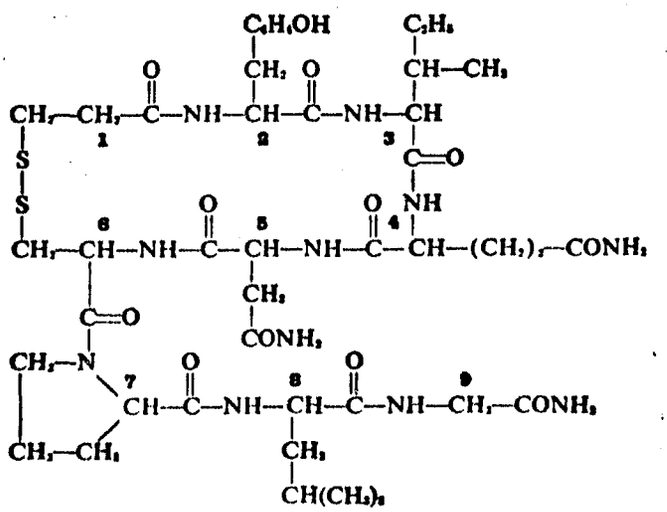


Figure 3: Structure of Deamino Oxytocin

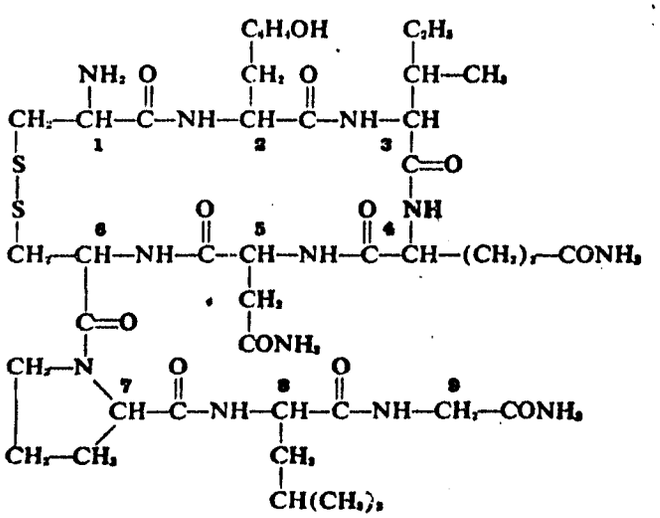


Figure 4: Structure of Oxytocin

2-D and L isomers, followed by the improved ion exchange method, resulted in the complete separation of the deprotected 2-L-isomer from a mixture of the undigested 2-D and L isomer. Although the digestion of the 2-L-isomer was incomplete, no evidence for the hydrolysis of the 2-D-isomer could be detected. Therefore, total rectification of a racemized residue after its incorporation into a peptide chain was demonstrated.

During the incorporation of tyrosine, an additional ion exchange method was developed which greatly simplified and improved the removal of picrate from the peptide.

The deamino oxytocin was obtained by known procedures after the incorporation of the last residue via S-Acetamidomethyl, 3-Mercaptopropionyl p-Nitrophenylate which has not been previously reported. The hormone analog possessed high biological potency.

EXPERIMENTAL

A. Analytical Methods:

1. Amino Acid Analyses:

In most cases, samples for amino acid analyses were dissolved in 6N HCl and hydrolyzed for 22 hours at 110° in sealed evacuated tubes. Hydrolyses of resin-bound peptides were performed in 1:1 12N HCl: dioxane (24). The hydrolysis time was extended to 48 hours for the single-chain peptides which contained isoleucyl residues, and 1 mg phenol per ml 6N HCl was used to hydrolyze the protected tyrosinyl peptide.

2. Thin Layer Chromatography:

The silica gel thin-layer plates were purchased from Analabs, Inc. (type Anasil G, 250 μ thickness) and used for ascending chromatography in the solvent system n Butanol:Acetic Acid:Water; 4:1:1 (BAW) unless another system is designated. Cbz-Arg-X-ONp picrates were run first in 20:1 Ethyl Acetate: 1N HCl/HOAc to dissociate the picric acid which appears in each plate (Fig. 13-18) at the solvent front. After drying, the plates were rechromatographed in BAW. This procedure was developed after attempts to dissociate and chromatograph the acid-stable products in the single system n BuOH:Formic Acid:H_2O (4:1:1) resulted in heavy streaking. Since the successful two-step technique was not introduced for some time, the Cbz-Arg-X-ONp picrates were stored for lengthy periods (room temperature) prior to their use for the chromatographic analyses presented. Although the tyrosinyl derivative resisted purification by recrystallization and the quantity of the remaining D-glutaminyl derivative was too minute for recrystallization, the L-glutaminyl and isoleucyl derivatives were each recrystallized from 95% ethanol and chromatographed for comparison with the original products. While all of the original

compounds apparently underwent some degree of decomposition on long standing, development of the plates, exclusive of that containing Cbz-Arg-Tyr-ONp picrate, with 5% KOH (not shown) produced yellow spots only at one R_f which corresponded to the correct product (except for a second spot corresponding to nitrophenol at the solvent front). The tyrosinyl derivative produced a faint yellow second spot below the primary component.

B. Model System:

1. Glutamine p-Nitrophenyl Ester Tosylates:

The tosyl salts of D- and L- glutamine nitrophenyl esters were prepared by the procedure of Stewart (21) and were recrystallized from methanol. For the D- and L- isomers, respectively* (unchanged by recrystallization from methanol): mp 186-7, 187-8; yield 63%, 65%; $[\alpha]_D^{25}$ -7.8, + 7.6 (c=2, DMF); elemental analysis C 49.19, 49.19 H 4.81, 4.90 N 9.51, 9.52 (requires C 49.19, H 4.83, N 9.56 for $C_{18}H_{21}N_3O_8S$).

2. Cbz-Arg-X-ONp Picrates:

Equimolar mixtures of Cbz-Arg-OH·HCl and an amino acid nitrophenyl ester salt (Tos, HCl, HBr) in a minimal volume of DMF† at 0° were treated with the stochiometric amounts of N-methylmorpholine and DCCI, then the reaction mixture was allowed to come to room temperature. After four hours precipitated dicyclohexylurea was removed by filtration, the filtered reaction mixture was reduced to an oil at room temperature on the rotary evaporator, and the residual oil was washed with cold 0.1N HCl.

*Stewart reports mp 164.5 - 166 and $[\alpha]_D^{25}$ + 9.0 (c=2, DMF) for the L isomer. We have observed that same melting point occasionally before recrystallization from methanol.

†The sparingly soluble tosyl salts of the glutamine nitrophenyl esters were only partially dissolved at the beginning of the reaction.

The crude product was taken up in 95% ethanol and treated with the theoretical amount of picric acid in ethanolic solution. Crystalline products which separated directly from the alcoholic solution or which formed from precipitated oils were analytically (C,H,N) pure except for Cbz-Arg-Ile-ONp picrate which required one recrystallization from ethanol. Yields obtained, optical rotations observed and elemental analyses for the various dipeptide nitrophenyl ester derivatives are given in Table I. I.R. Spectra for these compounds are shown in Figures 5-12. Thin-layer chromatography was performed as described and the results appear in Figures 13-18.

Table I

Cbz-Arg-X-ONp Picrates

X	% Yield	$[\alpha]_D^{25}$†	% Composition by Weight*		
			C	H	N
L-Gln	60	-16.5	(47.33, 47.47)	(4.36, 4.08)	(17.81, 17.54)
L-Ile	80	-14.6	(49.80, 49.69)	(4.83, 4.72)	(16.34, 16.18)
L-Tyr	70	-9.0	(51.16, 51.40)	(4.29, 4.50)	(15.34, 15.17)
D-Gln	42	+10.0	(47.33, 47.23)	(4.36, 4.46)	(17.81, 17.88)

*(calculated, found)

†c=1 in DMF

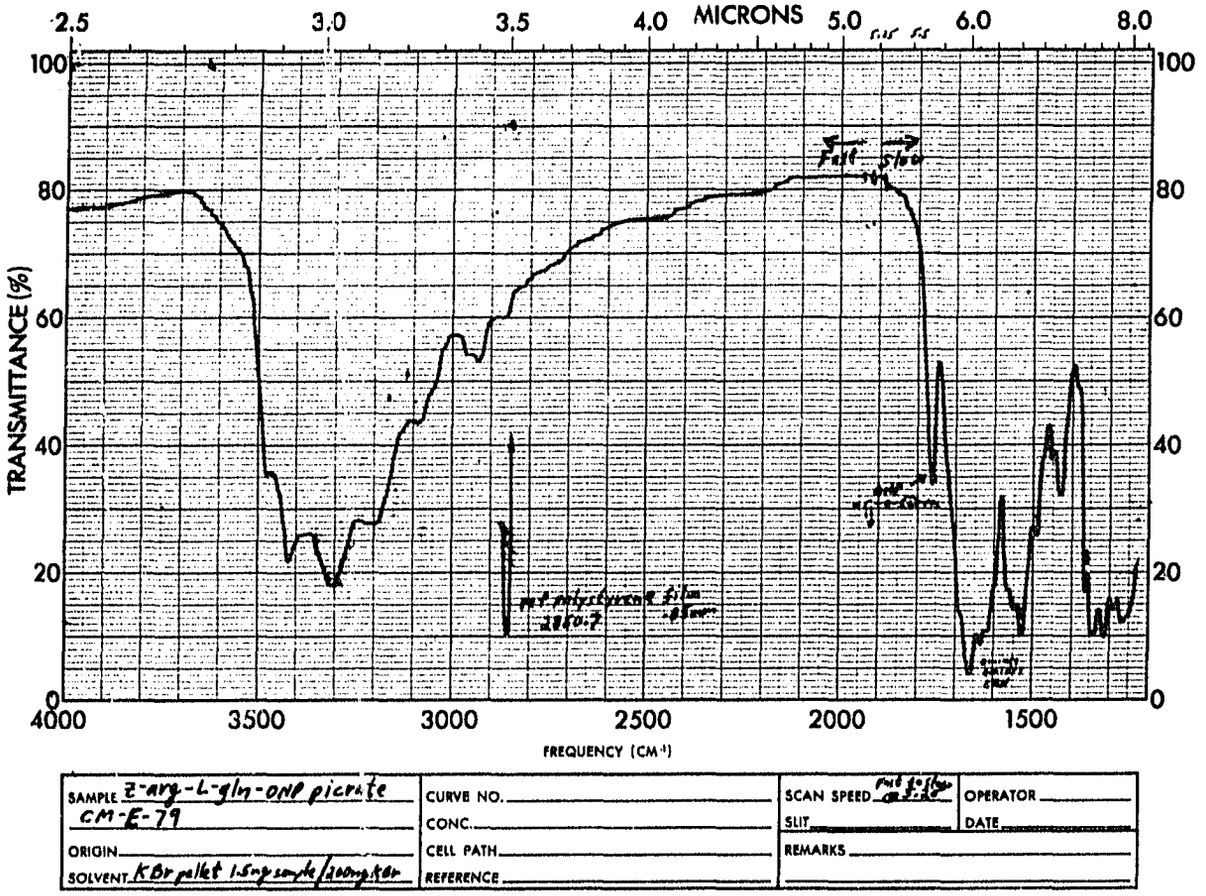


Figure 5: I.R. Absorption Spectrum of Cbz-Arg-L-Gln-ONp picrate at a concentration of 1.5 mg in 200 mg KBr compressed into a pellet.

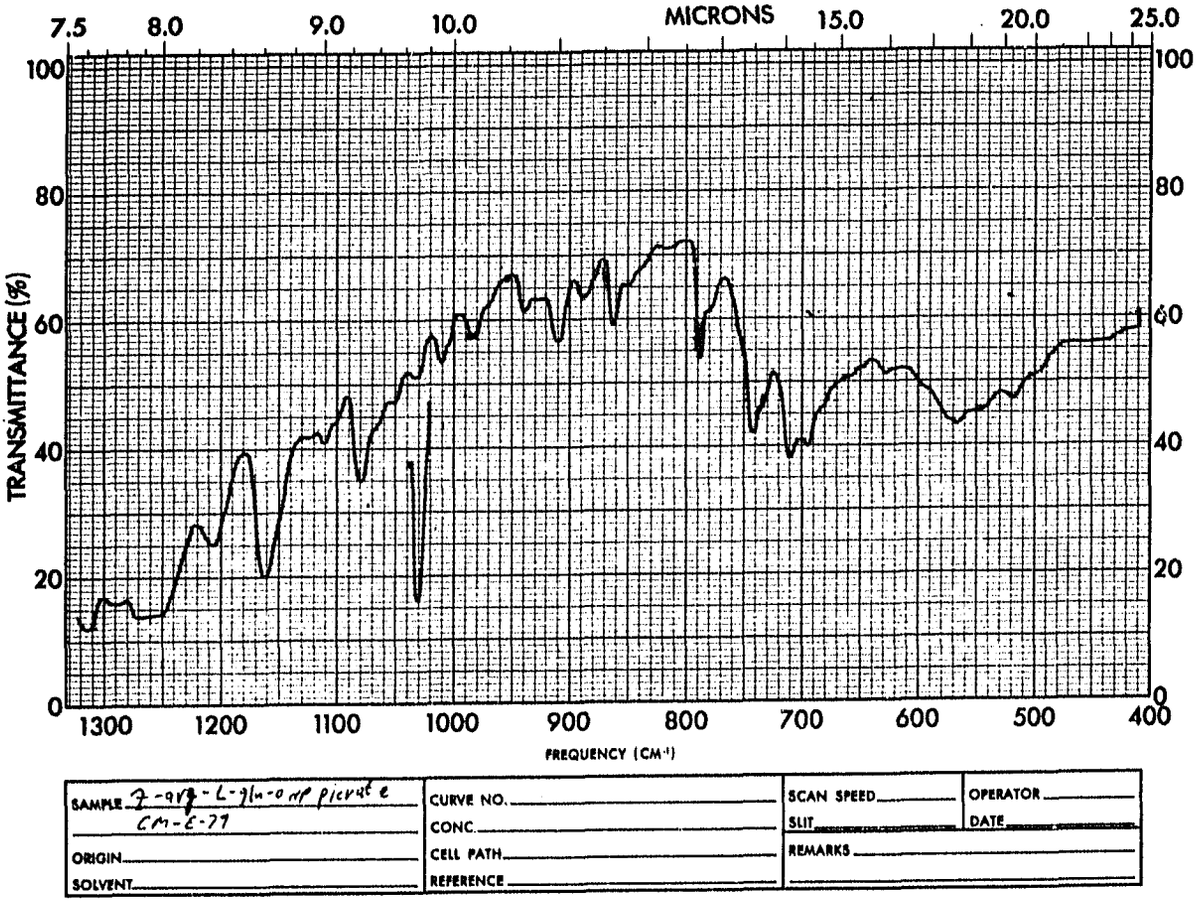


Figure 6: I.R. Absorption Spectrum of Cbz-Arg-L-Gln-ONp picrate ("Fingerprint" pattern) at a concentration of 1.5 mg in 200 mg KBr compressed into a pellet.

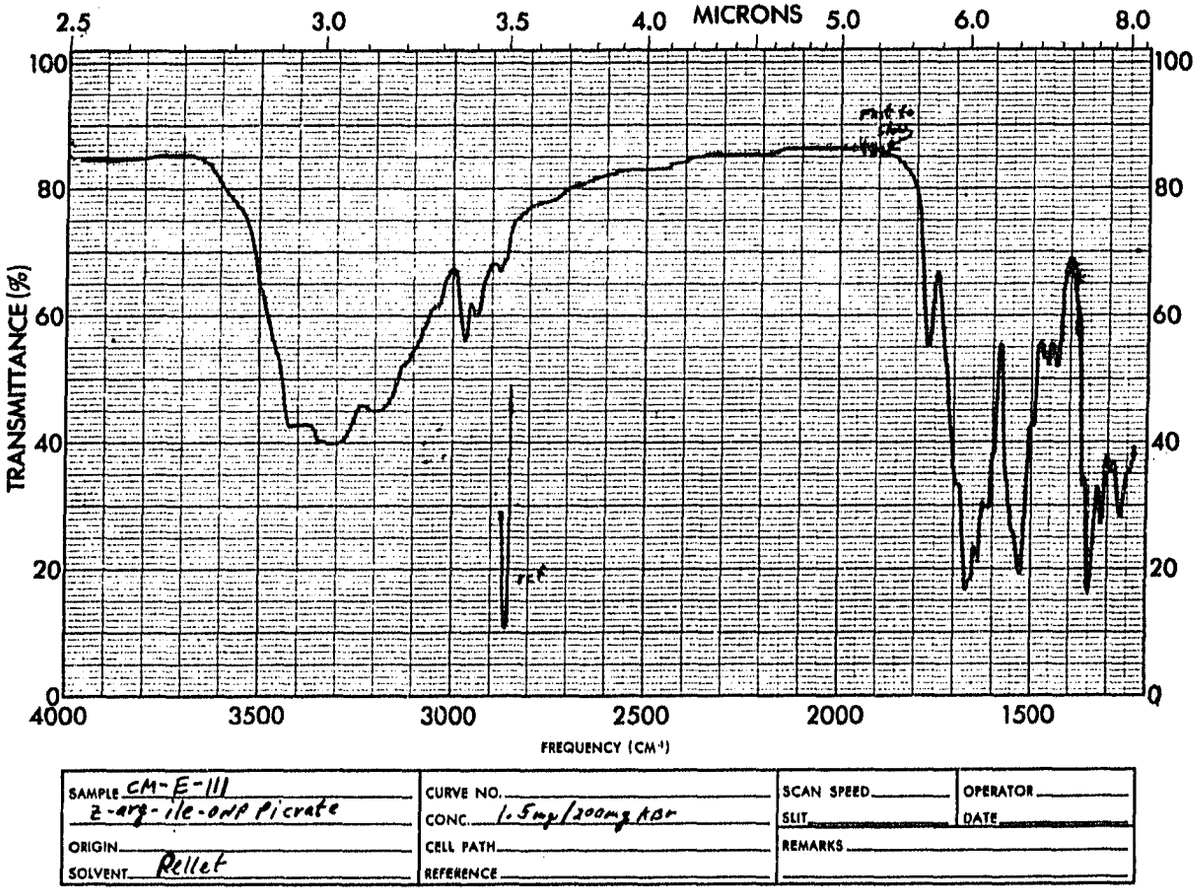


Figure 7: I.R. Absorption Spectrum of Cbz-Arg-Ile-ONp picrate at a concentration of 1.5 mg in 200 mg KBr compressed into a pellet.

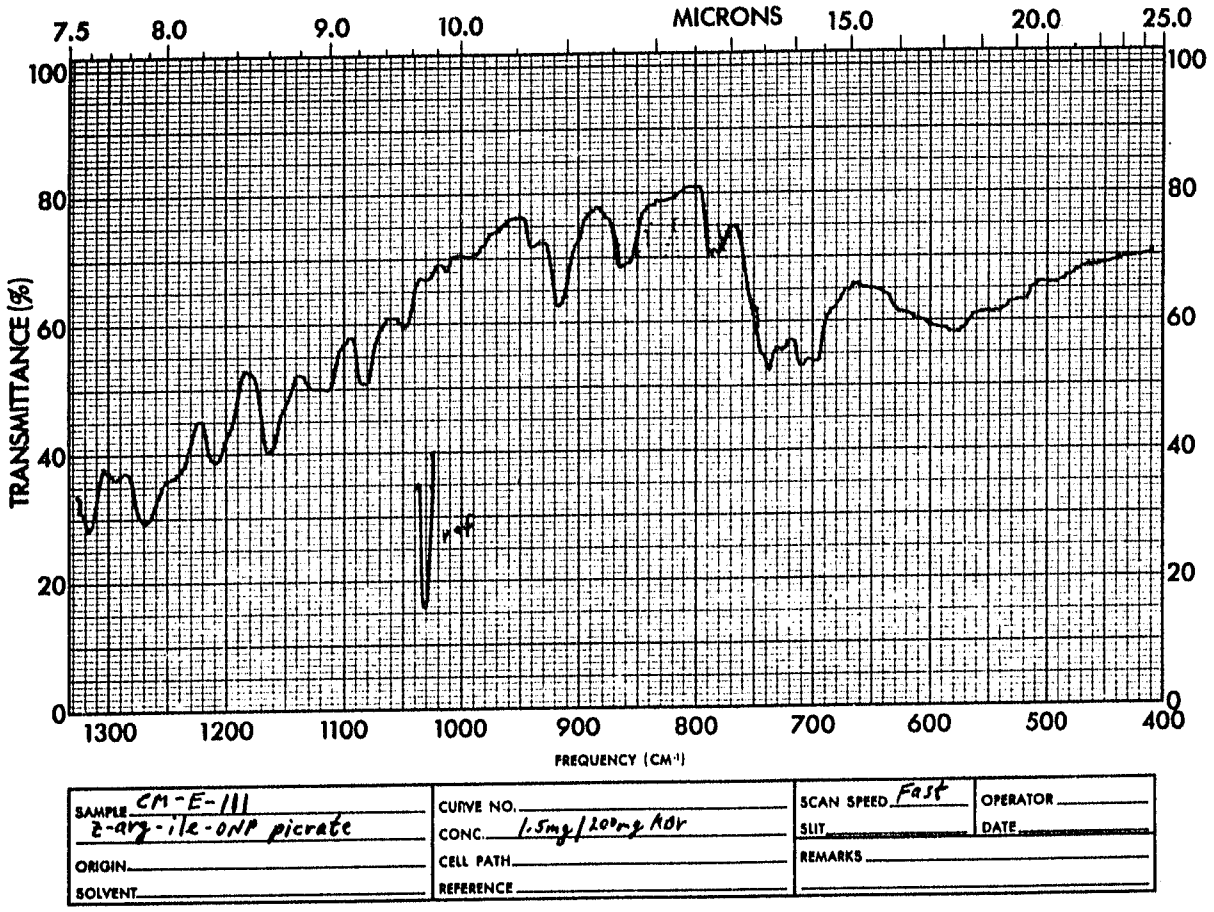


Figure 8: I.R. Absorption Spectrum of Cbz-Arg-Ile-ONp picrate ("Fingerprint" pattern) at a concentration of 1.5 mg in 200 mg KBr compressed into a pellet.

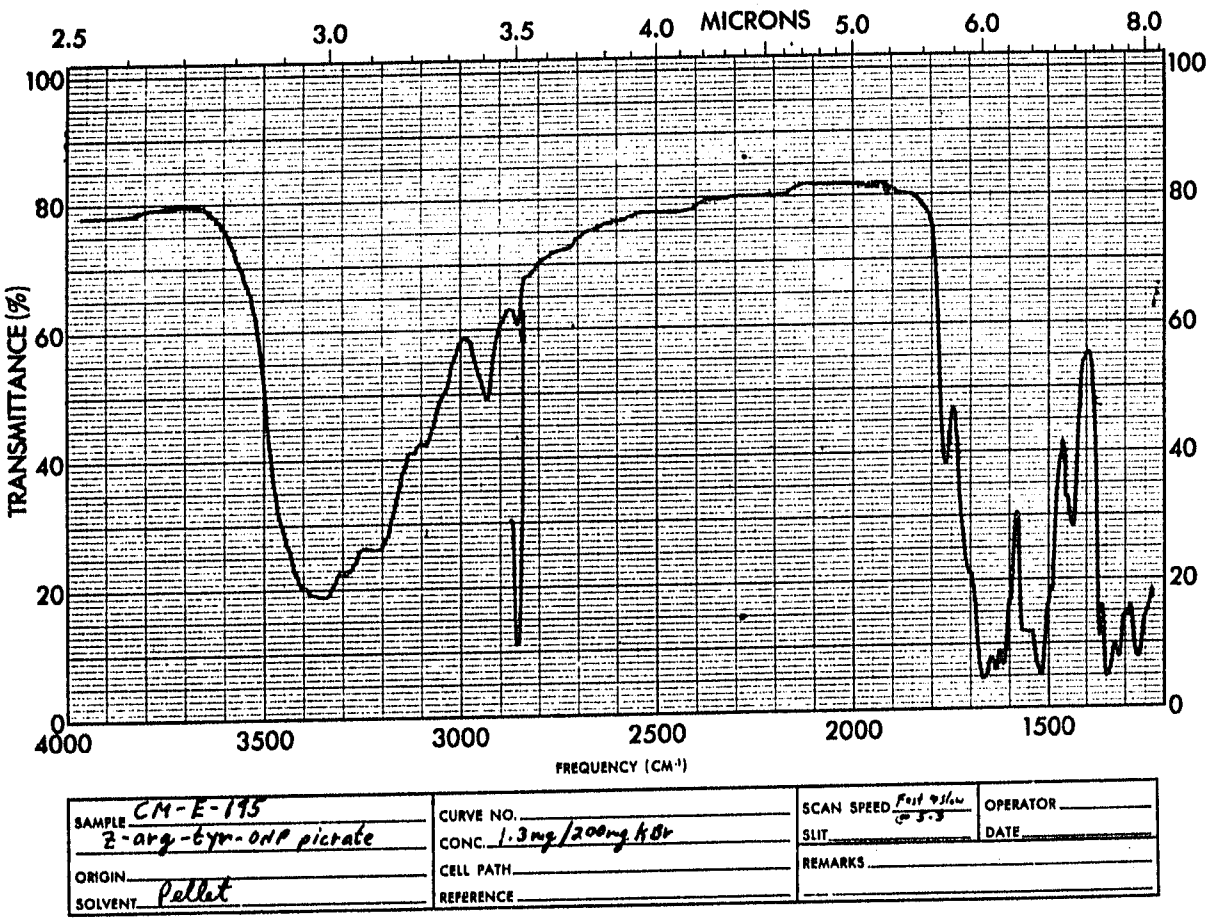


Figure 9: I.R. Absorption Spectrum of Cbz-Arg-Tyr-ONp picrate at a concentration of 1.3 mg in 200 mg KBR compressed into a pellet.

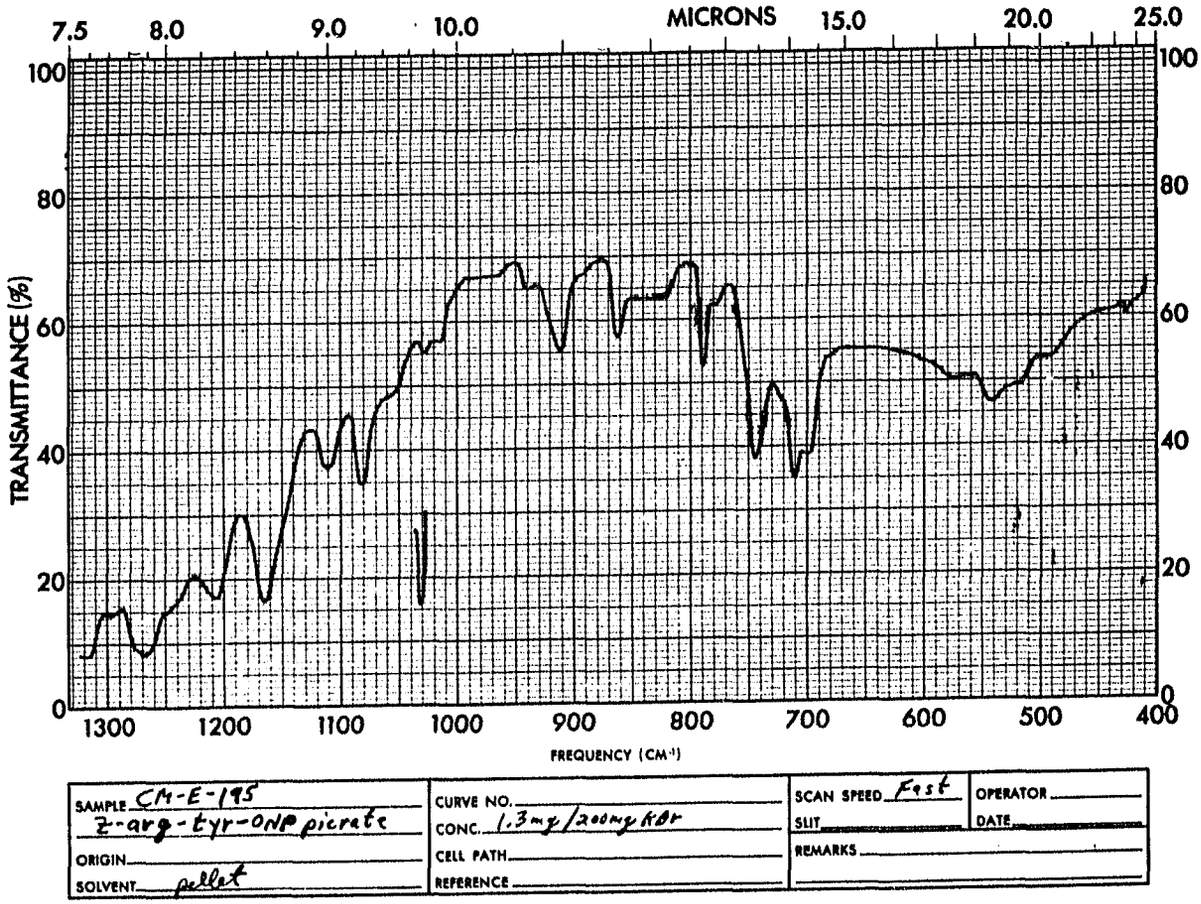


Figure 10: I.R. Absorption Spectrum of Cbz-Arg-Tyr-ONp picrate ("Fingerprint" pattern) at a concentration of 1.3 mg in 200 mg KBr compressed into a pellet.

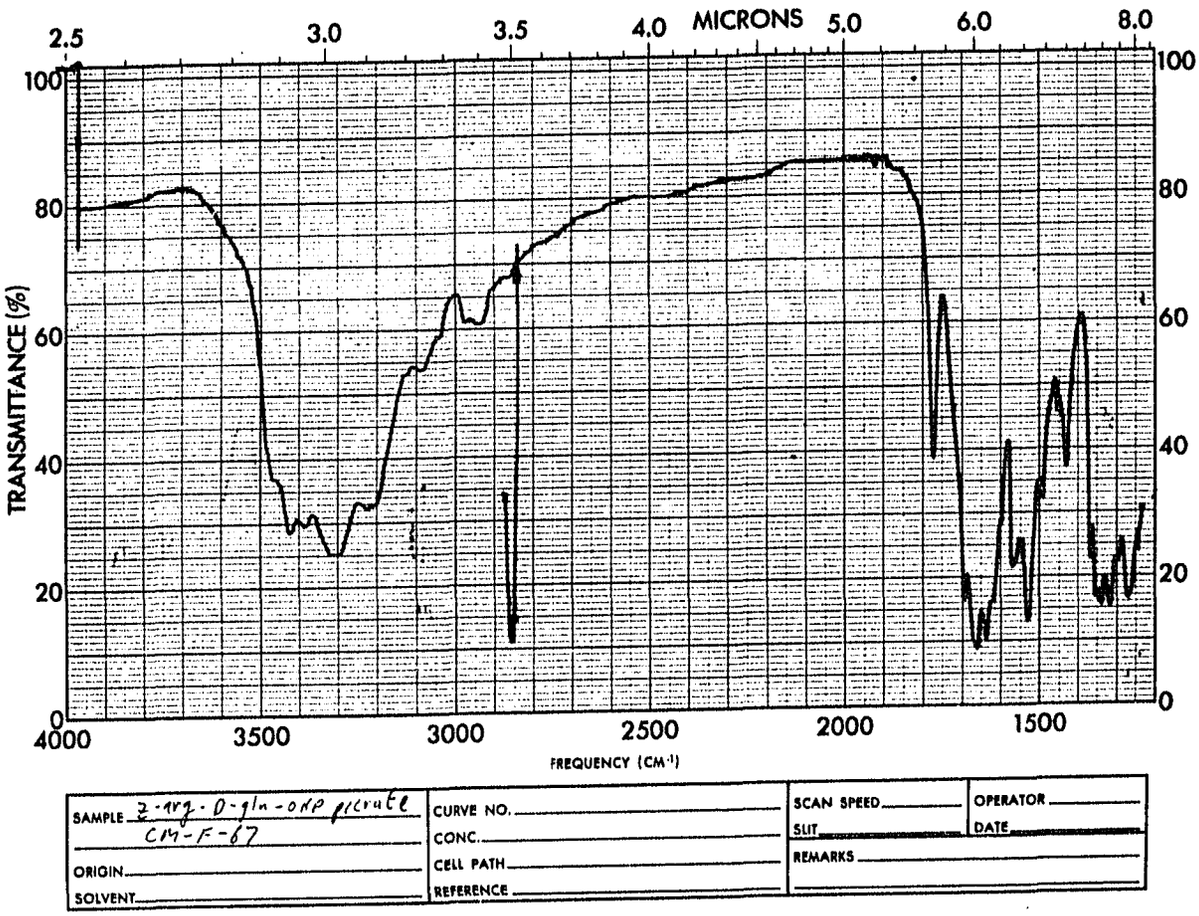


Figure 11: I.R. Absorption Spectrum of Cbz-Arg-D-Gln-ONp picrate at a concentration of 1.5 mg in 200 mg KBr compressed into a pellet.

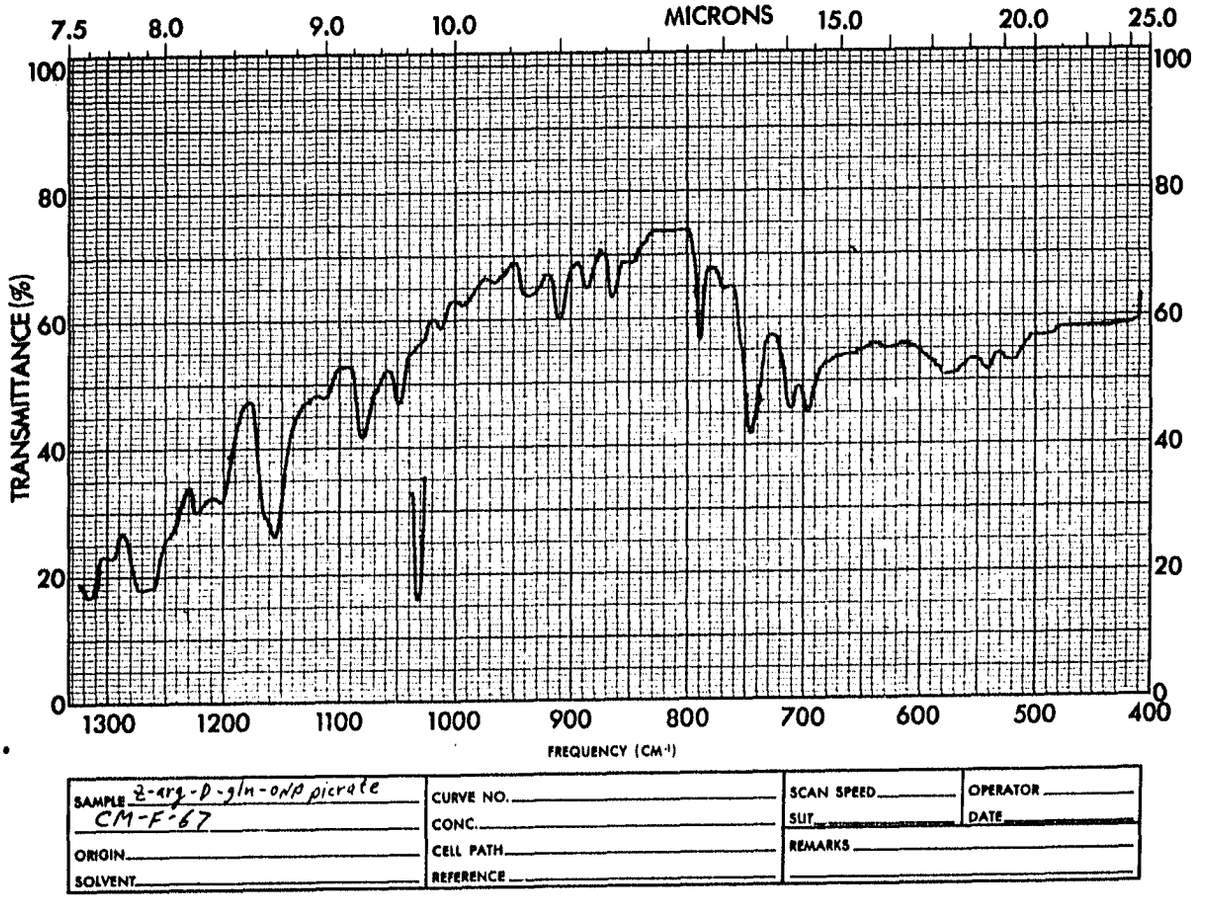


Figure 12: I.R. Absorption Spectrum of Cbz-Arg-D-Gln-ONp picrate ("Fingerprint" pattern) at a concentration of 1.5 mg in 200 mg KBr compressed into a pellet.

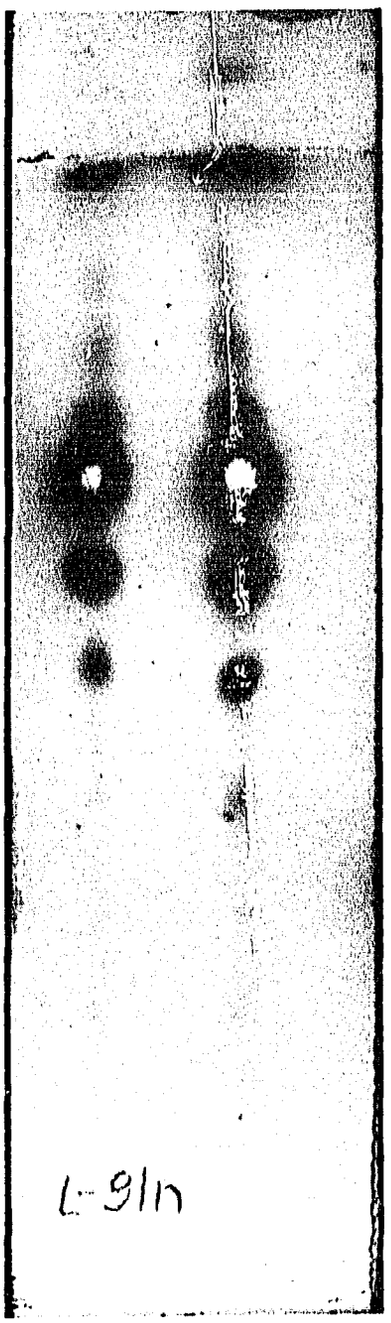


Figure 13: Cbz-Arg-L-Gln-ONp picrate after lengthy storage at room temperature. From Left to Right; 50 and 100 μg spots. The plate was run in 20:1 EtOAc:1N HCl/HOAc to dissociate picric acid (visible at solvent front just below nitrophenol), dried, and rerun in BAW. See text.

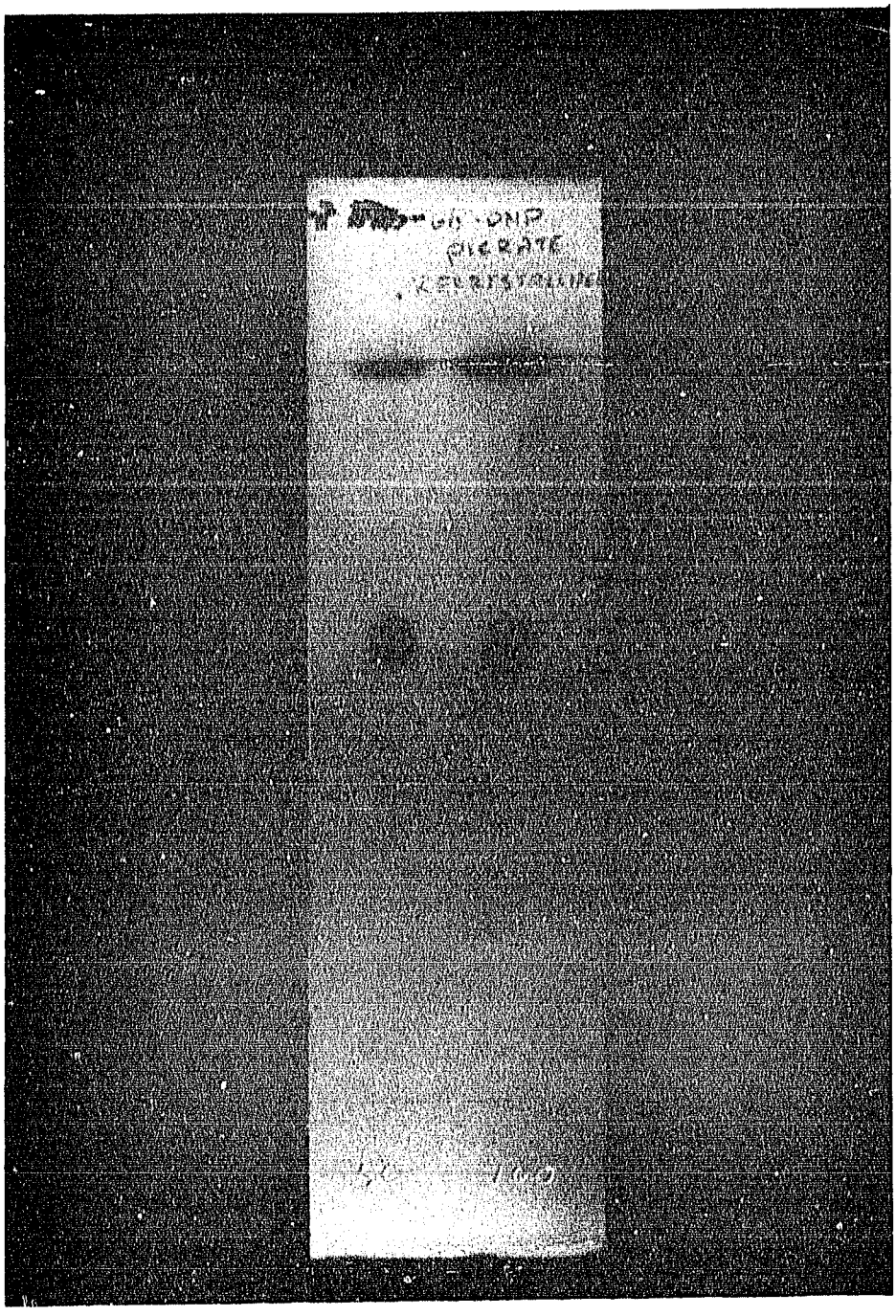


Figure 14: Cbz-Arg-L-Gln-ONp picrate freshly crystalized from 95% ethanol. From Left to Right; 50 and 100 µg spots. The plate was run in 20:1 EtOAc:1NHCl/HOAc to dissociate the picric acid (visible at solvent front), dried, and rerun in BAW. R_f 0.61. See text.

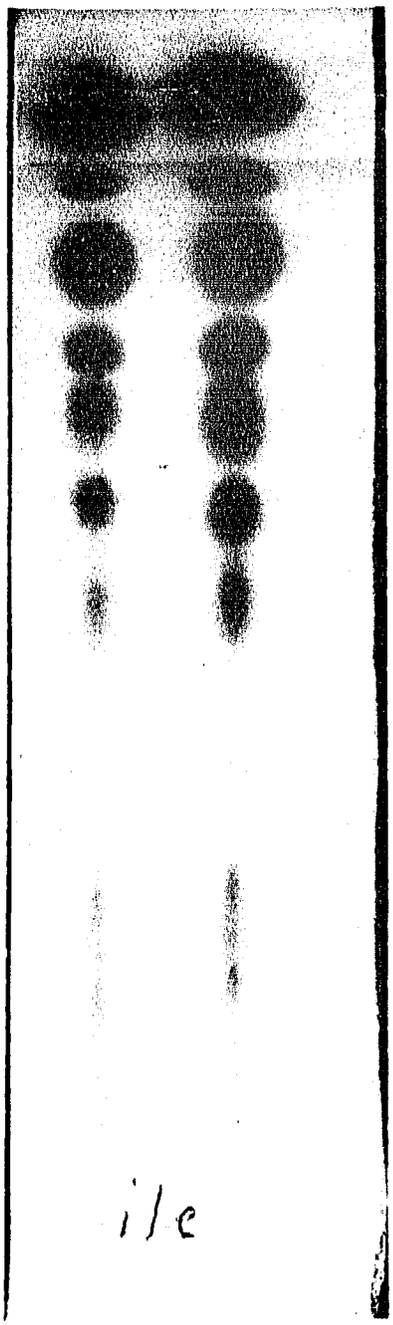


Figure 15: Cbz-Arg-Ile-ONp picrate after lengthy storage at room temperature. From Left to Right; 20, 50, 100 µg spots. The plate was run in 20:1 EtOAC: 1N HCl/HOAc to dissociate picric acid (visible at solvent front just below nitrophenol), dried, and rerun in BAW. See text.

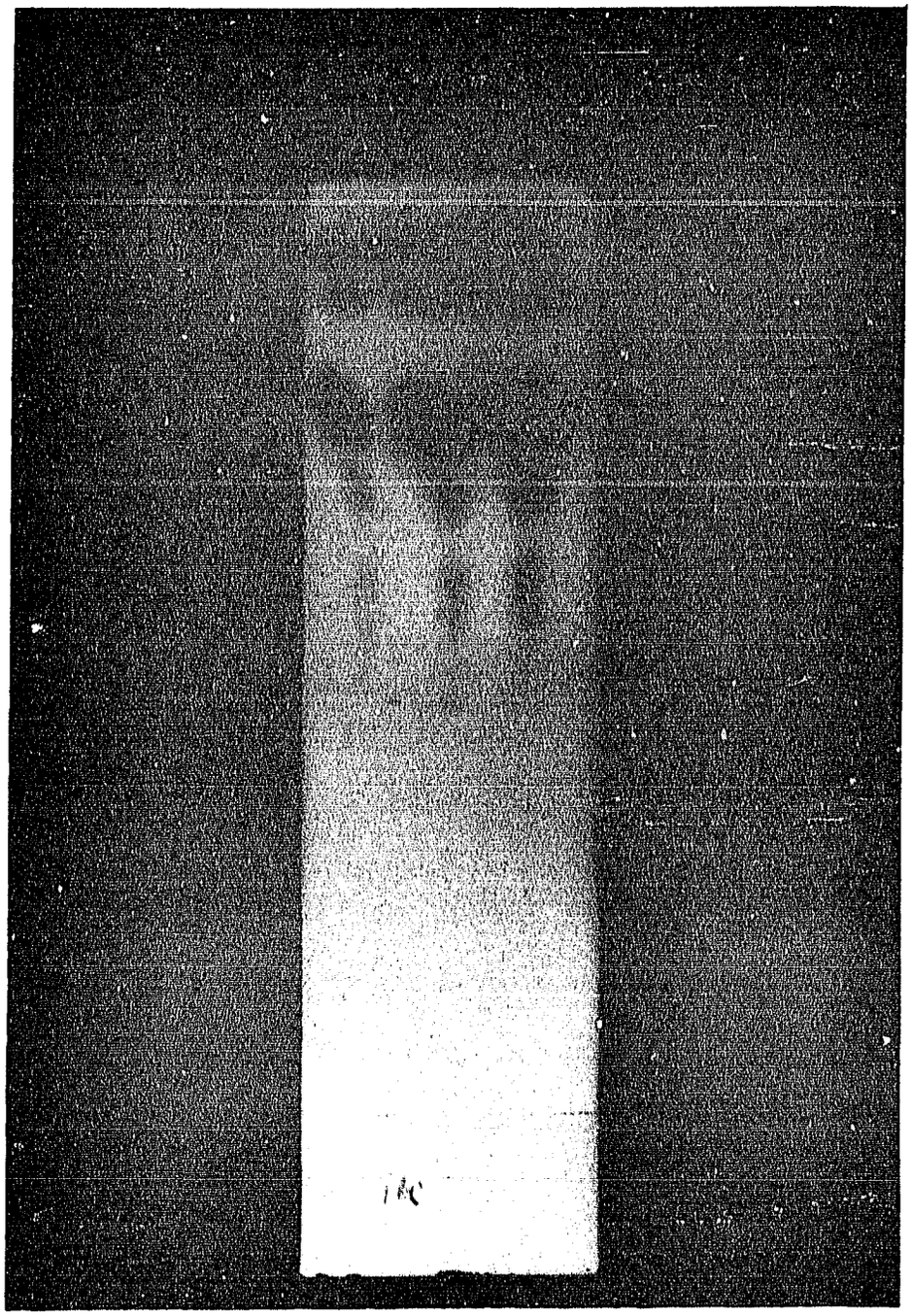


Figure 16: Cbz-Arg-Ile-ONp picrate freshly recrystallized from 95% ethanol. From Left to Right; 20, 50, 100 μg spots. The plate was run in 20:1 EtOAc: 1N HCl/HOAc to dissociate the picric acid (visible at solvent front just below nitrophenol), dried, and rerun in BAW. R_f 0.83 See text.

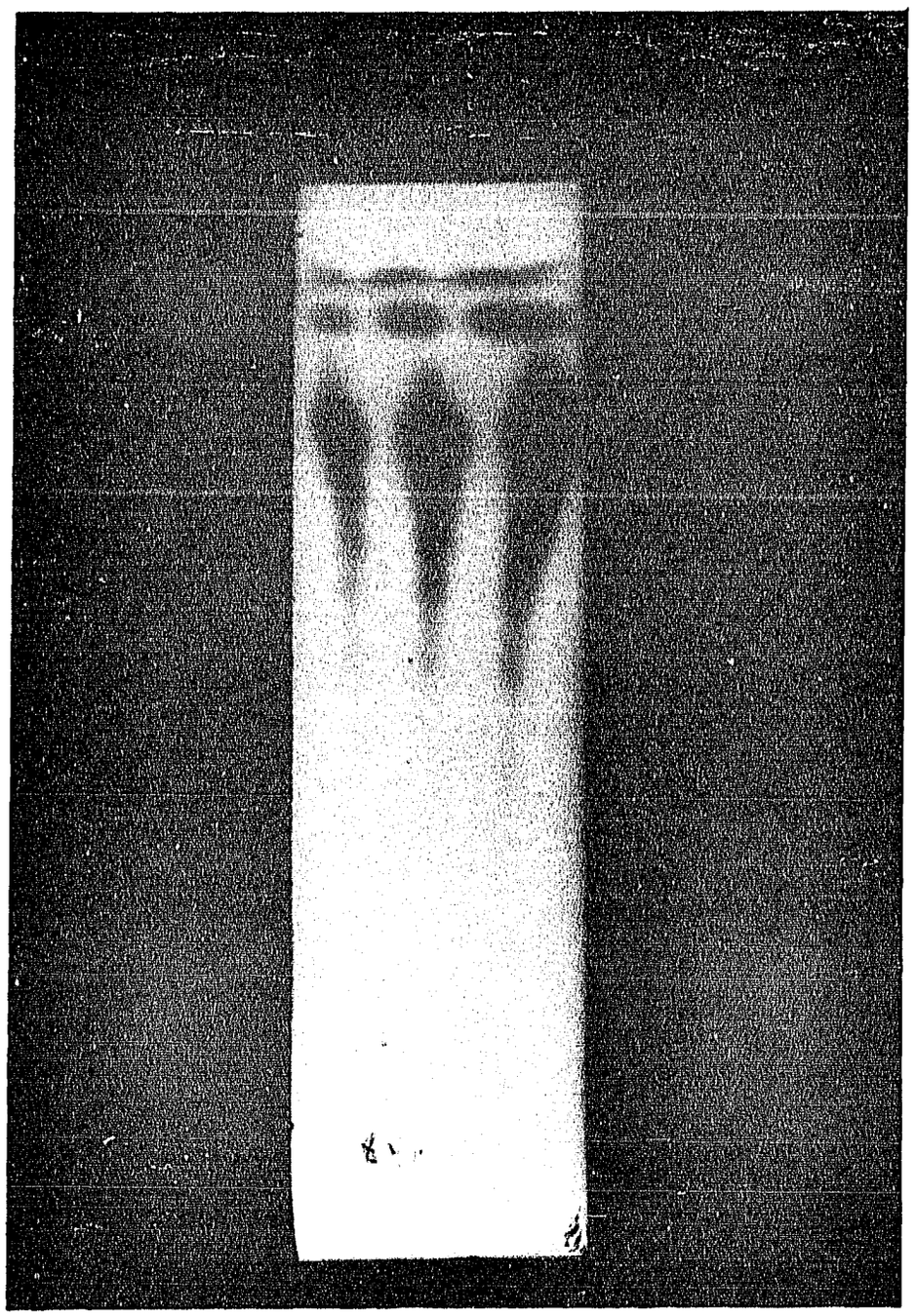


Figure 17: Cbz-Arg-Tyr-ONp picrate after lengthy storage at room temperature. From Left to Right; 20, 50, 100 μg spots. The plate was run in 20:1 EtOAc: 1N HCl/HOAc to dissociate picric acid (visible at solvent front just below nitrophenol), dried, and rerun in BAW. See text.

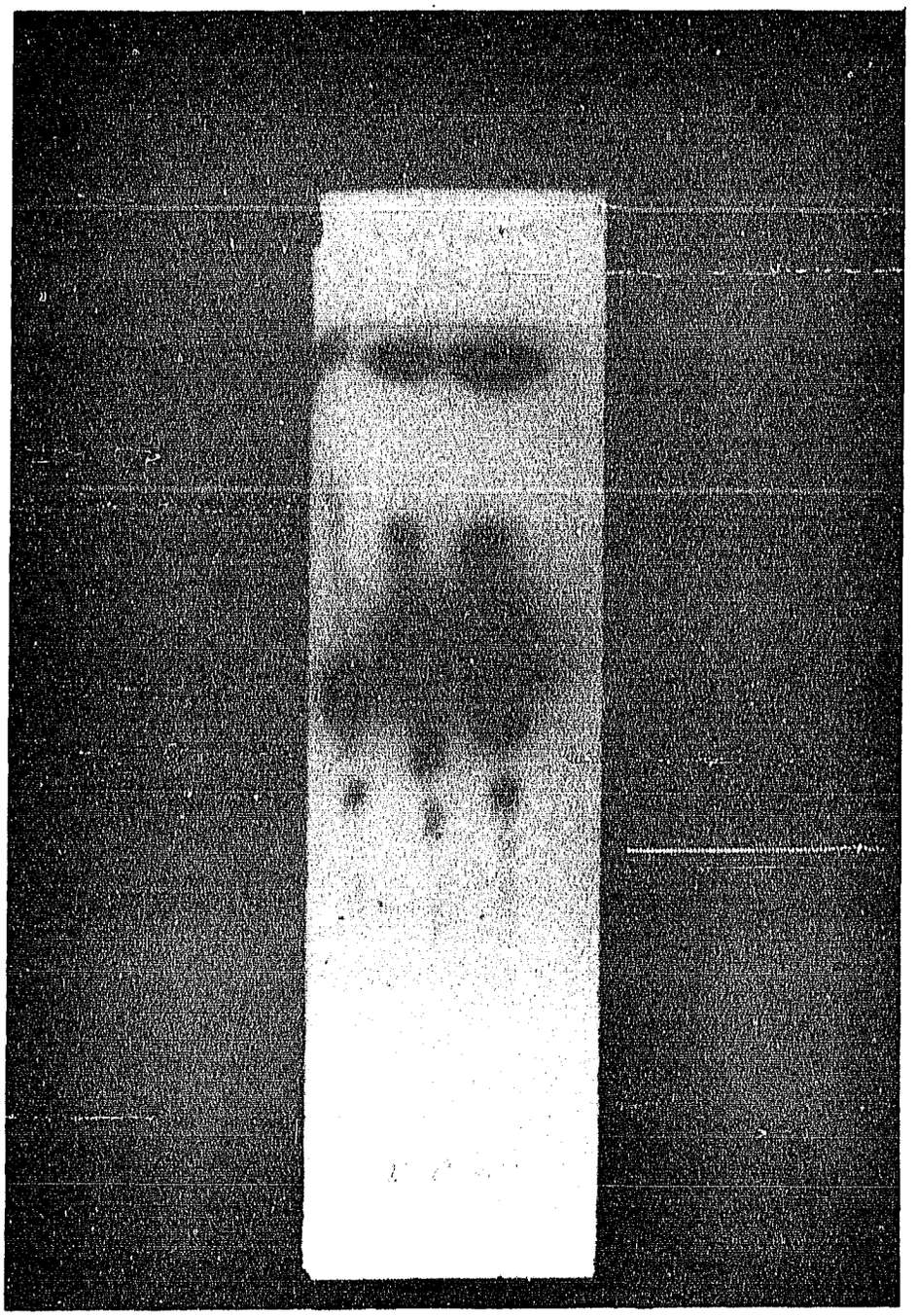


Figure 18: Cbz-Arg-D-Gln-ONp picrate after lengthy storage at room temperature. From Left to Right; 20, 50, 100 μg spots. The plate was run in 20:1 EtOAc: 1N HCl/HOAc to dissociate the picric acid (visible at solvent front just below nitrophenol), dried, and rerun in BAW. R_f of major component 0.58. See text.

3. Acylations of Glycine-Merrifield Resin Esters:

Boc-glycine, esterified with chloromethylpolystyrene-2% divinylbenzene resin (15) (0.27meq glycine/g resin ester was deprotected with N HCl in glacial acetic acid (22), washed with a DMF solution F in N-methylmorpholine and 0.5F in acetic acid (23), then washed with DMF. Each Cbz-Arg-X-ONp picrate (7μm) was added to a two fold molar excess of the prepared glycine-resin ester in 0.2 ml DMF. After 15 hours the resin samples were washed first with DMF, then with 1N HCl in acetic acid. The combined washings and the resin samples were separately taken to dryness under reduced pressure and hydrolyzed with 6N HCl and with 1:1, 12N HCl:dioxane (24) respectively. Amino acid analyses of the hydrolysates allowed approximate calculations of the degree of acylation of the glycine-resin ester compared to the theoretical degree of acylation (Table II).

4. (Cbz-Arg-(D and L)-Gln-Asn-Cys-Pro-Leu-GlyNH_2)$_2$:

(H-Asn-Cys-Pro-Leu-GlyNH_2)$_2$ • 2 TFA (16) was dissolved at a concentration of 30 mg/ml in aqueous 20% N-methylmorpholine, then the solution was frozen and lyophilized. To the lyophilized powder was added a 20% molar excess of either Cbz-Arg-D-Gln-ONp picrate or Cbz-Arg-L-Gln-ONp picrate in a minimal volume of DMF. After 48 hours glycine-substituted Merrifield resin (free base, 4X molar excess compared to the theoretical amount of residual dipeptide nitrophenyl ester) was added to the mixture. After an additional 15 hours the reaction mixture was filtered, the filtrate was concentrated to an oil, and the crude product was obtained as a solid by trituration with ether. The resultant yellow powder was mixed with a 2.5 fold molar excess of dicyclohexylamine hydrochloride and stirred at 45° for 30 minutes in H_2O (0.05 mmole peptide picrate/ml H_2O). The digest was filtered and the filter cake was twice reextracted with

Table II

Acylation of glycine-resin ester by

Cbz-Arg-X-ONp picrates

X	Calculated percent of coupling*	
	$100 \times \frac{2\ \text{Arg}}{\text{Gly}}$	$100 \times \frac{\text{Arg (bound)}}{\text{Arg (bound + free)}}$
L-Gln	73	81
L-Ile	72	80
L-Tyr	55	57
D-Gln	67	91

*Acylations of glycine-resin ester were carried out as described in the text. The extent of coupling was calculated by two methods (taking into account the two-fold excess of Gly): (1) on the basis of the ratio of Arg to Gly determined in the acid hydrolysate of an aliquot of the polymeric sample; (2) on the basis of the ratio of Arg determined in the acid hydrolysate of the supernatant and the acid hydrolysate of the polymeric sample. Calculations were based on Arg rather than "X" values because of the erratic recoveries of some of these amino acids, particularly glutamic acid (24) after hydrolyses in dioxane-HCl mixtures.

fresh aqueous dicyclohexylamine hydrochloride mixture. The filtered, combined aqueous extracts were adjusted to pH 4.0 with acetic acid and applied to a bed of Biorex 70 (23x2 cm) in the acid cycle. The column was washed with 0.2N acetic acid and water, then eluted with 30:4:66, pyridine:acetic acid:water (25,26). The products were recovered from the aqueous pyridine acetate solutions by rotary evaporation followed by lyophilization from frozen aqueous solutions. The purified "All-L-peptide" was obtained in 44% overall yield after precipitation from aqueous ethanol and the "2-D-peptide" was obtained in 48% overall yield after gel filtration on Sephandex G-10. Both products were characterized by amino acid analysis of acid hydrolysates (Table III).

5. Enzymatic Deprotections:

Samples of Cbz-Arg-protected peptides were dissolved at a concentration of 15 mg/ml in 0.02M $CaCl_2$ (27) at room temperature. The pH's of the substrate solutions were adjusted to 8.0 with aqueous NaOH, and 0.1 ml of stock trypsin solution (1 mg thrice-crystallized trypsin per ml 0.02M $CaCl_2$ in .001M HCl) was added for each ml of substrate solution. The stirred digest was maintained at pH 8.0 by automatic additions of 0.05N NaOH from a pH Stat (Fig. 19). In one experiment, a sample of "2-D-peptide" was incubated for an hour with trypsin as described, then an equal amount of "All-L-peptide" solution was added to the reaction mixture and the digestion continued (Fig. 20). In those cases where the degree of deprotection was determined by amino acid analysis, the cleaved and unreacted peptides were isolated together, but free of Cbz-arginine, by adjusting the pH of the reaction mixture to 3.0 with acetic acid followed by passage through a column of Sephadex G-10 (2.0x110 cm) in 0.2N acetic acid. Samples of the lyophilized

Table III

Amino acid compositions of peptides* before and after tryptic digestion†

	(1)	(2)	(3)	(4)
Arg	1.0	1.0	0.05	0.5
Glu	1.1	1.1	1.1	1.0
Asp	1.0	1.1	1.0	1.0
½Cys	1.0	1.0	0.9	0.9
Pro	1.0	1.0	1.0	1.0
Leu	1.0	1.0	0.9	1.0
Gly	1.0	1.1	1.0	1.0
NH_3	2.8	3.1	2.9	2.7

*(1) $(Cbz\text{-}Arg\text{-}\underline{L}\text{-}Gln\text{-}Asn\text{-}Cys\text{-}Pro\text{-}Leu\text{-}Gly\text{-}NH_2)_2$

(2) $(Cbz\text{-}Arg\text{-}\underline{D}\text{-}Gln\text{-}Asn\text{-}Cys\text{-}Pro\text{-}Leu\text{-}Gly\text{-}NH_2)_2$

(3) Tryptic cleavage product (5 hours) of (1)

(4) Tryptic cleavage product (10 hours) of (2)

†Tryptic digestions were carried out as described in the text; incubation time was 5 hours for the "All-L-peptide" and 10 hours for the "2-D-peptide". Tryptic digests were passed through a column (110x2.0 cm) of Sephadex G-10 in 0.2N HOAc to remove Cbz-Arg-OH from a mixture of unreacted material and N-deprotected product. Acid hydrolysates of Cbz-Arg-OH free mixtures were analyzed for their amino acid composition.

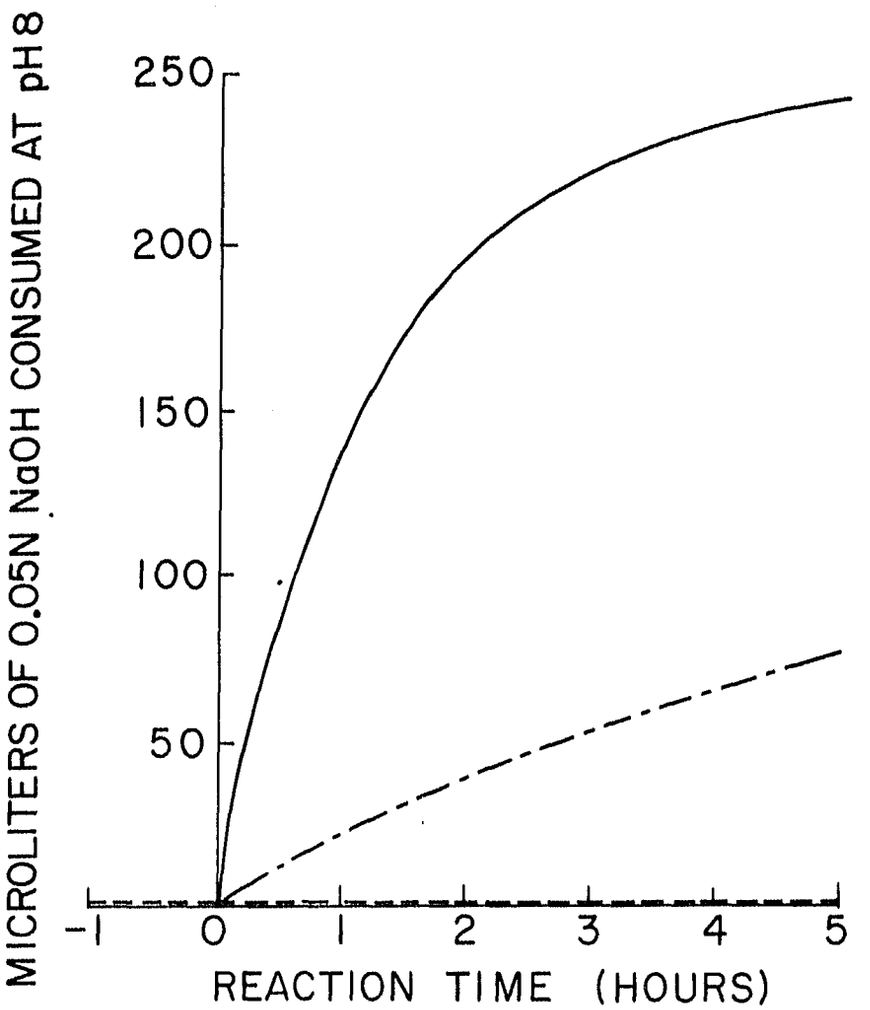


Figure 19: Tryptic Hydrolysis of "A11-L" and "2-D" Peptides:

"A11-L-peptide" (15 mg) (——) or 15 mg of "2-D-peptide" (—.—) was treated with 0.1 mg trypsin in a total volume of 1.0 ml 0.02 M $CaCl_2$, pH 8.0 for 5 hours. Trypsin incubated in the absence of peptide served as control (---). NaOH (0.05N) was added to maintain a pH of 8.0 and the amount of base added was recorded as a function of time.

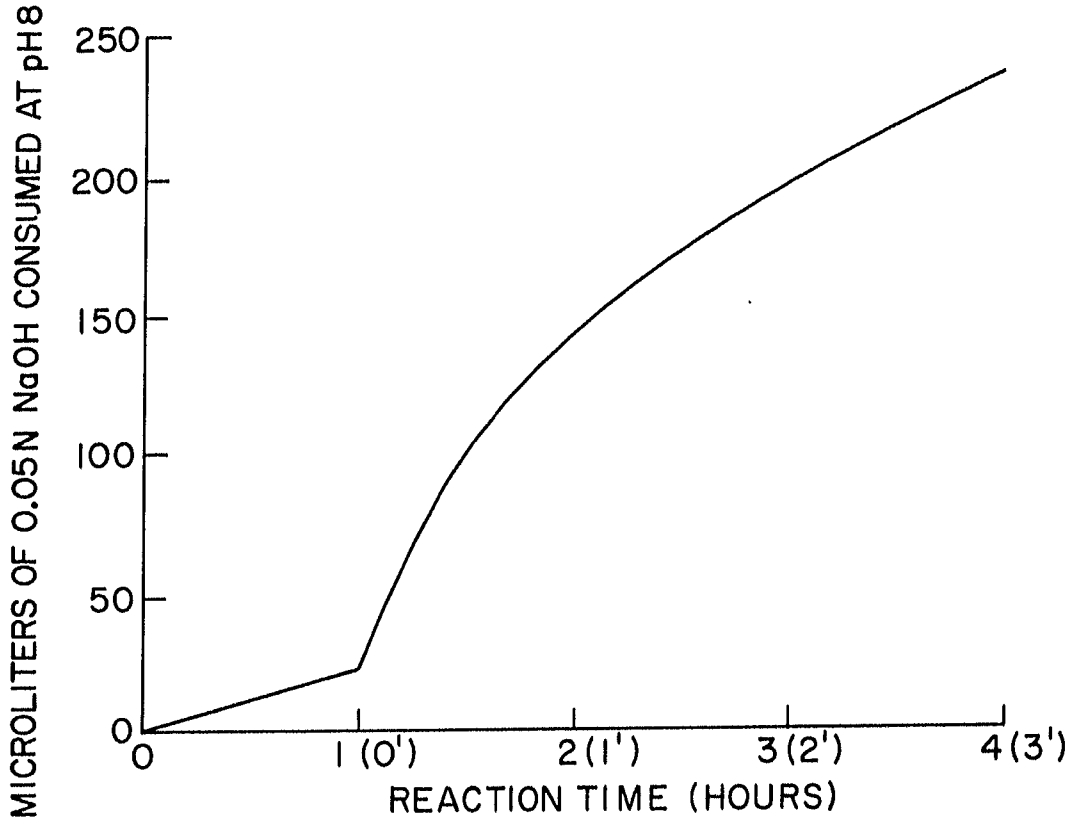


Figure 20: Tryptic Hydrolysis of "All-L-peptide" in the presence of "2-D-peptide":

"2-D-peptide" (15 mg) in 1.0 ml of 0.02 M $CaCl_2$ pH 8.0 was treated with 0.1 mg trypsin. After 1 hour "All-L-peptide" (15 mg) in 1.0 ml 0.02 M $CaCl_2$ was added to the foregoing reaction mixture. The uptake of base required to maintain a pH of 8.0 was recorded as a function of time throughout the course of the tryptic digestion.

products were hydrolyzed and analyzed for amino acid composition. Residual unreacted peptide was calculated from the arginine content of such hydrolysates (Table III).

C. Model Synthesis of Deamino Oxytocin:

1. Development of Sulfonic Acid Column Purification System:

Cbz-Arg-Ile-Gln-Asn-Cys (AcM)-Pro-Leu-GlyNH_2 (partial racemate at the Ile residue) (45 mg) was deprotected as described in the model system lyophilized, and dissolved in 0.5M NH_4OAc buffer at pH 4.6. The solution was added to a column of Bio-Rad AG50W -X4 sulfonic acid ion exchange resin (acid cycle) previously washed and equilibrated with the pH 4.6 buffer. As the digested peptide was eluted in the buffer, 6.1 ml fractions were collected automatically. After about 90 ml were collected, a linear gradient from pH 4.6 to 7 was started through the column by uniform mixing with a 0.5 M NH_4OAc buffer at pH 7. Samples from the tubes were removed for the Folin-Lowry reaction (28) and read at 750 mμ. The results shown in Fig. 21 indicated a single peak spanning tubes 15-33. The pH range found in these tubes was 4.6 to 4.8. Amino acid analysis of the acid hydrolysate of the lyophilized residue from pooled tubes 15-31 revealed no trace of Arginine or Alloisoleucine.

2. S-Acetamidomethyl, 3-Mercaptopropionyl-p-Nitrophenylate:

S-Acetamidomethyl, 3-Mercaptopropionic acid (2.84g, 16mM) (20) was stirred along with p-nitrophenyl trifluoroacetate (4.70g, 20mM) in 8 ml of pyridine freshly distilled from NaOH for 30 minutes. The addition of 70 ml of water precipitated the crude product as a solid which was crystallized from ethyl acetate. Yield 3.0g (63%); mp 119-121 elemental analysis for $C_{12}H_{14}N_2O_5S$: Calculated; C 48.31, H 4.73, N 9.39
Found; C 48.33, H 4.85, N 9.24.

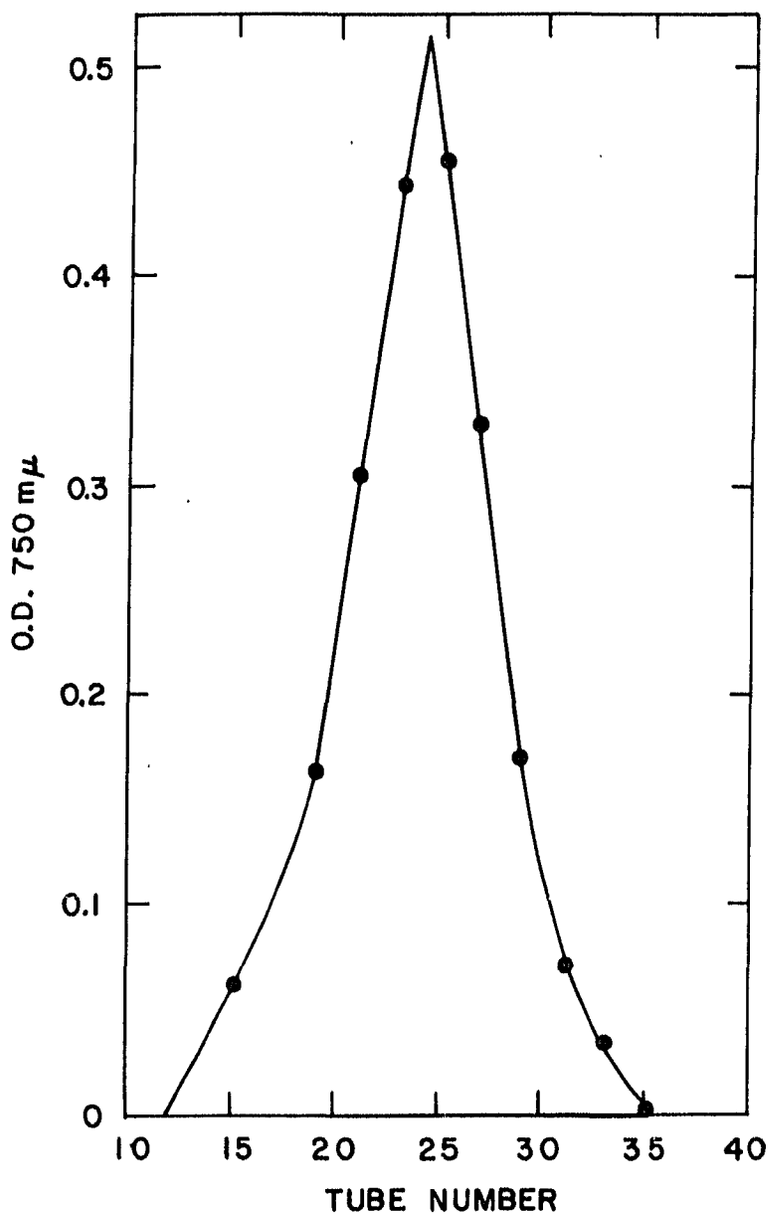


Figure 21: Folin-Lowry elution peak of H-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2 during development of ion exchange column procedure.

3. Cbz-Arg-Gln-Asn-Cys(AcM)-Pro-Leu-$GlyNH_2$:

H-Asn-Cys(AcM)-Pro-Leu-$GlyNH_2$ · HCl (20) was dissolved at a concentration of 50 mg/ml in aqueous 20% N-methylmorpholine, then the solution was frozen and lyophilized. To the lyophilized residue was added a 20% molar excess of Cbz-Arg-Gln-ONp picrate in a minimal volume of DMF. After 4 days, the gelatinous reaction mixture was triturated to a solid with ether. The resultant yellow powder was mixed with a 2.5 fold molar excess of dicyclohexylamine hydrochloride (DCHA·HCl) and stirred at 45° for 30 minutes in H_2O (0.03 mM peptide picrate/ml H_2O). The digest was filtered and the filter cake was twice reextracted with fresh aqueous DCHA·HCl mixture. The filtered, combined aqueous extracts were adjusted to pH 4.0 with 0.5 N NaOH and applied to a bed of Biorex 70 (23 x 3 cm) in the acid cycle. The column was washed with 0.2 N HOAc (500 ml) and water (500 ml) then eluted with 30:4:66, pyridine: acetic acid:water (25,26). The product was recovered from the aqueous pyridine acetate solution by rotary evaporation followed by lyophilization from a frozen aqueous solution. The purified peptide was obtained after gel filtration in 0.2N HOAc on Sephadex G-10 (2.0 x 110 cm); (Fig. 22) yield 83%; $[\alpha]_D^{25}$ -76.7 (c=0.5, 0.2N HOAc).

A hydrolyzed sample gave the following molar ratios of Amino Acids and NH_3: Arg, 0.9; Asp, 1.0; Glu, 1.1; Pro, 1.1; Gly, 1.0; Leu, 0.9 and NH_3, 3.5.

4. Cbz-Arg-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-$GlyNH_2$ (partial racemate at Ile residue)

H-Gln-Asn-Cys(AcM)-Pro-Leu-$GlyNH_2$ was prepared by tryptic hydrolysis of the Cbz-Arginyl group as described for the model system except that a 10 mg trypsin per ml 0.02M $CaCl_2$ in 0.001M HCl stock solution was used.

Figure 22: Cbz-Arg-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2. All spots are 50 μg. The three samples represent varying pooled fractions according to the Folin-Lowry elution peak from the Sephadex G-10 column. The fraction on the far right was from the trailing edge of the peak and was discarded.

The digestion curve is shown in Figure 23. After 25 minutes, the digestion mixture* was frozen and lyophilized to a powder which was partially dissolved in DMF and treated to a 20% excess of Cbz-Arg-Ile-ONp picrate. After five days, one-half the molar equivalent of N-methylmorpholine was added and after one more day the product was isolated from the mixture and the picrate removed as described for the protected glutamine peptide. Then the filtered combined aqueous extracts were adjusted to pH 4.6 with HOAc and applied to a bed of Bio-Rad AG50W-X4 (3 x 10 cm) in the acid cycle. The column was washed with 0.5M NH_4OAc (pH 4.6) and water, then eluted with the pyridine acetate buffer previously described. The product was recovered from the aqueous pyridine acetate solution by rotary evaporation followed by lyophilization from a frozen aqueous solution. The purified peptide was obtained after gel filtration in 0.2N HOAc on Sephadex G-15; yield 74%. A hydrolyzed sample gave the following molar ratios of Amino Acids and NH_3: Arg, 0.9; Asp, 1.0; Glu, 1.0; Pro, 1.1; Gly, 1.0; Alloisoleucine, 0.2; Ile, 0.7; Leu, 1.0; and NH_3, 4.0.

5. H-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2:

Cbz-Arg-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2 (partial racement at Ile residue) was deprotected by tryptic hydrolysis in the manner described for the model system. The digestion curve is shown in Figure 25. The pH of the digest was adjusted to pH 4.6 and applied to a bed of AG50W-X4 in the acid cycle. The product was eluted with 0.5M NH_4OAc (pH 4.6) and the eluate was concentrated in vacuo and lyophilized from water. The

*A small sample of the digestion mixture was passed through a Sephadex G-10 column and part was hydrolyzed for amino acid analysis in order to determine the extent of the deprotection reaction. No trace of Arginine was detected. The unhydrolyzed sample was spotted for TLC in BAW (Fig. 24) which revealed one ninhydrin positive component.

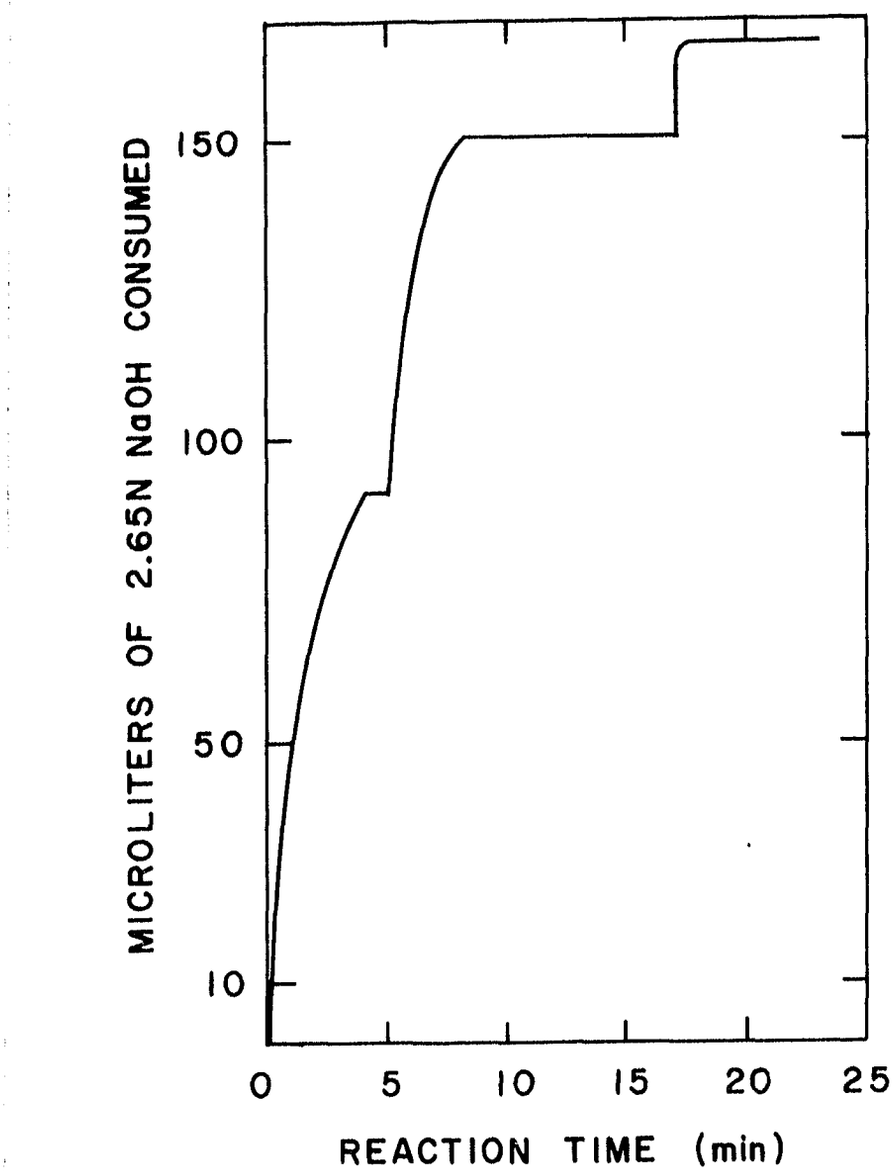


Figure 23: Tryptic digestion of Cbz-Arg-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2. Additional enzyme, equal to the initial concentration used, was introduced at 5 min. and again at 17 min.

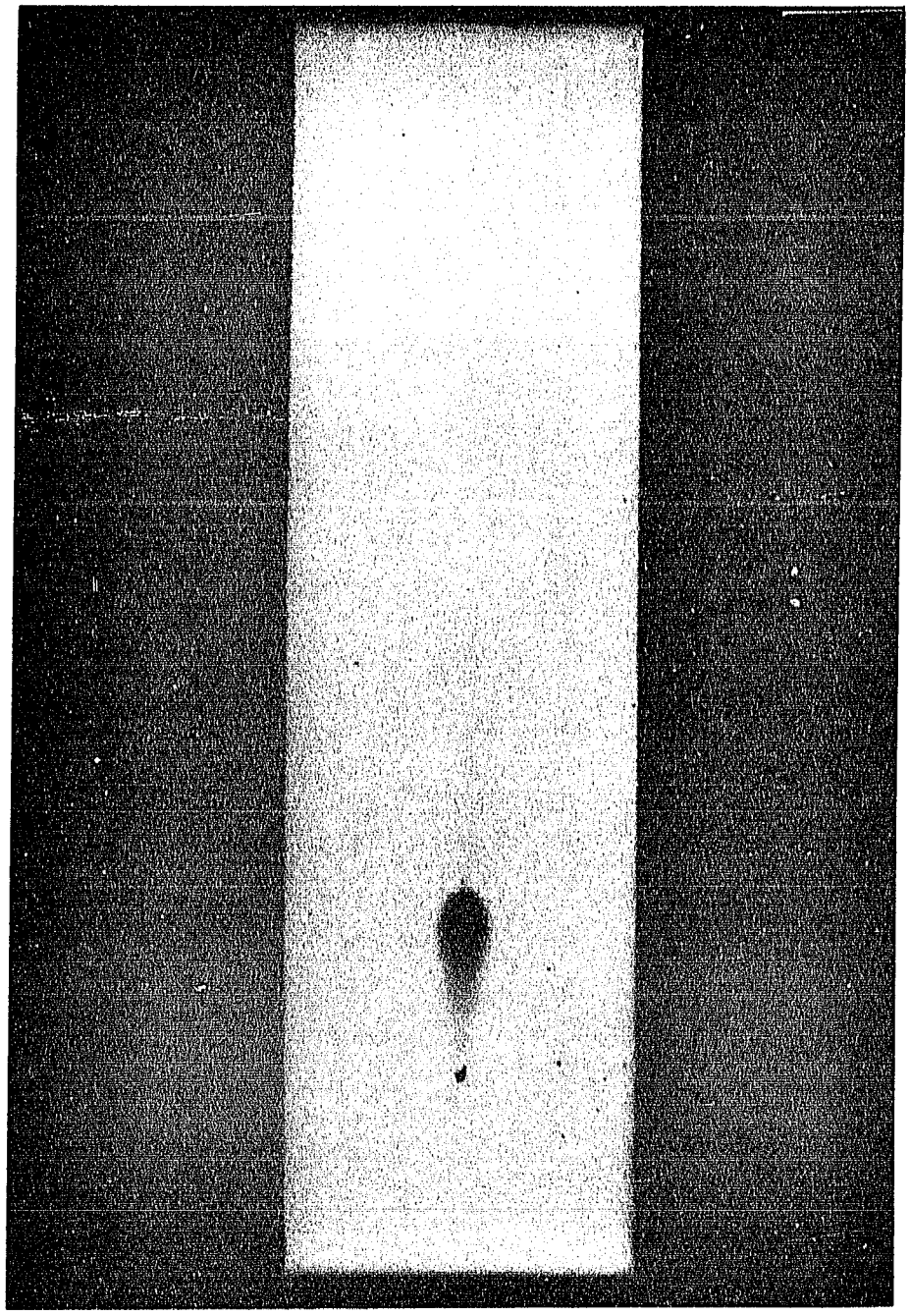


Figure 24: H-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2 (50 μg) purified on Sephadex G-10. The plate was run in BAW and developed with Ninhydrin spray reagent (134).

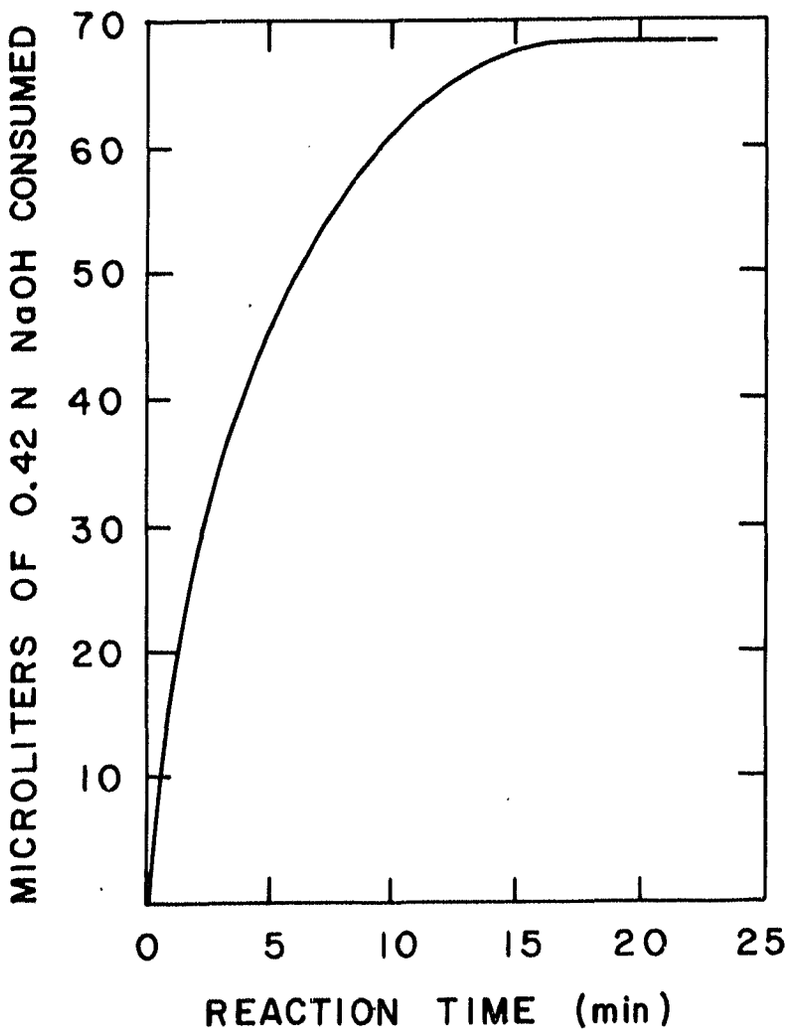


Figure 25: Tryptic digestion of Cbz-Arg-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-$GlyNH_2$ (partial racemate at Ile residue).

resultant powder was purified by gel filtration on Sephadex G-15 and converted to the free base by passage through a bed of Amberlyst-21 (basic form) in water followed by lyophilization of the frozen eluate. Yield 52%. Amino acid analysis; Asp, 1.0; Glu, 1.0; Pro, 1.1; Gly, 1.0; Ile, 1.0; Leu, 1.0; and NH_3, 4.0. No trace of Arginine or Alloisoleucine was detected. Thin layer chromatograms of the product are shown in Figures 26 and 27.

6. Cbz-Arg-Tyr-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2:

To the free base heptapeptide was added a 30% excess of Cbz-Arg-Tyr-ONp picrate in the minimal volume of DMF. After 2 days a 15% molar equivalent of additional activated tyrosine intermediate was added and one-half the stochiometric amount of N-methylmorpholine was added a day later. After a total of 4 days, the reaction solution was triturated to a solid with ether, and the resultant yellow powder was dissolved in 50% DMF:0.2N HOAc, filtered and applied to a bed of Amberlyst-21 (1.5 x 7 cm) previously equilibrated with the same solvent. The column was washed with 0.2N HOAc and the eluate was concentrated to dryness several times from water on the rotary evaporator. The residue was partially dissolved in 0.5 M NH_4OAc at pH 4.6, centrifuged, and the supernate was passed through the sulfonic acid column as described for the protected isoleucine peptide intermediate. The lyophilized product was then passed through a small Amberlyst-21 column in water and lyophilization of the frozen eluate yielded 40% of the product. TLC in BuOH/HOAc/H_2O (4:1:1) of the centrifuged sediment indicated that this material contained mostly the desired product. It was dissolved in a minimal volume of glacial HOAc, diluted with H_2O and centrifuged at 6000 rpm for ten minutes to remove the fine sediment. The supernate was passed through the sulfonic acid column and

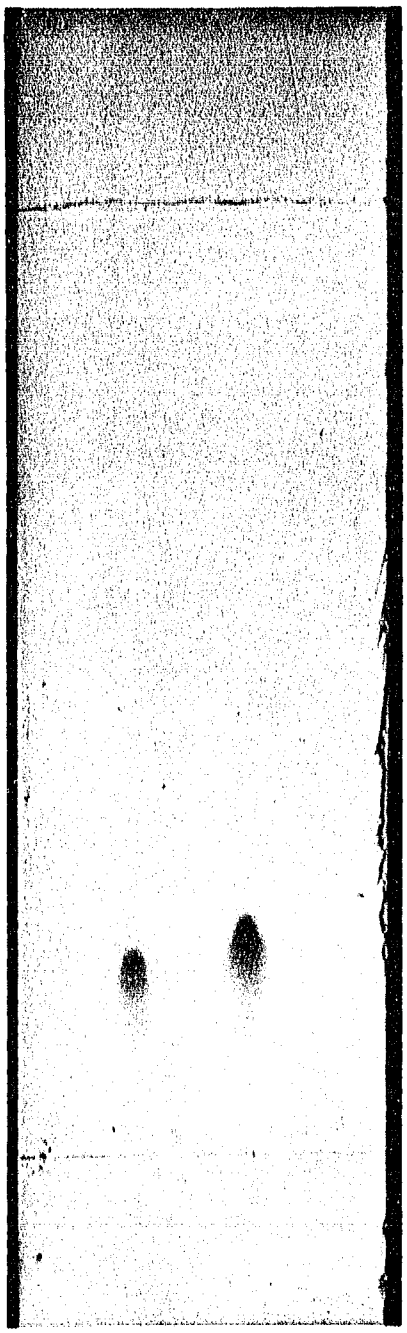


Figure 26: H-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH$_2$. From Left to Right; 50, 100 μg. Developed with Ninhydrin spray reagent (134). R_f 0.20

Figure 27: H-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2. From Left to Right; 50, 100 µg. R_f 0.20

the Amberlyst-21 column (in water) as previously described. The lyophilized product yielded an additional 40% of the product. Total yield, 80%. The product showed one major contaminant on TLC (Fig. 28). A hydrolyzed sample containing 1 mg phenol per ml 6N HCl gave the following molar ratios of Amino Acids and NH_3: Arg, 1.0; Asp, 1.0; Glu, 1.1; Pro, 1.1; Gly, 1.0; Ile, 1.0; Leu, 1.0; Tyr, 0.7; and NH_3, 4.1.

7. 3-Mercaptopropionyl (ACM)-Tyr-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2:

H-Tyr-Ile-Gln-Asn-Cys (AcM)-Pro-Leu-GlyNH_2, prepared from the corresponding protected peptide by tryptic removal of the Cbz-Arginyl group in the usual manner (Fig. 29), was partially dissolved in a minimal volume of DMF, then the stochiometric amounts of 3-Mercaptopropionyl (AcM)-ONp and N-methylmorpholine were added. After one day the reaction solution was concentrated on the rotary evaporator and the residue was washed well with 2-propanol. The crude product was dissolved in 50% aqueous DMF and passed through a mixed bed of Amberlyst-15 and Amberlyst-21 (20). The eluate was concentrated to dryness on the rotary evaporator, washed with 2-propanol and lyophilized from glacial HOAc. Yield, 67%. TLC in BAW showed one major component of R_f 0.47 (Fig. 30).

8. Deamino Oxytocin:

Removal of the Acetamidomethyl groups were performed as described by Marbach et al. (20). The reaction solution was concentrated in vacuo and placed directly on a Sephadex G-10 column (1.5 x 4 cm). The eluate was concentrated to a small volume which constituted the aqueous phase of the system nBuOH/Benzene/HOAc/Pyridine/H_2O (500:500:35:15:950) and it was applied in this two-phase system to a Sephadex G-25 partition column equilibrated with the upper phase of such a system. Fractions of 2.1 ml were collected and samples were removed from every third tube. These

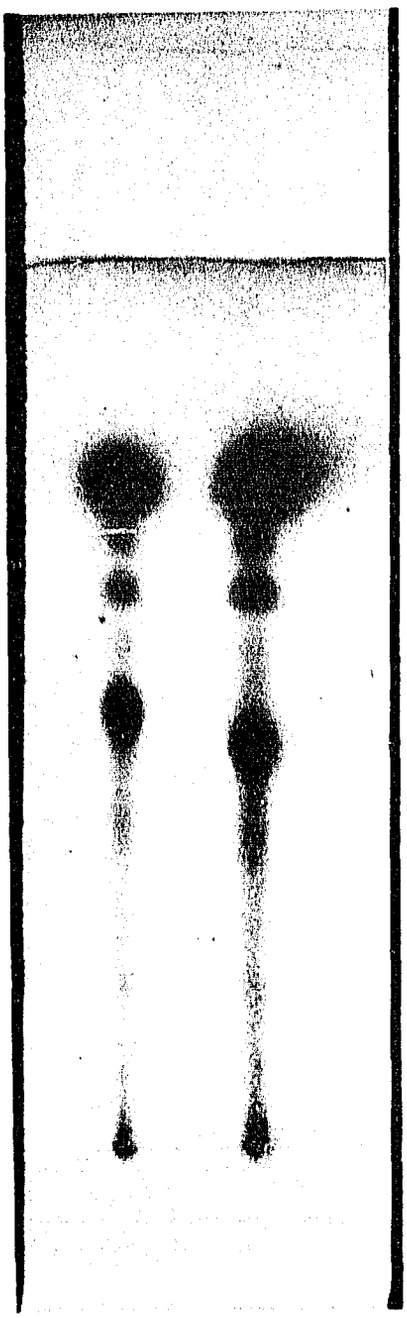


Figure 28: Cbz-Arg-Tyr-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2 (uppermost spot). From Left to Right; 50 µg, 100 µg.

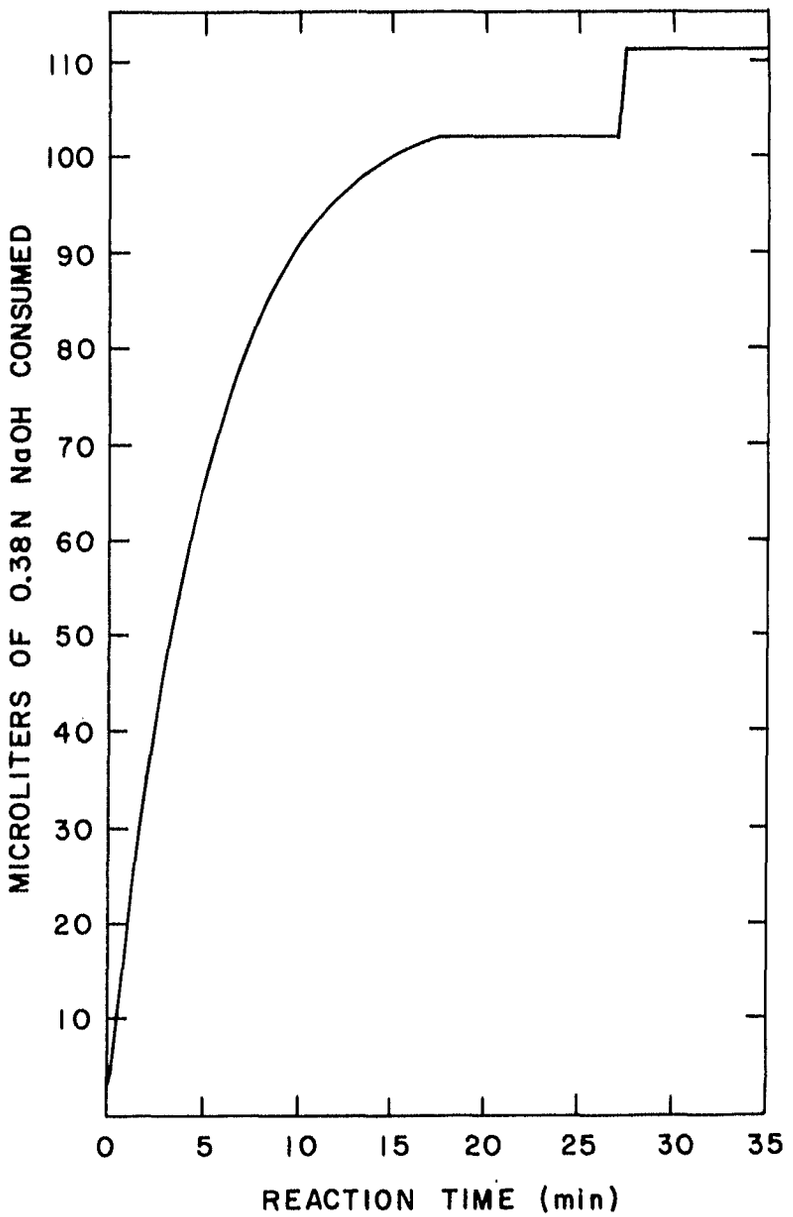


Figure 29: Tryptic digestion of Cbz-Arg-Tyr-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2. Additional enzyme, equal to the initial concentration used, was introduced at 27 min.

Figure 30: 3-Mercaptopropionyl (AcM)-Tyr-Ile-Gln-Asn-Cys(AcM)-Pro-Leu-GlyNH_2. From Left to Right; 5, 10, 50 µg spots. R_f 0.47

were evaporated to dryness and the major elution peak was determined by the Folin-Lowry method. The result is shown in Figure 31. The product was recovered from the concentrated eluate by lyophilization from a frozen aqueous solution. Yield 45%, $[\alpha]_D^{25}$-94.0 (c=0.33, 1N HOAc). TLC of this product showed only one spot of R_f 0.51 in the BAW solvent system (Fig. 32). When the analog was subjected to avian vasodepressor assay using the four-point design on four conscious chickens (29) according to the procedure of Coon (30), an activity of 880 units/mg was recorded.

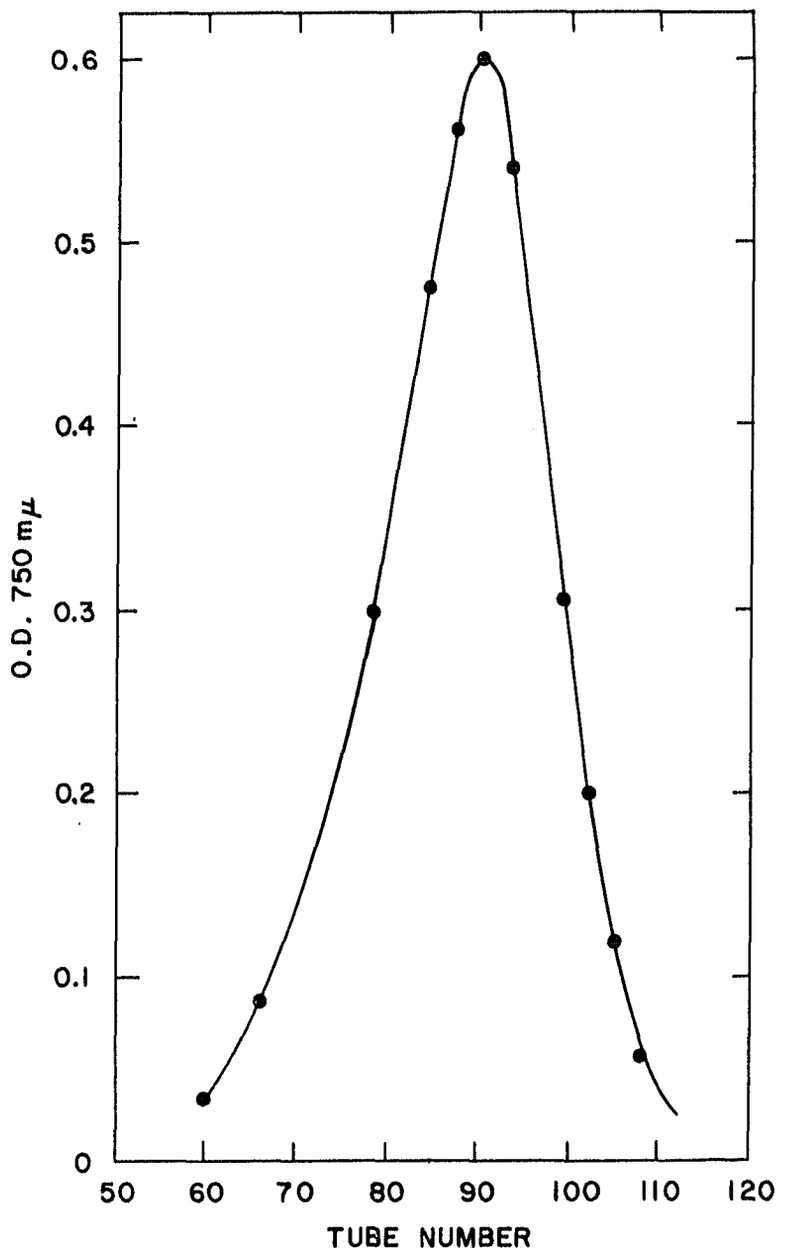


Figure 31: Folin-Lowry elution peak from partitiion column chromatography of Deamino Oxytocin.

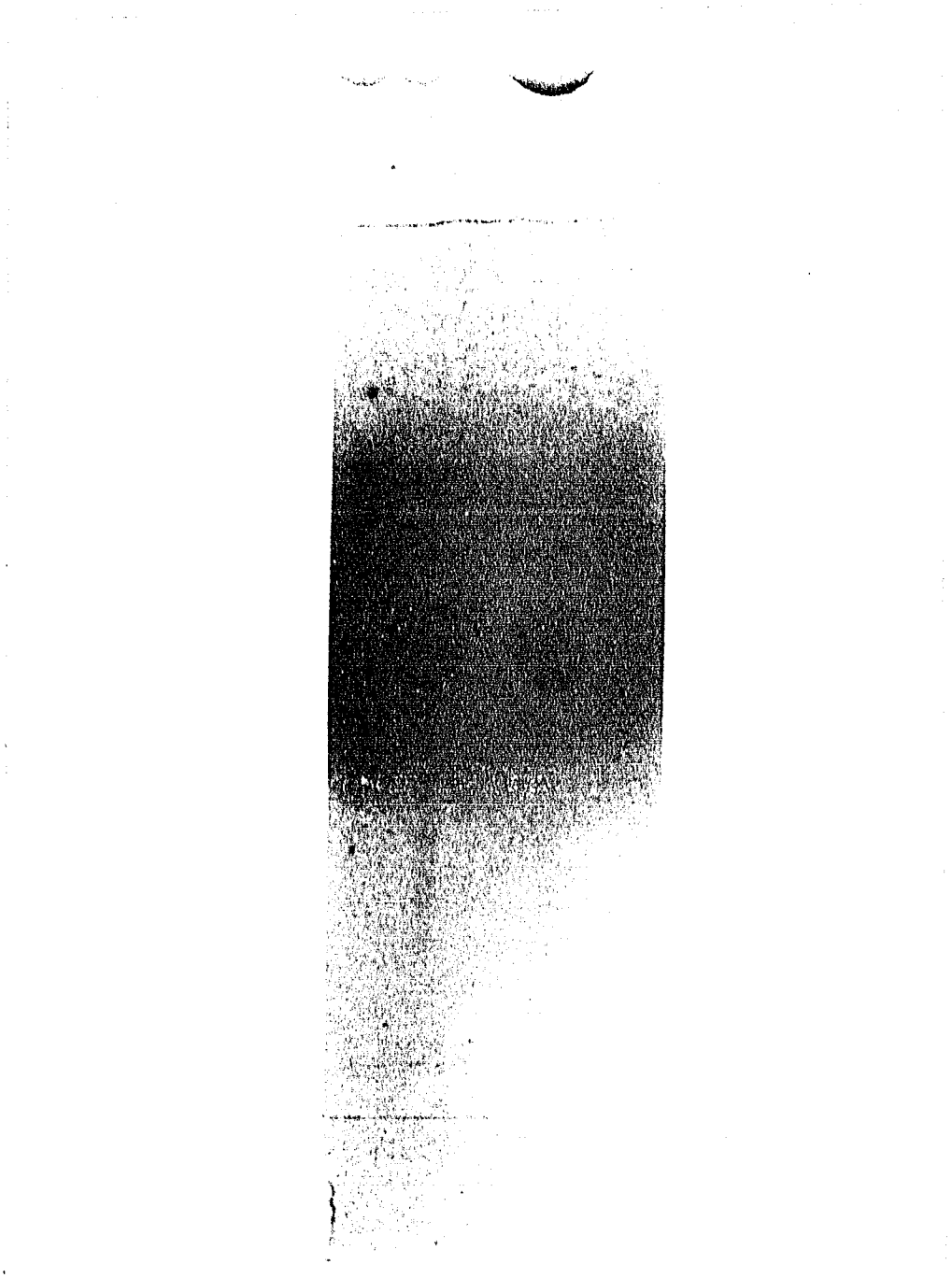


Figure 32: Deamino Oxytocin after purification by partition chromatography. From Left to Right; 100 µg, 50 µg spots. R_f 0.51

DISCUSSION

A. Evaluation of Experimental Results:

Several conclusions may be drawn from the first peptide synthesis using trypsin-labile Cbz-arginyl groups for the protection of α-amino functions. In those cases examined, the Cbz-Arg-X-ONp picrate intermediates were easily prepared from known compounds, they were conveniently purified, and they were obtained as pure, crystalline compounds. The Cbz-Arg-X-ONp picrates readily acylated amino compounds in the solid phase and in solution to give Cbz-arginylpeptide intermediates. The soluble protected intermediates were deprotected under tryptic catalysis with a high degree of chemical specificity and in the limited cases studied, with a useful degree of stereodiscrimination. The deprotection reaction was achieved in aqueous solution at pH 8 at room temperature; essentially physiological conditions. The course of the reaction was readily monitored by titration of liberated carboxyl groups, and the final degree of deprotection was determined with good sensitivity and precision by amino acid analysis of acid hydrolysates. Other types of peptide chains must be tested before a general application can be asserted for the rectification of racemization in a residue following its incorporation into the chain. However, the success encountered with the isoleucine residue during the synthesis of deamino oxytocin, coupled with the large rate differences observed during the digestion of the model D and L dimers, strongly indicates that the stereoselective properties of the enzyme will be widely applicable. A more detailed discussion of these possibilities will be presented.

Although the first experiments clearly indicated the potential strength of the tryptic deprotection scheme, several technical points

of the initial experimental format were imperfect. Without revision of the basic principles of the method, these technical points were refined to permit a standardized synthesis of a potent oxytocin analog. One modification involved the method for protecting the sulfhydryl groups of the cysteine residues. The initial use of symmetrical disulfides proved to be an inconvenient choice because the nature of the resultant peptide dimers is such that incomplete coupling can lead to heterogeneous products which are extremely difficult to separate. In contrast, single chain peptides generally will be coupled or uncoupled so that the products are either substituted or unsubstituted and their separation is then facilitated by their differing properties. Individual protecting groups for the sulfhydryl functions provided a clear cut solution to the problem by providing the appropriate single chains, but the nature of the group was a major consideration. In addition to being removable by a mild mechanism so as not to defeat one of the fundamental advantages offered by the model, the sulfur-protecting group had to be compatible with the aqueous solubility of the peptide in order to accommodate the enzymatic removal of the α-amino protecting groups. The S-acetamidomethyl group was selected since it was initially developed to operate in aqueous media (31), and especially because it had recently been utilized for a synthesis of deamino oxytocin where its removal by mild treatment with iodine was accompanied by simultaneous formation of the disulfide bridges (20).

The conversion to single-chain peptides permitted an investigation into the development of effective purification systems. The sequential introduction of protected amino acid residues through Cbz-Arg-X-ONp picrates generated a series of intermediates in which the guanido function of the protecting group alternated with the amino group of

deprotected intermediates. The large difference in pKa between ammonium and guanidinium ions provided the basis for the development of routine ion exchange methods for the systematic removal of incompletely coupled or incompletely deprotected fractions from primary products at each stage of the stepwise synthesis.

Another technical improvement was sought for the complete removal of picrate after each addition of a protected amino acid residue, as this was required to achieve aqueous solubility and to prevent enzyme inactivation. The initial method, involving exchange of picrate for hydrochloride ions introduced as dicyclohexylammonium hydrochloride, produced erratic results which were usually reflected as losses in yield presumably caused by incomplete exchange. The inefficiency of the ion exchange procedure is no doubt accounted for by the extremely low aqueous solubilities shared by the dicyclohexylammonium salts and peptide picrates. The method for picrate removal was greatly improved by passing the peptide picrates in solution through a basic anion exchange column. This was made possible by using an exchange resin which operates efficiently in aqueous DMF mixtures (20).

Initial attempts to characterize the Cbz-Arg-X-ONp picrates by thin-layer chromatography met with difficulty. The components streaked on the plate presumably owing to partial dissociation of the picric acid in the acidic solvent systems which were employed (the dipeptide derivatives are stable in acidic environments). The problem was overcome by the development of a two-step technique in which the picric acid was first completely exchanged and carried to the top of the plate in 20:1 EtOAc: 1N HCl/HOAc, then the dipeptide was chromatographed by rerunning the dried plate in BAW. At the time of this development, some of the dipeptide intermediates

had been stored for lengthy periods (approximately one year) at room temperature in screw-cap vials. Some decomposition had occurred in all of the older Cbz-Arg-X-ONp picrates judging from the new chromatographic system. It is significant that except for Cbz-Arg-Tyr-ONp picrate, when these components were developed with 5% KOH, only one yellow spot appeared in addition to that corresponding to p-nitrophenol at the solvent front. The spot appears yellow from the formation of nitrophenol upon alkaline hydrolysis of the nitrophenyl ester. Therefore, even though some decomposition may occur after long storage periods, in general, only the correct component would be expected to be capable of acylating amine components. Moreover, the L-Gln and Ile dipeptide intermediates were strikingly purified by recrystallization from 95% ethanol. The tyrosinyl dipeptide intermediate resisted purification by recrystallization which was not surprising because it was the most difficult to crystallize initially (crystals formed from precipitated oils) and because it exhibited the most extensive degree of decomposition. The quantity of Cbz-Arg-D-Gln-ONp picrate available was too minute for recrystallization. The results indicate that minor decomposition of the Cbz-Arg-X-ONp picrates will generally not give rise to undesirable acylated amine components during the appropriate coupling reactions, and that pure products may sometimes be recovered from the partially decomposed mixtures by recrystallization. The effect of storage at low temperatures and in vacuum dessicators on the rates of decomposition of the intermediates has not been determined.

Although the substrate specificity of trypsin for bonds involving the carboxyl groups of lysyl or arginyl residues has been well established (10,11), investigations into the stereospecificity exhibited by trypsin

towards optical isomers of these residues have appeared infrequently in the literature. Based on the early observations that poly-L-lysine was freely hydrolyzed while poly-D-lysine was resistant to attack, the assumption was made that L-derivatives of lysyl and arginyl residues were required for tryptic hydrolysis (11). In 1958, Laskowski, et al., discovered a lack of absolute stereospecificity in trypsin when they observed some hydrolysis in α-N(p-toluene) sulfonyl D-arginine methyl ester (D-TAME) (32). Further support for this contention came in 1963 when Trowbridge, et al., reported, without experimental detail, that both L- and D-lysine ethyl esters were hydrolyzed (33). Finally, in 1972 Purdie, et al., conducted specific experiments to obtain detailed information regarding the stereospecificity of trypsin towards esters of lysine and arginine. They concluded that "...Trypsin catalyses the hydrolysis of esters of the D-forms of basic amino acids at moderate rates..." (34). They further noted that acylation of the α-amino group of lysine or arginine esters increased the stereoselective capabilities of the enzyme, and they tentatively pointed out that trypsin exhibits a greater stereospecificity towards arginine esters than towards lysine esters; in their case, six times greater (34).

While the foregoing studies described some stereoselective properties of the enzyme towards arginine and lysine compounds, there have apparently been no reports dealing with the effects of optical isomers in the adjacent amino acid donating its amino terminus to the susceptible peptide bond. The work presented here represents the first such demonstration and may be of interest to many investigators studying the mechanism of action of this enzyme and to peptide chemists interested in the use of the enzyme as a reagent. A pair of diasteriomeric peptides in which

the arginyl residue was of the L form and the adjacent amino acid, in this case glutamine, was either the D or L isomer were synthesized for this purpose. Several important conclusions emerge from the results of this demonstration:

1) The enzyme is capable of differentiating between the optical forms of an amino acid contributing its N-terminus to the peptide bond formed with an L-Arginyl residue. This differentiation is expressed by variable rates in the hydrolyses of the bonds, rather than by an all-or-nothing effect. In assessing the potential of exploiting these rate differences for effective stereodiscrimination, it is important to consider the experimental results in conjunction with certain features of enzyme action in general and trypsin in particular. For example, it is not obvious from Figure 19 that the differential hydrolyses of the diasteriomers reflect a sufficient difference to be of practical value. However, the graph measures the rates of hydrolysis for equal concentrations of the "All-L-" and "2-D" peptides incubated separately. The actual hydrolysis rates in a mixture of diasteriomers is affected partly by the relative concentration of the two species. The fact that the rate of hydrolysis of the "All-L-peptide" is markedly faster than that of an equal concentration of the "2-D" peptide indicates that either a) the enzyme binds the L-diasteriomer more readily than the D-derivative, b) the enzyme catalyses the hydrolysis of the L-diasteriomer more efficiently than it does the "2-D-" peptide once the enzyme-substrate complex is formed, or c) the enzyme preferentially binds and hydrolyzes the "All-L" substrate. Since the presence of the "2-D" peptide did not noticeably impede the digestion of the "All-L-" peptide (Fig. 20), the enzyme appears to have both a higher affinity and a greater catalytic efficiency towards the "All-L-" derivative.

Under these circumstances, if the concentration of the D-diasteriomer was very small compared to the "All-L" substrate, the amount of cleavage of the former would be very low during the initial phase of the digestion of the other isomer. With such disproportionate concentrations, the initial cleavage of only the L-peptide would have probably taken place even if the relative affinity of the enzyme had been the same for the two substrate species. This is so because the enzyme would form a complex with the most readily available specie; in this case the L-peptide present in much higher concentration. The binding affinity is apparently lower for the "2-D" peptide, so that in very low concentrations this diasteriomer would form relatively few enzyme-substrate complexes. Even if this were the only factor - and it is not - which enabled the observed stereoselective properties to be used to practical advantage, it would be sufficient for recovering greatly purified L-intermediates from partially racemized peptide mixtures. A compromise in yield would be necessary in this case since the hydrolysis of the suppressed diasteriomer would increase as the concentration of the predominant peptide substrate diminished. By terminating the enzymic reaction early (eg just prior to or during the transition from a first to second order reaction as judged by the titration curve), maximal stereoselection would be insured.

Many reagents and reaction conditions for incorporating amino acids into peptide chains have been developed and are popular because their use is accompanied by a low tendency towards racemization during the activating and coupling steps. The concentration of the undesirable diasteriomer introduced by racemization at each addition cycle is therefore generally very small in proportion to the predominant peptide, making the foregoing procedure a practicable technique rather than just

a theoretical possibility.

2) Since it has been demonstrated that trypsin can distinguish between optically active amino acid residues on either side of the susceptible peptide bond, it is valid to conclude that there exist at least five functional points of enzyme interaction with the peptide substrate. Not all of these sites would be implicated during the hydrolysis of a particular diasteriomeric peptide, so the possibility exists for partially inhibiting the enzyme by blocking some, but not all of the multiple sites. If suitable inhibitors are found for this purpose, undesirable hydrolytic reactions can be selectively suppressed during the digestion of the desired substrate. A systematic search for such partial inhibitors is warranted, especially since these studies may also shed light on the mechanism of action of trypsin.

3) In addition to the above conclusions, there is an additional factor which bears mention but it is based on preliminary experimental evidence and its actual operation is speculative. It has been previously shown that carbobenzoxyarginine is a trypsin inhibitor (35). Since this moiety is released from the protected peptides during the enzymatic deprotection reaction, some revealing experiments were run to determine the effect of such inhibition on the cleavage of the model diasteriomers. The digestion of the "2-D" peptide was completely inhibited on the addition of a less than one-half molar equivalent of carbobenzoxyarginine, yet the digestion of the "All-L-peptide continued at a respectable rate even in the presence of five times the molar equivalent of the inhibitor. It has already been noted that a digestion of a mixture of the diasteriomers, where the initial concentration of the L-derivative is substantially greater than that of the D-diasteriomer, would begin with the prefer-

ential cleavage of the "All-L-peptide". Before the peptide was half cleaved, the Cbz-Arginine being released from the reaction would completely prevent the start of any hydrolysis of the "2-D-peptide"- even when the cleavage of the L-derivative was complete. Thus, in a situation where moderate racemization has occurred, the routine enzymatic deprotection step should provide a built-in selective inhibitor and total stereoselection might be possible even when the deprotection reaction is driven to completion.

Support for this may be indicated by the complete lack of any detectable hydrolysis of the racemized isoleucine peptide during the deprotection step in the course of the synthesis of deamino oxytocin. The opportunity for this practical application of the stereoselective nature of the enzyme presented itself when a problem arose during the isolation of the N-deprotected single-chain C-terminal hexapeptide of oxytocin. After the enzymatic removal of the Cbz-Arginyl protecting group from all of the other intermediate fragments, the digestion mixture was routinely acidified to pH3 and passed through a Sephadex column for desalting. Whenever the H-Gln-Asn-Cys-(AcM)-Pro-Leu-GlyNH_2 was recovered by this procedure it consistently failed to incorporate any reasonable amount of Cbz-Arg-Ile during the appropriate coupling reaction, even in the presence of large excesses of Cbz-Arg-Ile-ONp picrate. The ninhydrin test for free amino groups was in each case negative prior to terminating the coupling reaction. This fact was consistent with the well known tendency of free glutaminyl residues to form cyclic pyrrolidonecarboxylyl residues when they occur at the N-terminus of polypeptides (36-38). Lyophilization of the enzymic deprotection reaction mixture without prior adjustment of the pH followed by its direct entrance into the coupling reaction

allowed the unaltered intermediate to couple efficiently with the protected isoleucine nitrophenyl ester. Amino acid analysis of the resultant purified protected isoleucine peptide revealed an extra peak corresponding to D-alloisoleucine. The ratio of isoleucine to alloisoleucine was 4:1 and the sum of the two peaks equaled the expected value for isoleucine alone.

The direct coupling of the unisolated deprotection product resulted in heavy salt contamination in the reaction mixture which in all likelihood facilitated the observed racemization*. Since 80% of the protected isoleucine peptide was the desired diasteriomer, it seemed worthwhile to attempt its isolation from the racemized peptide, especially since this offered an excellent test of the stereoselective capability of the enzyme in a practical situation. In addition, the complete separation of the optical isomers of isoleucine on the amino acid analyzer offered a convenient and sensitive method for monitoring the degree of product separation.

As already mentioned, the actual separation of the diasteriomeric isoleucine peptides was based upon the large difference in pKa between guanidinium and ammonium ions of the protected and deprotected products respectively. The requirement for selectively deprotecting only the desired all L peptide in the presence of the racemized derivative rested solely upon the enzyme. After the routine digestion of Cbz-Arg-Ile-Gln-Asn-Cys·(AcM)-Pro-Leu-GlyNH_2 (partial racemate at Ile residue) was carried out, it was found that the best reproducible separation of the products

*The quality of the Cbz-Arg-Ile-ONp picrate was checked by coupling it to the glycine-resin ester followed by hydrolysis and amino acid analysis as described previously. No alloisoleucine was detected. In addition, when the experiment was repeated with a high salt concentration introduced into the coupling reaction, extensive racemization of the Ile residue was observed.

was achieved on a sulfonic acid ion exchange column in a 0.5M ammonium acetate buffer at pH 4.6. Under these conditions, the products which escaped cleavage by trypsin remained on the column along with the Cbz-Arg-OH which arose from the deprotected products. The free N-terminal peptide, recovered by lyophilization from the eluate, gave the expected values for H-Ile-Gln-Asn-Cys (AcM)-Pro-Leu-GlyNH_2 upon amino acid analysis. No trace of either arginine or D-alloisoleucine could be detected*. Thus, the rectification of a partially racemized residue in a peptide chain was clearly demonstrated for the first time.

It is noteworthy that the enzymic digestion was allowed to proceed to its apparent completion. In fact, additional enzyme was added in order to favor completion. If the protected D-alloisoleucine peptide is a trypsin substrate (a point which remains to be demonstrated), then this example gives support to the actual operation of the principles which were enumerated for the suppressed hydrolysis of substrates present in low concentrations and for the built-in selective inhibitory system provided by released Cbz-Arg-OH.

B. Scope and Limitations of the System:

The underlying principles of the new method of amine deprotection described provide the basis for the development of peptide synthetic procedures with capabilities not previously available. The combination of chemical specificity, the mild conditions for removal of amine-protecting groups in aqueous solution, and the degree of stereoselectivity in the deprotection reaction observed in the experiments presented can

*Usually one ninhydrin negative contaminant in the free heptapeptide was detected by TLC in BuOH/HOAc/H_2O (4:1:1). Gel filtration on Sephadex G-15 afforded the pure product.

probably not be duplicated by any other scheme currently in the literature. The system is designed to be integrated into a system of peptide synthesis which will operate exclusively or partially in aqueous media under essentially physiological conditions. There is inherent within the scheme a convenient and sensitive method for monitoring the course and extent of the deprotection reaction. In conventional methods, if incomplete deprotection is detected, the only recourse is usually repetition of the reaction whereas the enzymatic method provides an effective method for removing the uncleaved products. Such products in all likelihood would have escaped cleavage in the case of the enzymatic deprotection method, because they had become racemized; the enzymatic deprotection scheme therefore provides a routine method for removing peptide chains in which recently incorporated residues have become racemized. The mildness of the enzymic deprotection reaction permits the practical development of additional blocking groups which can be removed by significantly more mild methods than those which are currently in use. As in the introduction of any substantially innovative operation, there is much room for refinement in the experimental format and for expanding the techniques to include, for example, other enzymes and substrates. Modifications of certain existing synthetic methods which would render them compatible with the enzymatic procedures are equally justified for they would result in the development of extremely versatile and mild techniques which would produce sophisticated products of consistently high quality. These possibilities will be discussed in the light of recent progress toward that goal, and in conjunction with the potentials of existing reagents for hastening the transition.

1) Alternative Enzyme - Substrate Systems:

Perhaps the most obvious difficulty in the method described is its limited application for the preparation of polypeptides containing lysyl or arginyl residues. The use of conventional blocking groups for their side-chain functions would compromise the mild conditions offered by the enzymic method, although its stereoselective capabilities might still be useful if the intermediates retained water-solubility. In principle, a much better alternative would be to employ a different protecting group which could be hydrolyzed by an enzyme with a different substrate specificity than trypsin. The new group could be used to protect the side-chains of the lysyl or arginyl residues and would be subjected to the appropriate enzymic deprotection only after completion of the desired polypeptide. Conversely, the new enzyme-substrate combination might be chosen as the repetitive α-amino blocking system, especially if the substrate was not found as a residue in the desired product or if the enzyme was an exopeptidase.

Alternative enzymes are selected on the basis of the same requirements which led initially to the use of trypsin: 1) The enzyme must exhibit a very high degree of substrate specificity so that there is no damage to the peptide chain. This is of special importance when the enzyme will be required for repetitive steps. 2) The enzyme must be highly purified to prevent side reactions. 3) It is desirable for the enzyme to be commercially available and inexpensive in order that it be widely accessible for general application. 4) The enzyme should be reasonably stable and resistent to inactivation if it is to be successful as a routine reagent.

Trypsin was an obvious choice from the beginning. This enzyme exhibits one of the highest degrees of substrate specificity known among

the purified peptidases (39,40), hydrolyzing only those bonds involving the carboxyl groups of lysyl or arginyl residues. Bovine trypsin may be purchased inexpensively from commercial sources in highly pure, crystalline form, and it can be easily handled with normal care at room temperature without loss of activity. There has been some chymotryptic activity attributed to bovine trypsin (41-43) which could present problems of chemical specificity in the synthesis of particularly chymotrypsin-sensitive sequences; however, studies with porcine trypsin (44) and with carefully prepared bovine enzyme (45) suggest that such difficulties can be minimized.

The type of acylating group employed is dictated exclusively by the specificity of the enzyme selected for its hydrolysis. Multiple substrates for one enzyme permit some opportunity for choice, as does chemical alteration not affecting the substrate recognition features. Within these narrow limits, the decision to use carbobenzoxyarginine was settled upon according to the considerations which follow.

Trypsin is an endopeptidase and will only very slowly hydrolyze bonds which are formed from arginyl or lysyl residues with a free amino terminus. The acylation of this amino group renders the bond susceptible to cleavage as is evidenced by the use of tosyl arginine methyl ester (TAME) as a routine standard (33). The carbobenzoxy group is a common amino acylating group and N^{α}-carbobenzoxyarginine and N^{α}-Cbz-Lysine are readily accessible (46-48). The ε-amino group of lysine is fully capable of forming amide bonds with activated carboxyl groups and must therefore be protected prior to the use of an N^{α}-substituted lysine as a blocking group for the system envisioned. Seely and Benoiton (49) showed that various ε-N-Methyllysine ester and amide derivatives were only very poor substrates

for trypsin. The ε-di- and ε-trimethyl derivatives tested were completely resistant to tryptic hydrolysis. While this tends to discourage further investigation into the use of ε-N-protected lysine derivatives as trypsin-labile blocking groups, it is noteworthy that Heller, et al., (50) observed tryptic hydrolysis at what was presumed to be a lysyl residue in position 72 of Cytochrome c from Neurospora crassa and then they later discovered that the residue was actually ε-N-trimethyllysine (51). This suggests that the behavior of trypsin substrates involved in ester or amide bonds may not be analogous to those which form peptide bonds.

Whether or not an N^{α}-, N^{ε}-, substituted lysine would serve as an enzymatically labile amine protecting group, the advantages offered by carbobenzoxyarginine make it the better of the two choices. The pKa of the guanido group is so much higher than the amino group that the former can be protected by protonation (12,13) while the free amine reacts. Not only does this obviate the need for altering a recognizable portion of the substrate, but the differences in pKa also permitted the development of the effective ion exchange procedures previously described. In addition, several reports indicate that arginine derivatives are generally better trypsin substrates than those of lysine (49), and that the stereospecificity constants (52) are greater between L and D arginine derivatives than between L and D lysine derivatives (34).

A chemical analog of arginine in which the α-amino group is replaced by hydrogen has the potential of being a markedly improved trypsin-labile amine protecting group. The carbobenzoxylation required for arginine would of course be eliminated, but the major advantage lies in the loss of the asymmetric center at the α-carbon of arginine. Any racemization occurring during the formation or attachment of the Cbz-Arginyl protecting

group is reflected by the resistance of D-arginyl bonds to be hydrolyzed by trypsin.

The proposed chemical modification would create a compound which may loosely be regarded as "deamino-arginine". The structure would be that of 5-guanidinovaleric acid, and its synthesis from 5-aminovaleric acid and 2-methyl-2-thiopseudourea would be rather simple and straightforward since it has an analogous precedent in the conversion of ornithine to arginine (53). Preliminary results indicate that this compound was successfully prepared by the above method in our laboratory. It is not certain whether the 5-guanidinovaleryl residue will in fact be a trypsin substrate; a trial system similar to the model developed for Cbz-Arginyl derivatives would be required.

Unfortunately, there are very few enzymes which are nearly as suitable as trypsin for use as a deblocking reagent. Although many enzymes are available in pure, crystalline form, their substrate specificities are generally too broad for them to be exploited for stepwise peptide synthesis. At present there is at least one enzyme which may soon become applicable for the deblocking procedure, and others might prove useful as they are discovered, purified or tested. The one which is promising is pyrrolidonecarboxylyl peptidase which has been partially purified from a fluorescent pseudomonad and from several other sources (54-57). This enzyme specifically releases pyrrolidonecarboxylic acid (PCA) from the N-terminal of amino acids or peptides which contain the pyrrolidonecarboxylyl residue (Fig. 33).

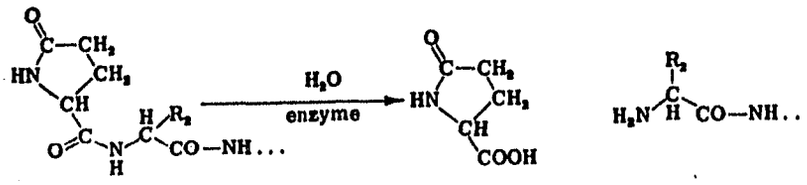


Figure 33: Action of Pyrrolidonecarboxylyl Peptidase on Pyrrolidonecarboxylyl Peptides

The apparent fact that pyrrolidonecarboxylyl peptidase is an exopeptidase makes it particularly well suited as a reagent for the removal of α-amino protecting groups. The enzyme preparation with the highest degree of purity which could be obtained was rather unstable, but an intermediate preparation which could be stabilized was shown to specifically hydrolyze the appropriate bond without detectable damage to the rest of the protein. Although the enzyme is not yet commercially available, the bacterial strains can be obtained for culture or can be purchased as freeze-dried suspensions. The isolation, purification and assay procedures are explicitly described in the literature (56). Since this enzyme is gaining increasing recognition for its importance in protein sequence work (56,57), it is likely to become further refined and readily available in the near future. The pyrrolidonecarboxylyl blocking group may provide several of the advantages which were offered by the Cbz-Arginyl group. PCA, which arises from the cyclization of free glutamic acid and from glutamyl or glutaminyl residues (36-38), is readily obtained commercially. A suitable charge difference for the development of ion exchange separation methods will exist between the nearly neutral protected intermediates and the more basic deprotected products which possess free amino groups. The enzymatic digestion of the PCA group occurs at nearly neutral pH (56),

and it is readily detectable as glutamic acid in amino acid analyses of acid hydrolysates. In addition, PCA does not have any acylatable groups and therefore requires no modification prior to its use as a protecting group.

In further analogy to the trypsin/Cbz-Arginyl system, the PCA group possesses a disadvantageous asymmetric center, and stereoselection was demonstrated on both sides of the susceptible peptide bond (58). However, the presence of the D-isomer on either side of the bond resulted in negligible enzyme activity, so the enzyme appears to be specific for residues of the L configuration only. The presence of either type of D-isomer did not impede the hydrolysis of the all L derivative (58). Whether this group will be a substrate when it occurs on amino acid side-chains, especially those of arginine and lysine, remains to be explored.

The enzyme which removes the N-formylmethionine residue from the amino terminus of newly synthesized bacterial protein has not yet been isolated, but efforts in this direction are continuing. In view of its proposed physiological role, this enzyme is likely to exhibit an especially high degree of substrate specificity. In fact, it is apparently capable of differentiating between the formylated methionine and unsubstituted methionyl residues in the same protein chain. This property undoubtedly classifies the enzyme as an exopeptidase. As was pointed out in the case of pyrrolidonecarboxylyl peptidase, this is a distinct advantage over the use of an endopeptidase such as trypsin for the purpose of removing α-amino protecting groups.

There is some question as to whether or not the enzyme is capable of recognizing small peptides, since it removes the formyl methionine from nearly completed or totally completed proteins *in vivo*. However,

the N-terminus of the smaller protein chains may be buried within the ribosome so that further chain elongation is required to expose the N-terminus to the enzyme (59).

2) Existing Reagents Which Are Potentially Compatible with the Enzymic System:

While the new system described for the enzymatic removal of protecting groups in fully aqueous solutions represents a substantial departure from the conventional synthetic procedures, reagents and techniques have been developed which have the potential to readily adapt the remaining features of the stepwise synthetic reactions to this environment. The most important and promising of these will be presented along with proposals for their immediate and future applications to expanding and improving the present scheme.

a) Coupling Reagents:

(1) Carbodiimides: Sheehan and Hess introduced N,N' dialkylated carbodiimides as carboxyl activating agents for the synthesis of peptide bonds (60). The most successful of these reagents was N,N' dicyclohexylcarbodiimide which is still among the most important and widely used condensing methods in peptide chemistry. Its preparation is very simple, it offers rapid peptide bond formation with very little tendency toward side-reactions or racemization, and it is not sensitive to moisture. In fact, it can be used in aqueous solutions. From the overall reaction shown in Fig. 34, it is apparent that the reaction is accompanied by the formation of N,N' dicyclohexylurea.

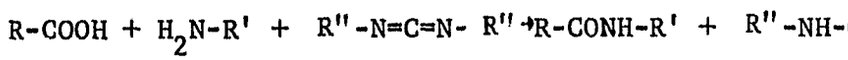
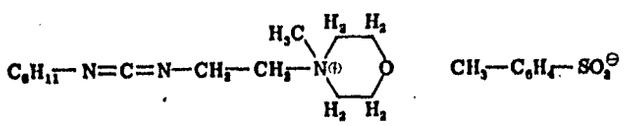


Figure 34: Overall coupling reaction of a carboxylic acid with a primary amine by the action of N,N' Dicyclohexylcarbodiimide.

This by-product has a very low solubility in most organic or aqueous solvents, so that it is well suited to removal by filtration from products which are soluble in these media. However, in cases where the desired peptides and the dicyclohexylurea have similar solubility properties, product purification becomes more difficult. This situation is especially prevalent in the synthesis of high molecular weight peptides. For this reason, Sheehan and co-workers (61,62) undertook the development of new carbodiimides featuring tertiary or quaternary amine substituents which rendered them as well as their corresponding urea derivatives soluble in dilute acid or in pure water respectively. The best reagents to emerge from this effort were 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide, 1-cyclohexyl-3-(4-diethylaminocyclohexyl) carbodiimide and their corresponding metho p-toluenesulfonate salts.



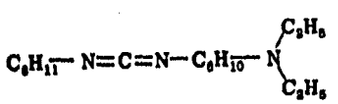


Figure 35: Water Soluble Carbodiimides. Above: 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho p-toluenesulfonate. Below: 1-cyclohexyl-3-(4-diethylamino cyclohexyl)carbodiimide.

These reagents have been used successfully for aqueous peptide synthesis (5,63,64) and for the cyclization of peptide chains (65).

Ultimately, the enzymatic deprotection scheme will operate within the realm of the automated solid phase technique to be described later. Given suitable solid supports, the synthesis will proceed entirely in aqueous media. The water-soluble carbodiimides should prove effective for this purpose during the coupling steps, and the urea by-products will be readily removed by washing the resin-bound product with water. The transition to complete aqueous peptide synthesis will be hastened by the existence of these reagents, especially since they promise also to be important for preparing activated dipeptide intermediates which operate in aqueous media.

(2) N-Hydroxysuccinimide Esters: One of the most important advances in peptide chemistry was the ability to preactivate the carboxyl group of a protected amino acid or peptide in the form of stable, often crystalline activated esters. The method was introduced by Wieland, et al. (66). It meant that each specie could be isolated, purified and characterized before its addition to the growing peptide chain. Preactivation also often eliminates the requirement for C-terminal or side-chain carboxyl protecting groups.

Several types of reagents which form activated esters have been developed, of which only a few operate efficiently in water and give rise to water-soluble by-products. Of these, the best characterized and most widely used are those esters formed by the coupling of amino acid carboxyl groups with N-hydroxysuccinimide (67,68). The coupling can be performed by the action of carbodiimides. The substitution of succinimide esters for p-nitrophenyl esters as the activating specie for the protected dipeptide intermediates is likely to result in well-characterized crystal-

line intermediates capable of coupling in aqueous media. The N-hydroxysuccinimide reagent has also recently been used in situ with carbodiimide to promote better yields with virtually no detectable racemization even in the coupling of peptide fragments (69,70). N-hydroxybenzotriazole has been used for this purpose, also with great success (71). Since this latter reagent apparently works through an activated ester intermediate, it may also be of use in aqueous couplings, although its suitability for this purpose has not yet been explored. It is a great tactical advantage to possess preactivated water-soluble N-protected amino acid esters which form water-soluble by-products. In conjunction with the water soluble carbodiimides, these devices promise to launch the coupling of peptides in aqueous media at a respectably sophisticated level.

(3) N-carboxyanhydrides: Hirschmann and co-workers have developed an elegant method of controlled stepwise peptide synthesis using amino acid N-carboxyanhydrides (Leuch's anhydrides, NCA's) and amino acid N-thiocarboxyanhydrides (72,73) in an aqueous medium. The success of the method depends primarily upon the very careful control of pH, temperature, and rate of mixing. The crystalline N-carboxyanhydride typically reacts in only two minutes with a free amino acid or peptide in a cold, basic, rapidly mixed aqueous solution. Immediately after the condensation reaction, the amino group of the newly incorporated residue is prevented from further reaction by its formation of a carbamate ion. Acidification releases the amino group in its protonated form so that readjustment of the pH to basic conditions prepares the newly formed peptide for coupling with another NCA (Fig. 36).

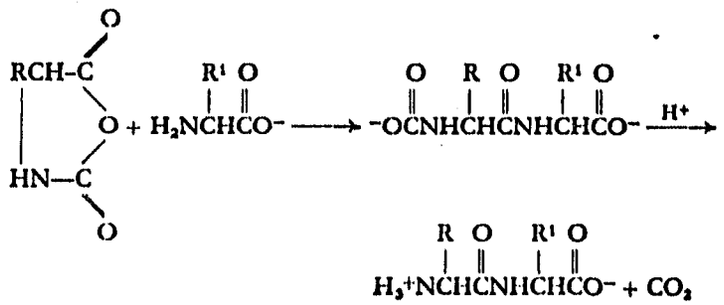


Figure 36: N-carboxyanhydride method of peptide synthesis.

With this method, only lysine and cysteine require side-chain protection when they occur as the nucleophilic component (72). The general use of unprotected components enables free peptides to be prepared directly in a very short time with a minimal number of steps, as well as permitting the peptides to be elaborated in their native aqueous environment. To date, the formation of hydantoic acids and overreaction have prevented the nearly 100% reaction necessary at each step to extend the method to the routine synthesis of long-chain peptides without isolation of intermediate products (74). Short fragments prepared this way have been used as intermediates in the synthesis of proteins, as illustrated by the preparation of bovine ribonuclease S by Hirschmann, et al. (75-80). Although the method succeeds in aqueous solution, the pH is high (usually between 10 and 10.2) (72).

Leuchs anhydrides offer the possibility of some intermediate technical improvements in the preparation of the activated dipeptide intermediates. The reaction of an NCA with N-hydroxysuccinimide, for example, would produce the corresponding free amino acid succinimide ester which could be directly coupled to Cbz-Arg-OH, or some other enzyme-labile

derivative. This is a convenient alternative to the more expensive process exemplified by the preparation of the glutamine-p-nitrophenyl ester-p-toluenesulfonates. Although the laboratory preparation of the NCA's might best be derived from carbobenzoxyamino acids (81) industrial preparation may well proceed via treatment of free amino acids with phosgene (72). In the latter case, the necessity of employing one type of amino blocking group only to replace it subsequently with a different group would be avoided.

b) Non-Enzymatically-Labile Blocking Groups:

(1) Acetamidomethyl Group: As techniques evolve for performing the synthetic steps in aqueous solutions, greater emphasis is being placed on minimizing the need for protecting groups. This was well illustrated by the NCA method which under appropriate circumstances required protection for only two amino acid residues. While this is certainly the proper direction, it is helpful to exploit existing compatible groups for the transition. The S-acetamidomethyl group (AcM), developed by the same laboratory that introduced the NCA method of synthesis, was designed specifically to protect the cysteine side-chain (Fig. 37) during protein elaboration by the newly devised NCA technique (31).

$$CH_3CONHCH_2SCH_2CH(NH_2)COOH$$

Figure 37: S-Acetamidomethyl cysteine.

The new group does not hinder the aqueous solubility of the protein, and it can be removed by mercuric ion at pH 4 and room temperature. Subsequently, it was found that the group is also removed by iodine (82). These properties were demonstrated by the synthesis of ribonuclease by

the Merck group (75-80) and during the model synthesis of deamino oxytocin.

There has been a general lack of well-suited sulfur protecting groups for cysteine which can withstand the normal conditions of synthesis and then be removed by mild treatments. The AcM group has several advantages and is a welcome alternative to many means of other S-protection for preparations of polypeptides in aqueous solutions where mild conditions are stressed.

(2) Nitrophenylsulfenyl (NPS) and 2,4-Dinitrophenylsulfenyl (DNPS) Groups: When the NPS (83,84) and the DNPS (85) groups were introduced into peptide chemistry for use as amine protecting groups, they featured removal by unusually mild acid conditions, or by desulfuration with Raney nickel (86). It was later found, during studies to circumvent problems encountered by both methods, that the two groups could be removed by treatment with thiophenol or thioglycolic acid in organic solvents to give the free amino acid or peptide and a disulfide (Fig. 38).

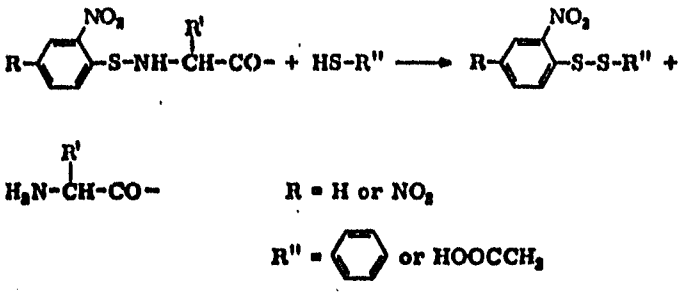


Figure 38: Removal of NPS and DNPS from the N-terminus of an amino acid residue by thiols.

In addition, it was noted that, "The deblocking reaction occurs also using ter-butyl and isopropyl mercaptans in pyridine solution, or thiosulfate in aqueous alcohol using acetic acid as catalist" (87). The sulfur displace-

ment reaction will be shown to be of central importance to the strategy envisioned for expanding the mild synthetic techniques to include the automated solid phase method to be described.

There is a likely possibility that replacement of the nitro group(s) with a methyl-sulfonyl group(s) may adequately impart a hydrophilic nature to these protecting groups without altering the mechanism for their removal, since the proposed replacement moiety is also capable of "activating" the phenyl ring (88). These groups, appropriately modified if necessary, may serve to protect the side-chain of lysine (and possibly arginine) during the stepwise elaboration of a peptide containing these residues when trypsin-labile N-protection is employed. The side-chain would merely be freed by mild thiolysis upon completion of the peptide sequence.

(3) Miscellaneous Blocking Groups: While the most immediately important groups were treated first, there are several others which may have future potential, although they either require further investigation due to their recent development, or they have serious limitations which have so far prevented them from being generally accepted. These groups will be briefly described.

The trifluoracetyl group (TFA) deserves mention primarily because it can be removed by mild alkaline hydrolysis and is stable to acid (89). It can be introduced into amino acids without racemization. Although the TFA group has been known for over twenty years (89) it has infrequently been selected for use because it has some major drawbacks. Difficulties are encountered in the introduction of the group to several amino acids (eg, serine, threonine, lysine, ornithine) and it cannot, in most cases, be added to free peptides (90-92).

Citraconic anhydride (2-Methylmaleic anhydride) is a commercially

available reagent which was shown by Dixon and Perham (93) to preferentially react with the amino groups of proteins and be removed by incubation at room temperature in a pH 3.5 buffer overnight. Although this mild cleavage reaction is beneficial, the introduction of a free carboxylic acid is a serious disadvantage, especially since it would participate in the carboxyl activation of protected peptide fragments. In general, it complicates the already difficult problem of reversible carboxyl protection for the side-chains of aspartic and glutamic acids as well as for the C-terminus of polypeptides.

Photosensitive protecting groups are at a very preliminary stage of investigation, but the early findings suggest that they may become useful since they offer yet another mechanism of mild deprotection. Wieland and co-workers described both the 3,5-Dimethoxybenzyloxcarbonyl group which was shown to be photo- and acid-labile (94), and the α, α-Dimethylbenzyloxycarbonyl group (94,95) which was labile to heat and acid. By combining the two systems, they later readily prepared α, α-Dimethyl-3,5-dimethoxybenzyloxycarbonyl azide (Fig. 39) which was used to acylate amino acids in yields of 60-85% (96).

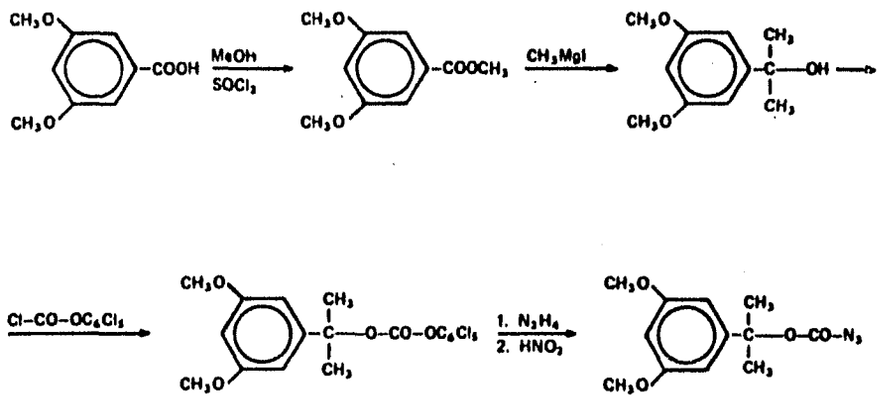


Figure 39: Preparation of α-α-Dimethyl-3,5-dimethoxybenzyloxycarbonyl azide.

The protecting group, for which these workers proposed the abbreviation Ddz, was reported to be quantitatively removed from the corresponding amino acid or peptide by exposing them in solution to ultraviolet light. The Ddz group was also cleaved rapidly with 5% trifluoroacetic acid in dichloromethane at 20°.

Several reports indicated the successful use of the photolabile o-nitrobenzyl group for protection of aldehyde, amino-, and carboxy-groups (97-100) which prompted Rich and Gurwara (101) to prepare analagous resins for anchoring N-protected amino acids during stepwise solid phase peptide synthesis. The resulting carboxyl attachment to the modified resin through a nitrobenzyl ester was quantitatively cleaved by photolysis (3500 Å) in methanol under anaerobic conditions over a period of 12-17 hours. The authors reported good yields of the freed N-protected products without racemization or damage to aromatic rings.

There is a side-chain protecting group for arginine residues with questionable application to peptide synthesis in its present form, but it deserves mention because it reacts specifically with the guanido group at pH 7 to 8 and 25° and comes off slowly under neutral or weakly alkaline conditions. The group is phenylglyoxal which can be obtained commercially and may be readily radiolabeled. The protected residue contains two phenylglyoxal moieties per guanido group, and this derivative is stable below pH 4 (102). The group was used to limit tryptic hydrolysis exclusively to lysine residues, although the phenylglyoxal presumably reacted only with the accessible arginine residues in the protein tested. Treatment of proteins with a large excess of reagent and prolonged reaction time resulted in the substitution of lysine residues.

A group which can be added to arginine residues, and remain in place

while the conditions are below pH 4 (eg, during isolation of the protein or fragment) but be removed in good yield under neutral conditions, operates by a mechanism which warrants attention. In its present state, phenylglyoxal is useful for the purposes described by the author (102) and might possibly be extended to the protection of arginyl residues in a peptide chain being synthesized with trypsin-labile protecting groups.

3) Immediate Applications of the Enzymatic Deprotection Scheme:

a) Incorporation of Fragile Residues: One of the incentives for the undertaking of a project which would introduce a new degree of mildness to a vital step in the current methodology of peptide synthesis was its potential for allowing desirable but highly labile residues to be incorporated into peptide sequences. It should be emphasized that it is a long range goal which has been envisioned for the integration of the enzymatic deprotection scheme with other elements to produce a system of stepwise synthesis in environments closely approximating those of physiological systems. Nevertheless, the amino deblocking system as it has been presented has been shown to be compatible with the stepwise incorporation of amino acid residues into peptide chains under very mild conditions.

By itself, the method is not immediately intended for use in the total stepwise elaboration of peptides, although this is by no means excluded if it is deemed practical for a particular situation. Rather, one of the immediate strengths of the method lies in its ability to be used for the incorporation of one or several residues which are unusually sensitive to the conventional treatments for the deblocking of α-amino groups. As an example, an analog of oxytocin in which one desired modification is the substitution of the glutaminyl residue by an O-diazoacetyl serinyl (azaserinyl) residue is of interest to those who are

attempting to isolate the oxytocin receptor and to study the hormone-receptor interactions. Azaserine was first discovered as an antibiotic (103,104) and its structure was determined to be that shown in Figure 40.

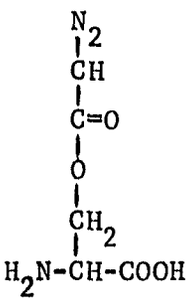


Figure 40: Structure of Azaserine.

It was found that azaserine inhibited a particular glutaminyl amido transferase which recognized the aliphatic diazo groups as though they were amide groups. The interaction of the enzyme with the antibiotic, notably at sulfhydryl functions, resulted in the formation of a covalent bond (105). It is possible that if azaserine replaced glutamine in an analog of oxytocin, that the resultant derivative would bond covalently with the oxytocin receptor. Since the modified serine side-chain is unstable to acidic environments such as those commonly produced during the conventional repetitive deblocking of α-amino functions, it has not been feasible to attempt the synthesis of a peptide hormone analog containing azaserine. The availability of the enzymatic deprotection scheme which operates under essentially physiological conditions should provide the necessary mildness for the preparation of the desired analog. Inasmuch as the azaserinyl residue would be introduced into position number four of oxytocin, the C-terminal pentapeptide of the hormone could be prepared completely by established procedures. The enzymatic deblocking procedure would only be required four times (three times for "deamino" analogs)

during the preparation of the nonapeptide.

The preparation of a Cbz-Arg-Azaserinyl-ONp picrate intermediate would need to be accomplished by a more indirect method than that used to obtain the dipeptides in the model. This inconvenience results from the inability to use an acid treatment for the formation of the free amino acid nitrophenyl ester of azaserine. Considering that serine should be compatible with the general procedure for making the dipeptide intermediates, and that 2,4 dinitrophenyl esters (ODNp) rather than nitrophenyl esters have been used for the carboxyl activation of serine derivatives (106,107), a good route to the desired dipeptide intermediate might be via Cbz-Arg-Ser-ODNp prepared analogously to the Cbz-Arg-X-ONp's. This intermediate could be reacted with the N-thiocarboxyanhydride of glycine (108)* to form the corresponding O-glycyl serinyl derivative. That this reaction would proceed in the presence of protonated guanido functions is supported by the conversion of the N-carboxyanhydride of ε-Cbz-Lysine to ε-Cbz-Lysine methyl ester hydrochloride in methanolic HCl (109). Moore, et al., have formed the O-diazoacetyl derivative from the O-glycyl derivative with $NaNO_2$ in bicarbonate buffer solution (108). Analogous treatment of the Cbz-Arg-Ser (O-Gly) - O DNp should yield the desired Cbz-Arg-Azaserine-ODNp.

It should be noted that the conversion of 4 - serine oxytocin to 4-azaserine oxytocin would be impractical because the conversion is not quantitative and purification and characterization of the final product by available methods would be exceedingly difficult.

*Hirschman and co-workers have had far greater success with coupling reactions using the N-thiocarboxyanhydride of glycine rather than the N-carboxyanhydride.

b) Incorporation of Optically Pure Radiolabeled Amino Acids: Radiolabeled amino acids in peptides and proteins have been widely used as tags during their isolation and purification as well as to aid in studies concerning their mechanisms and types of action and their physical and chemical composition. Often, the preparation of optically pure labeled amino acids or of the peptides which contain them is expensive and difficult. In the case of labeled peptides, optical purity may be diminished by racemization occurring during the incorporation of the labeled amino acid.

The procedures described in this thesis may provide a viable solution to both the expense of preparation and the losses in optical purity cited. Depending upon the results of further investigations into the stereoselective capabilities of trypsin, it may be possible to label racemic mixtures of amino acids, introduce them into the growing peptide as α-Cbz-Arginyl-protected intermediates, and following tryptic digestion, isolate the deprotected all-L-peptide from the protected 2-D-peptide by ion exchange chromatography. If this proves infeasible, there is still a strong likelihood that a moderate degree of racemization occurring during the incorporation of optically pure labeled amino acids can be conveniently rectified by this technique.

c) Synthesis of Sequences Containing D-Amino Acid Residues: Occasionally, the synthesis of peptides are desired which contain one or more residues of the D-configuration. It is interesting to note that the differential cleavage rates observed between the model 2-D and all-L diasteriomers (Fig. 19) are not so great as to preclude the use of the enzymatic deprotection method for the introduction of D-residues. Operationally, the contamination by any peptide chains containing the

L-isomer of the desired D-amino acid residue could be removed by the normal cycle of enzymatic deprotection and ion exchange chromatography. The protected 2-D-peptide could be recovered and resubjected to incubation with trypsin (perhaps in greater concentration than is generally employed for the deprotection of all-L-peptides) to accomplish the deprotection of the 2-D-diasteriomer.

On the other hand, several points in the discussion, notably the inhibitory effects of Cbz-Arg-OH were developed which indicated that in some cases, adequate deprotection of 2-D-peptides may not be possible*. In some situations it might still be worthwhile to take advantage of the stereoselection provided by the enzyme for the isolation of the 2-D protected diasteriomer but then remove the carbobenzoxy group by conventional methods (eg, catalytic hydrogenolysis; HBr/HOAc) and subject the peptide to one cycle of the Edman degradation (110,111) normally used for end group analysis of amino acids.

d) Synthesis of "Hormonogens" and Other Latent Biologically Active Peptides: Biological systems have long exhibited the recurrent theme of producing active molecules which themselves often have another physiological role. Precursor molecules which give rise to active hormones are generally referred to as prohormones. Often, the enzymic splitting of one or several specific covalent bonds in a peptide or protein prohormone releases the hormone.

Based upon this process, various pharmacological agents have been developed which become activated upon their interaction with certain agents found in various body tissues so that the release of the active

*Dilution of the reaction mixture may promote the continued digestion of the substrate by diminishing the effective concentration of the inhibitor.

substance is controlled with regard to time, concentration, or confinement to specific areas. For example, synthetic analogs of oxytocin in which the chain was extended at the amino end with additional amino acids displayed protracted effects in vivo (112). The properties of these analogs are such that they agree with the proposal that they act as synthetic "hormonogens", and that the active hormone is produced by enzymic action on the hormonogen (112-115). Recently, studies have been made on the effects which the added amino acids have on analogs of lysine vasopressin (116).

In the cases cited, the activating enzyme also attacks the released hormone. Perhaps the incorporation of the cysteinyl residue in position 1 of oxytocin via Cbz-Arg-Cys (AcM)-ONp would result in an improved synthetic hormonogen. Trypsin and trypsin-like enzymes are present in many tissues, and the enzyme which activates the hormonogen would not damage the product. In addition, purification of the Cbz-Arg-oxytocin would be facilitated by ion exchange procedures owing to the presence of the guanidinium ion.

The above is only one example in the case of one hormonogen, but the principle might be extended to other types of biologically active peptides (eg, antibiotics) as well as to other hormonogens.

The effects which Cbz-Arg-OH may have on the biological systems tested would need to be investigated. Other enzyme-labile groups, such as the PCA group mentioned earlier, may be superior for synthetic prohormones since the activating enzyme would be an exopeptidase and the released substance may be a more passive co-product of the reaction than Cbz-Arg-OH.

4) Extension to Solid Phase Synthesis: The solid phase method

of peptide synthesis developed by Merrifield (117) has provided a relatively rapid and simplified route to the stepwise elaboration of polypeptides of designed sequence (Fig. 41). By utilizing an insoluble resin support partially substituted with reactive groups, an N-protected amino acid was successfully attached covalently to the resin by esterification. After the selective removal of the α-amino protecting group, a second N-protected amino acid was coupled to the resin-bound amino acid. Repetition of the cycle would, in principle, extend the chain to the desired length at which time the completed peptide would be released from its support and purified by conventional techniques.

By anchoring the C-terminal amino acid to an insoluble support, the entire synthesis could be performed in a single vessel where the by-products and unreacted materials would simply be washed away by filtration. The system was therefore well suited to automation (118). In addition to the speed and ease of operation gained by the solid-phase technique, the problems of solubility often encountered during solution syntheses are avoided. It must be noted that the method has some major drawbacks which prevent its general application to the stepwise elaboration of very long chain polypeptides and true proteins. These problems stem from the fact that the intermediate fragments are not isolated and purified. Under such circumstances, quantitative coupling at each step is required to prevent a final mixture of sequences which are too closely related for separation by conventional techniques. Side reactions occurring during each coupling and deprotection step and during the cleavage of the final product from the resin all contribute to the formation of heterogenous products. However, improvements in the method are steadily being introduced and the solid phase preparation of many biologically active peptides

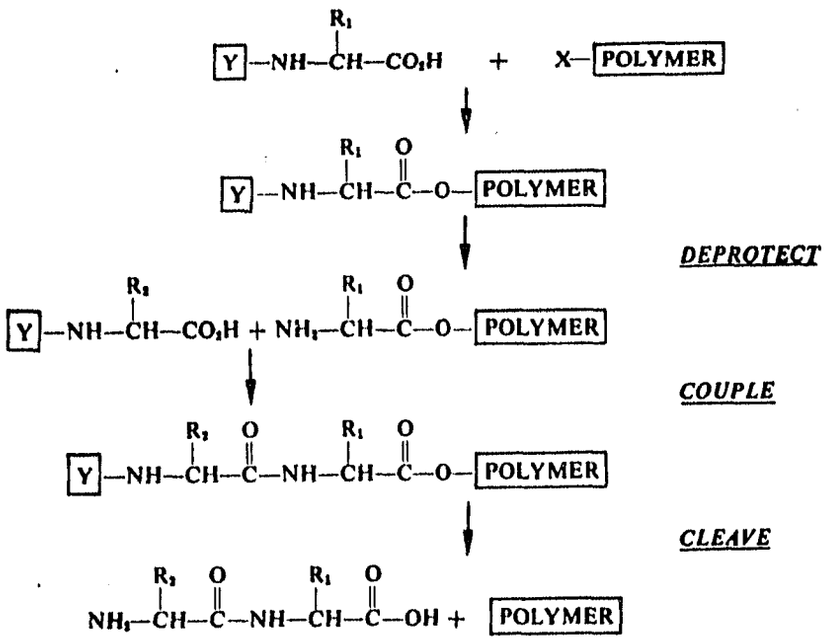


Figure 41: General scheme of Solid Phase peptide synthesis.

has been routinely achieved with great success (eg, 119-126).

A good deal of unwanted reactions in this method undoubtedly arise from the relatively harsh conditions of synthesis which result from the use of multiple selectively removable protecting groups and from the consequently severe conditions of deprotection and removal from the resin.

If solid phase synthesis (and particularly semisynthesis) could be performed entirely in aqueous solution and with mild deprotection and cleavage from the resin, this rapid and simple automatic technique might well be extended to the synthesis of highly pure polypeptides with many more residues than is possible by the conventional method.

Obviously, an enzymatic N-deprotection system based on the principles of the one advanced herein is the method envisioned for extending the repetitive deprotection reaction of the solid phase system to essentially physiological conditions. While this satisfies one major part of an integrated system of mild, aqueous solid phase peptide synthesis, other required components must be developed to achieve such a goal. Efforts in this direction are highly encouraging, as will be discussed.

With several useful reagents already available as previously described, and with the advent of the enzymatic system for the repetitive deblocking steps, the major obstacles which remain in the creation of a mild, aqueous automatic system of peptide synthesis are; a) the existing harsh mechanisms for removal of the completed chains from the solid support, and b) the nature of the insoluble supports conventionally employed.

One alternative to the severe cleavage of the peptides from the resin was that developed by Glass, et al., (127) in which the starting amino acid was anchored by its side-chain to the solid support through

a thiol-labile bond (128). This was accomplished via a dinitrophenylene bridge as demonstrated in Figure 42. In addition to offering the mild thiolytic mechanism for removal of the peptide from the resin, the method permits the bidirectional elaboration of peptide sequences (which has several potential advantages that are discussed by the authors) as demonstrated by the syntheses of thyrotropin releasing hormone (127,130), oxytocin (129,130) and deamino oxytocin (129,130). In addition, the side-chain attachment to the resin has the secondary function of side-chain protection for whichever amino acid is utilized.

Another interesting idea for the mild release of peptides from their supports is the use of enzyme-labile linkages. Blecher and Pfaender (8), for example, attached a model tripeptide to a water-soluble polymer through an arginine bridge. Treatment with trypsin resulted in the release of the expected tetrapeptide containing the C-terminal arginyl residue. Incubation of the free tetrapeptide with carboxypeptidase B, which specifically removes C-terminal arginyl and lysyl residues, quantitatively afforded the model tripeptide and arginine.

Whichever mild methods for peptide release from the resin are chosen, their use in conjunction with the enzymatic N-deprotection scheme is only possible if the insoluble support is compatible with the elaboration of peptides in aqueous media. Moreover, the enzyme must have ready access to the growing chains. The conventional polymeric resin introduced by Merrifield (117) and subsequently widely employed for most solid phase syntheses* is incompatible with both of these requirements. The common resins swell only in certain organic media which allows the reagents to

*Many modifications of the original Merrifield resin have been described, although most have retained the basic features which render them inadequate for use in aqueous media.

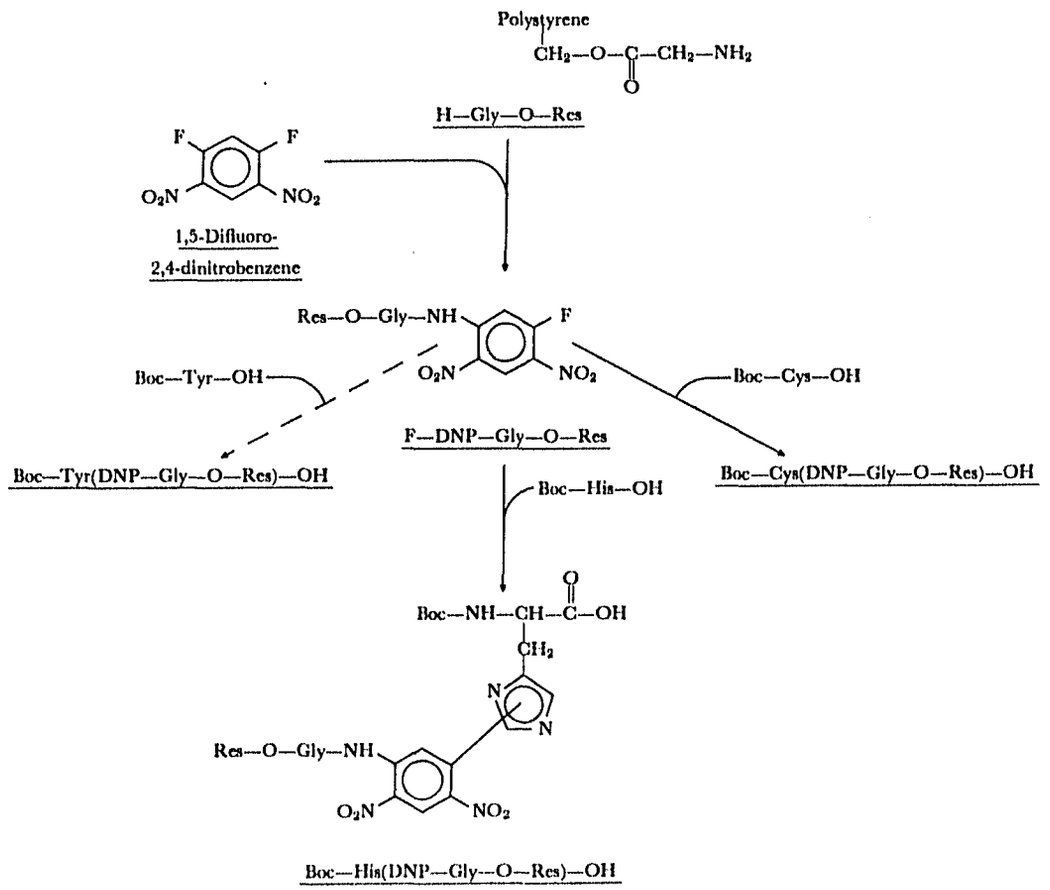


Figure 42: Thiol-labile attachments of amino acids through their side-chains to solid supports.

penetrate the interstices of the polymeric matrices where the growing points of many of the peptide chains are located. Substitution of the polymer by groups which cause it to swell in aqueous media may provide some advancement toward aqueous solid phase synthesis (130), but enzymatic N-deprotection would remain a problem since it is doubtful that an enzyme could penetrate even a swelled matrix and still operate efficiently in hydrolyzing its substrate.

The best approach to suitable solid supports will likely be in the area of substituted glass beads. In one case, a special pellicular resin was prepared by polymerizing a thin layer of polystyrene onto glass beads (131). Graft polymers have also been prepared in which polystyrene branches are substituted directly onto the surface of the beads (132). Glass beads which have been directly substituted with functional groups such as propylamine are commercially available. The main feature of the above supports is that the peptide chains all grow from the surface of the individual beads. There is no requirement for swelling, aqueous media may be utilized, and the growing chains are more accessible to enzymes. It is important to recognize that the shift to aqueous synthesis will be gradual and the present methodology will be heavily relied upon throughout this development. It is therefore important for the solid supports to function in both aqueous and non-aqueous media so that they are compatible with the operational intermediate stages of development towards the ultimate goal. In this regard, the inert resins mentioned above should prove to be more immediately useful than those few which operate exclusively in aqueous media (eg, polyethyleneamine)(eg, 8). It is notable that trypsin is capable of functioning in partially aqueous solutions (even in 50% DMF) (133) and may therefore be quickly adaptable

to the intermediary stages of the new methodology.

In our laboratory, early attempts are underway to modify the thiol-labile amino acid side-chain resin attachments to include substituted glass beads. In a preliminary experiment, histidine was successfully attached via its side-chain to the propylamine glass beads through a thiol-labile dinitrophenylene bridge. The histidine was recovered by thiolysis with 2-mercaptoethanol. Both the attachment to and cleavage from the resin were performed in aqueous bicarbonate buffer. Similarly, tert-Butyloxycarbonyl-cysteine-OH was attached and removed from the resin, although the coupling reaction was performed in DMF. Although the substitution of propylamine on the starting resin and the subsequent amino acid substitutions were low; early investigations indicate that this shortcoming will soon be overcome.

The stepwise elaboration of complete peptides by the enzymatic deprotection approach could become feasible on solid support surfaces which are compatible with the conditions for enzyme activity. However, far more emphasis should be placed upon perfecting the use of such a system in conjunction with semisynthetic techniques. Usually, specific changes in relatively few portions of peptides and proteins are desired for synthetic analogs, rather than totally revolutionary sequences. Naturally occurring fragments can be obtained in many cases which may be used as synthetic precursors to such analogs. To date, the lack of synthetic techniques which operate in an essentially physiologic environment has largely contributed to the slow progress of semisynthesis. The mild conditions demonstrated during the operation of the enzymatic deprotection method, especially when combined with the envisioned extension of the method to solid phase synthesis, provides a genuine foundation

for the advancement of the art of semisynthesis. In addition, the advent of solid particles substituted with functional groups which are specific for particular amino acid side-chains (eg, cysteine, histidine, tyrosine) should aid the development of unique and powerful isolation and purification methods.

A subtle feature inherent within the technique of solid phase synthesis is that the extra steps required to remove picrate ions during synthesis in solution is eliminated. The picrate can be exchanged for alternate counter ions while the peptide is anchored to the solid support and the free picrate salts can be washed away in appropriate solvents by filtration. However, the exchange might be unneccessary altogether if the activated dipeptide intermediates were introduced as their amorphous hydrochloride salts; this would be especially helpful during peptide elaboration in solution.

C. Conclusion:

The foregoing work provides the foundation for the development of significantly improved peptide synthetic procedures. The combination of chemical specificity, the mild conditions for removal of amine-protecting groups in aqueous solution, and the degree of stereoselectivity in the deprotection reaction has not been previously available. The course of the deprotection reaction can be conveniently monitored and controlled, and the extent of the reaction can be sensitively determined by amino acid analysis. Powerful routine ion exchange purification techniques were developed for the removal of incompletely coupled or incompletely deprotected components from primary products at each stage of the stepwise synthesis. The removal of incompletely deprotected peptides implies the separation of optically distinct components under appropriate circumstances.

The system may be immediately applicable to particular synthetic problems, especially when some of the suggested refinements are instituted. Ultimately, the principles underlying the enzymatic removal of amine protecting groups may be broadened to include other types of protecting groups and other types of functional groups. The mild conditions for the removal of these groups should accommodate the increased use of existing non-enzyme-labile but mildly removable protecting groups and encourage the development of new ones. Further, it is suggested that the method can be integrated into a system of peptide and protein synthesis which will operate partially or exclusively in aqueous media under essentially physiologic conditions. The synthesis may be accomplished either by the solution or the solid-phase method, or by the two in combination. Peptide chain elaboration could be performed stepwise and by fragment coupling of synthetic or semisynthetic products.

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