Identification of Asparaginyl and Glutaminyl Residues in *endo* Position in Peptides by Dehydration–Reduction[1]

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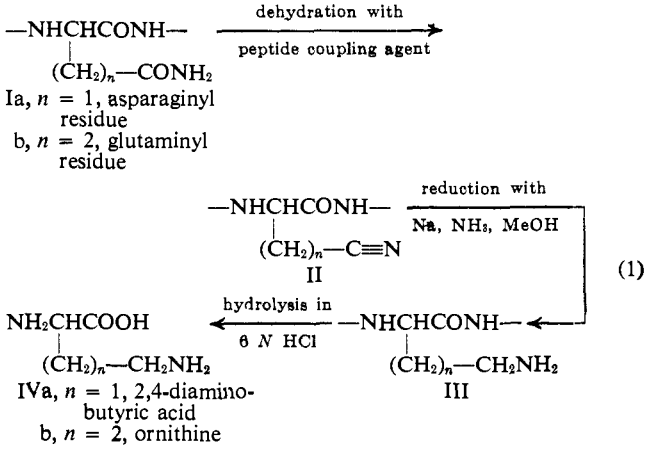
Abstract: A procedure has been developed for ready identification of asparaginyl and glutaminyl residues in the *endo* position in peptides. The peptide amides are dehydrated on a microscale in triethyl phosphite, with the peptide coupling agent ethylene chlorophosphite, to yield the corresponding cyanopeptide derivatives which are then subjected directly to a micro-Birch reduction. The treated peptides are hydrolyzed, and products are determined on the automatic amino acid analyzer. *endo*-Asparaginyl and -glutaminyl residues give rise to 2,4-diaminobutyric acid and ornithine, respectively. Several isoasparaginyl and isoglutaminyl residues examined gave rise to β-alanine and γ-aminobutyric acid through hydrogenolysis of the respective intermediate α-cyanoamino acid derivatives. The dehydration–reduction procedure has been applied to polypeptides ranging in molecular weight from 771 to 1422, including natural oxytocin, synthetic isoglutamine-4-oxytocin, synthetic isoasparagine-5-oxytocin, natural evoldine, and commercial tyrocidin and bacitracin, as well as several model compounds of smaller size. The results confirm the presence, in evolidine, of an asparaginyl residue and, in oxytocin and tyrocidin, of asparaginyl and glutaminyl residues, all in *endo* position. Of four structures that have been suggested elsewhere for bacitracin, the present findings are consistent only with that one having an asparaginyl residue joined by an α→ε linkage to lysine within a cyclopeptide of seven amino acid residues. Formation of amino alcohols in high yield has been found to accompany reductive cleavage of peptide proline bonds in the Birch reduction. Synthesized as new model compounds were carbobenzoxy-β-cyano-β-L-alanine and carbobenzoxy-β-cyano-L-alanylglycine benzyl ester.

In structural studies with proteins, peptide antibiotics, and other similar natural substances, the need for a convenient procedure for identifying residues of asparagine and glutamine (I) has become evident. Ammonia in such substances generally is assigned to the aspartic acid and glutamic acid residues, and usually it is assumed that these are present as residues of asparagine and glutamine rather than as residues of isoasparagine and isoglutamine. When more residues of dicarboxylic acids than ammonia are present, the exact location of the amides frequently remains unknown or sometimes is assigned on the basis of the electrophoretic behavior and the composition of degradation fragments.

During the synthesis of certain peptides related to oxytocin, a side reaction resulting in the conversion of an asparagine residue to one of 2,4-diaminobutyric acid (IIIa) was encountered.[3] Since then it has been established that carbobenzoxy-L-asparagine and carbobenzoxy-L-glutamine can be readily dehydrated to the β-cyanoalanyl or γ-cyanoaminobutyryl derivative by the peptide-coupling agents N,N′-dicyclohexylcarbodiimide and alkyl chloroformates.[4–6] On reduction with sodium in liquid ammonia, the ω-cyanoamino acid derivative gives rise to the readily identifiable basic amino acid 2,4-diaminobutyric acid (IVa) or ornithine (IVb).[4,7] ω-Nitrile formation is now regarded as a likely side reaction in the synthesis of asparagine and glutamine peptides. The present paper reports a closer study of the dehydration and reduction reactions and their application as an analytical procedure for identifying residues of asparagine and glutamine in peptides. The over-all procedure can be represented as reaction 1.



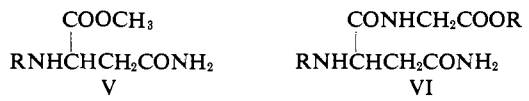
(1)

Peptide syntheses showing ω-nitrile formation as a side reaction (*cf.* ref 8b for a review) seem to have employed mainly tetraethyl pyrophosphite or N,N′-dicyclohexylcarbodiimide for coupling derivatives having the asparagine and glutamine residues in C-terminal position, It has recently been established that dehydration of asparagine amide with N,N′-dicyclohexylcarbodiimide requires the assistance of the asparagine carboxyl group.[6,8,9] Two independent studies of this reaction with the oxygen-18 technique suggested a mechanism in which the carboxyl group and the carbodiimide react to form an intermediate O-acylurea adduct from which a dicyclohexylurea anion is lost by intramolecular displacement by the carboxamide oxygen. The formed aminoisosuccinimide derivative on base-catalyzed ring opening could yield the β-cyanoalanine derivative.[8,9] Thus, the side reaction with dicyclo-

(1) Aided by U. S. Public Health Service Grant NB 04316 and by Muscular Dystrophy Associations of America.
(2) Visiting Research Fellow, 1961–1962.
(3) C. Ressler, *J. Am. Chem. Soc.*, **78**, 5956 (1956); see also D. T. Gish, P. G. Katsoyannis, G. P. Hess, and R. J. Stedman, *ibid.*, **78**, 5954 (1956).
(4) C. Ressler and H. Ratzkin, *J. Org. Chem.*, **26**, 3356 (1961).
(5) C. H. Stammer, *ibid.*, **26**, 2556 (1961).
(6) B. Liberek, *Bull. Acad. Polon. Sci., Ser. Sci. Chim.*, 10, 227 (1962).
(7) M. Zaoral and J. Rudinger, *Collection Czech. Chem. Commun.*, **24**, 1993 (1959).
(8) (a) R. Paul and A. S. Kende, *J. Am. Chem. Soc.*, **86**, 741 (1964); (b) *ibid.*, **86**, 4162 (1964).
(9) D. V. Kashelikar and C. Ressler, *ibid.*, **86**, 2467 (1964).

hexylcarbodiimide is expected to be confined to coupling reactions at the carboxyl group of the asparagine and glutamine residues. Although this reagent might be useful in connection with identifying asparagine and glutamine residues in C-terminal position, its requirement for a free carboxyl group for amide dehydration makes it unsuitable for identifying the majority of asparagine and glutamine residues bound in the *endo* position in peptides.

Various simple N-protected asparagine and glutamine esters have been smoothly dehydrated to ω-cyano ester derivatives with *p*-toluenesulfonyl chloride in the absence of neighboring carboxyl group assistance.[7,10] For synthesizing nitriles from primary carboxamides unrelated to amino acid amides a variety of dehydrating agents have been used. These include phosphorus pentoxide, phosphorus oxychloride, thionyl chloride, phosphorus pentachloride, sulfamic acid, aluminum chloride, acyl and arylsulfonyl chlorides,[11a] amidochlorides,[11b] and dihalotriphenylphosphoranes.[11c] Several such reagents were therefore examined for suitability for dehydrating peptides containing fully bound asparagine residues to the corresponding β-cyanoalanine peptides. For these experiments carbobenzoxy-L-asparagine methyl ester (V), carbobenzoxy-L-asparaginylglycine benzyl ester (VI), and evolidine (XI) served as chief model compounds.



V VI

R = carbobenzoxy ($C_6H_5CH_2OCO$)
R′ = benzyl ($C_6H_5CH_2$)

A preliminary attempt to dehydrate evolidine with dimethylformamidochloride (thionyl chloride in dimethylformamide)[11b] at 25° for 1 hr resulted in a dark brown reaction mixture. *p*-Tosyl-L-asparagine treated with *p*-toluenesulfonyl chloride[7] or carbobenzoxy-L-asparagine with an excess of this reagent was known to yield tarry products. This made these reagents less suitable for the desired analytical purpose, in part because the colors tended to obscure the progress in the subsequent sodium–ammonia–methanol reduction step which is judged by the behavior of a blue color. In several early unpublished attempts to synthesize certain asparagine peptides from carbobenzoxy-L-asparagine with ethylene chlorophosphite (ethylene phosphorochloridite) as coupling agent[12] yields of the desired asparagine peptides were particularly low with poor recoveries of starting material. If the side reaction occurring with ethylene chlorophosphite were dehydration of asparagine amide[10] this coupling agent would be potentially useful for converting asparagine-containing peptides to the corresponding β-cyanoalanyl peptides. Experiments with V and ethylene chlorophosphite in pyridine now show that dehydration of asparagine amide proceeds readily with this reagent. Unlike dicyclohexylcarbodiimide, ethylene chlorophosphite does not require participation of a neighboring carboxyl group for amide dehydration. Moreover, the crude dehydration product is readily obtained from the reaction mixture since excess reagent can be decomposed with ethanol or removed under high vacuum. The dehydration of V with ethylene chlorophosphite has been examined under a variety of conditions of temperature, solvent, and amount of reagent. With 4 moles of ethylene chlorophosphite in pyridine at 90–100° for 30 min, V, on a 1-g scale, gave carbobenzoxy-β-cyanoalanine methyl ester in 80% yield. Yields were lower with a lower reaction temperature or with triethyl phosphite replacing pyridine even when the reaction time was extended. Dimethylformamidochloride at a low reaction temperature also effectively dehydrated V to the β-cyanoalanine ester. Tetraethyl pyrophosphite[13] in close to molar amount in pyridine did not result in appreciable dehydration of V within 3 hr at 100°, although dehydration was significant with a large excess of the reagent in the absence of solvent or base.

For purposes of analysis of peptide ω-amide, several milligrams of VI was heated in a small closed test tube with ethylene chlorophosphite in triethyl phosphite at 100° for varied periods. This solvent was used to avoid the bright orange colors resulting frequently with pyridine. Excess reagent and solvent were removed. The residue representing the crude protected β-cyanoalanine dipeptide was reduced without purification in the same reaction tube with sodium–ammonia–methanol to the 2,4-diaminobutyric acid peptide which was then hydrolyzed in 6 *N* HCl. The hydrolysate was examined by paper electrophoresis for the appearance of 2,4-diaminobutyric acid and the disappearance of aspartic acid. It was then analyzed quantitatively for 2,4-diaminobutyric acid on the automatic amino acid analyzer.[14] Optimum conditions on a scale of 4 to 6 μmoles of VI were 3–5 hr at 100° with 50 μl of ethylene chlorophosphite in 200 μl of triethyl phosphite for the dehydration, and 100 μl of methanol for the reduction. Hydrolysates showed glycine, aspartic acid, and 2,4-diaminobutyric acid in the molar ratios 1.00:0.09:0.88. Yields of 2,4-diaminobutyric acid were 61 to 73%. Experiments with evolidine indicated that it was advantageous to extend the dehydration time to 24 hr for larger peptides. Reduction mixtures were frequently desalted before hydrolysis, although it was possible to examine hydrolysates by paper electrophoresis or on the amino acid analyzer without desalting.

All reductions were carried out under the conditions of the Birch reduction.[15] In earlier experiments with carbobenzoxy-β-cyano-L-alanine it had been noted that by-product formed with sodium in ammonia was absent in reductions carried out in the presence of methanol.[4] Quantitative examination of the reaction with pertinent published and new model cyanoamino

(10) Subsequent to undertaking this work, *p*-tosyl-L-glutamine methyl ester was dehydrated with the peptide coupling agent *as*-dichlorodimethyl ether (K. Poduska and H. Gross, *Chem. Ber.*, 94, 527 (1961)) and carbobenzoxy-L-asparagine methyl ester and certain N-protected amino amides were dehydrated with phosphorus oxychloride (B. Liberek, *Bull. Acad. Polon. Sci., Ser. Sci. Chim.*, 10, 407 (1962); B. Liberek, A. Nowicka, and J. Szrek, *Roczniki Chemii*, 39, 369 (1965)).

(11) (a) B. Rickborn and F. R. Jensen, *J. Org. Chem.*, 27, 4608 (1962), and references cited therein, and in ref 4 and 7; (b) H. Eilingsfeld, M. Seefelder, and H. Weidinger, *Angew. Chem.*, 72, 836 (1960); (c) L. Horner, H. Oediger, and H. Hoffmann, *Ann.*, **626**, 26 (1959).

(12) R. W. Young, K. H. Wood, R. J. Joyce, and G. W. Anderson, *J. Am. Chem. Soc.*, 78, 2126 (1956).

(13) G. W. Anderson, J. Blodinger and A. D. Welcher, *ibid.*, 74, 5309 (1952).

(14) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, 30, 1190 (1958).

(15) A. J. Birch, J. Cymerman-Craig, and M. Slaytor, *Australian J. Chem.*, 8, 512 (1955).

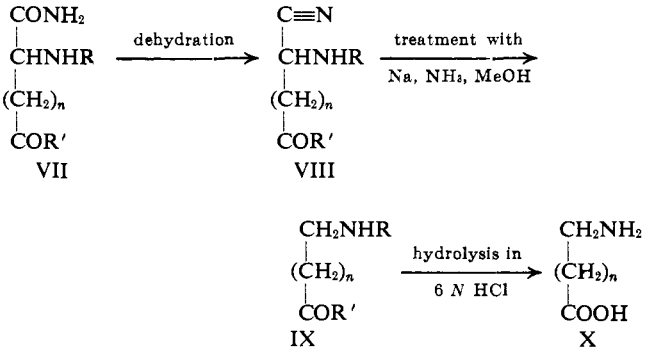
Table I. Reaction of α-, β-, and γ-Cyanoamino Acids or Derivatives with Sodium–Ammonia–Methanol. Chromatographic Ninhydrin Analyses[a]

Substance	Wt, mg	Condition[b]	System[c]	Yield,[d] %	Amino acids and NH_3 in product (molar ratios)
β-Cyano-α-L-alanine[e]	0.87	Reduction	C	102	2,4-Diaminobutyric acid (2,4-DAB)
Cbz-β-cyano-α-L-alanyl-glycine benzyl ester	1.63	Hydrolysis	A, B	103	Gly:Asp:NH_3 1.00:1.00:1.05
	1.12	Reduction, hydrolysis	A, C	72	Gly:2,4-DAB:Asp 1.00:0.92:0.02
	1.88	Reduction, desalting, hydrolysis	A, B	90	Gly[f]: 2,4-DAB:Asp 0.94:1.00:<0.01
γ-L-Glutamyl-β-cyano-α-L-alanine dicyclohexyl-ammonium salt[g]	2.28	Reduction, desalting, hydrolysis	A, B	89	Glu:2,4-DAB:Asp 0.94:1.00:<0.01
γ-Cyano-α-L-amino-butyric acid[h]	1.10	Reduction	B	98	Ornithine
Cbz-β-cyano-β-L-alanine	2.56	Hydrolysis	A, B	95	Asp:NH_3 1.00:0.98
	2.74	Reduction	A, B	63	β-Alanine
α-Cyanoglycine[i]	2.28	Reduction	A	96	Glycine

[a] See ref 14. [b] Treatment before amino acid analysis. Reductions carried out near the boiling point in 1–5 ml of liquid NH_3 containing 100 μl of methanol, except for 200 μl with β-cyanoalanine and α-cyanoglycine. [c] See Analysis of Residues in the Experimental Section. [d] Yield of reduction product or average recovery on hydrolysis only. [e] Data with natural amino acid: C. Ressler, *J. Biol. Chem.*, **237**, 733 (1962). [f] Also present: 5% unidentified material eluted 23 ml after 2,4-DAB or 18 ml before NH_3 in position of ethanolamine. Occasional reductions had up to 26%. [g] Data of ref 16. [h] See ref 4. [i] Mp 124°, synthesis to be published.

acid and dipeptide derivatives (Table I) now shows that reduction of the ω-cyano group to the ω-aminomethyl group with sodium–ammonia–methanol takes place very readily and in high, if not quantitative, yield. β-Cyanoalanine and γ-cyano-α-aminobutyric acid were converted within half a minute quantitatively to 2,4-diaminobutyric acid and ornithine, respectively. Carbobenzoxy-β-cyano-L-alanylglycine benzyl ester, synthesized from carbobenzoxy-β-cyano-L-alanine and glycine benzyl ester with N,N′-dicyclohexylcarbodiimide, and showing the expected amino acid and ammonia composition, on reduction and hydrolysis gave 2,4-diaminobutyric acid in 72% yield. The yield increased to 90% when the reduced material was freed of salts with a small column of Dowex 50-X2 before hydrolysis. Glycine and the formed 2,4-diaminobutyric acid were present in close to equimolar amount. This procedure was used recently in establishing the composition of the naturally occurring β-cyanoalanine peptide isolated from seeds of *Vicia sativa* (common vetch), γ-L-glutamyl-β-cyano-L-alanine.[16] The dicyclohexylammonium salt of the latter, when reduced, desalted, and hydrolyzed, gave 2,4-diaminobutyric acid in 89% yield.

It might be expected that the dehydration step with ethylene chlorophosphite would convert an isoasparaginyl residue in peptide linkage to a residue of β-cyano-β-alanine. To provide analytical evidence for the isoasparaginyl residue analogous to the formation of 2 4-diaminobutyric acid from asparagine, it was desired to prepare this new type of amino acid incorporating an α-aminonitrile moiety, or a derivative of it, and to determine its behavior under the conditions of the sodium–ammonia–methanol reaction. Carbobenzoxy-β-cyano-β-L-alanine (VIIIa) was synthesized from carbobenzoxy-L-isoasparagine (VIIa) by the route used to obtain carbobenzoxy-β-cyano-L-alanine from carbobenzoxy-L-asparagine, *i.e.*, by dehydration with N,N′-dicyclohexylcarbodiimide in pyridine.[4] VIIIa formed a mole each of aspartic acid and NH_3 on hydrolysis in 6 *N* HCl and was converted to isoasparagine with hydrogen bromide in acetic acid. When VIIIa was treated with sodium–ammonia–methanol, β-alanine (IXa) appeared as the single, recognized product in 63% yield. α-Cyanoglycine [(COOH)CHNH$_2$C≡N], available from other studies, gave glycine in 96% yield under these conditions. Isoasparagine-5-oxytocin (XX, VIIb) and isoglutamine-4-oxytocin (XXI, VIIc), model synthetic octapeptides having an isoasparagine and an isoglutamine residue in an *endo* position, showed in hydrolysates after dehydration–reduction 0.53 mole of β-alanine (Xb) and 0.42 mole of γ-aminobutyric acid (Xc), respectively (Table V, columns 2 and 3).



VIIa, VIIIa, R = $C_6H_5CH_2OCO$; R′ = OH; *n* = 1
VIIb, VIIIb, IXb, R = COCHNHR‴;
R′ = NHCHR‴CO; *n* = 1
VIIc, VIIIc, IXc, R = COCHNHR‴;
R′ = NHCHR‴CO; *n* = 2
IXa, R = H; R′ = OH; *n* = 1
Xb, *n* = 1
Xc, *n* = 2

The α-aminoacetonitrile linkage in VIIIa, in α-cyanoglycine, and those formed *in situ* on dehydration of the

(16) C. Ressler, S. N. Nigam, Y.-H. Giza, and J. Nelson, *J. Am. Chem. Soc.*, **85**, 3311 (1963).

oxytocin isomers XX and XXI appear to have undergone significant cleavage of the cyano group under the conditions of the Birch reduction. Analogy for this reaction may exist in the formation of toluene, benzyldimethylamine, and benzylethyldimethylamine on treatment of phenylacetonitrile,[17a] α-dimethylaminophenylacetonitrile,[17b] and α-dimethylamino-α-ethylphenylacetonitrile,[17b] respectively, with sodium in liquid ammonia. Hydrogenolysis of a number of substituted α-aminonitriles has been observed also with sodium in alcohol or in moist ether,[17c] and with lithium aluminum hydride.[17d] The scope of this reaction in the amino acid field remains to be explored.

The dehydration–reduction reaction developed does not appear to be directly applicable to the identification of C-terminal asparagine amide. Reaction of carbobenzoxy-L-asparagine and the α- and β-benzyl esters of carbobenzoxy-L-aspartic acid led mainly to unidentified products whose chromatographic behavior on the amino acid analyzer tentatively suggests that ethylene chlorophosphite reacts with free carboxyl groups to form phosphorylated derivatives that are incompletely hydrolyzed under the conditions used in the acid hydrolysis step. Consistent with this is the observation that similar treatment of carbobenzoxy-L-prolyl-L-leucylglycine resulted in appreciable loss of glycine. The nature of the products of reaction of ethylene chlorophosphite with carboxyl groups and the possible application of this reaction to the identification of C-terminal amino acid residues in peptides are under investigation.

A convenient general procedure is presented for microanalysis of peptide ω-amide in the *endo* position. Details are given for dehydration, reduction, desalting, and analysis of residues. A modified chromatographic system is described for use with the automatic amino acid analyzer, which affords good resolution of 2,4-diaminobutyric acid and ornithine, derived from the ω-amides, β-alanine and γ-aminobutyric acid, derived from the dehydrated isoasparaginyl and isoglutaminyl residues, as well as other substances frequently present in reduction mixtures and physiological fluids. The procedure has been applied to natural oxytocin, natural evolidine, commercial natural tyrocidin, and bacitracin, as well as to the model synthetic peptides isoasparagine-5-oxytocin and isoglutamine-4-oxytocin. At the time these experiments were completed, except for the oxytocins, the exact nature of the amide-bearing groups in these was either unknown or based on indirect evidence. The deductions permissible on the amide-bearing aspartic acid and glutamic acid residues on the basis of the present results have been discussed for each polypeptide. Side reactions in the dehydration–reduction reaction have also been noted. In evolidine, conversion of a residue of leucine to one of leucinol was encountered, apparently the result of reductive fission of the leucylprolyl bond. Formation of amino alcohols on reductive cleavage of peptide acylproline bonds with sodium–ammonia–methanol was established also with several simpler proline peptides.

(17) (a) L. H. Baldinger and J. A. Nieuwland, *J. Am. Chem. Soc.*, **55**, 2851 (1933); (b) G. F. Morris and C. R. Hauser, *J. Org. Chem.*, **27**, 465 (1962); (c) W. McMeeking and T. S. Stevens, *J. Chem. Soc.*, 347 (1953); (d) G. Chauviere, B. Tchoubar, and Z. Welvart, *Bull. Soc. Chim. France*, 1428 (1963), and references cited therein.

Discussion

Evolidine

cyclo[L-Ser-L-Phe-L-Leu-L-Pro-L-Val-L-Asp(β-NH_2)-L-Leu]

XI

The structure of evolidine, a polypeptide from the leaves of the plant *Evodia xanthoxyloides*, was proposed by Law, Millar, and Springall as the cyclic heptapeptide amide XI.[18] In their degradative studies the position of the mole of ammonia had not been determined experimentally, and it was assumed that the ammonia was bound to the single residue of aspartic acid as an amide. It may be noted, however, that the ammonia can be reassigned instead to the carboxyl group of the leucine residue linked through its amino group to the aspartic acid residue. This would allow another structure (XII), having a C-terminal leucinamide residue, to be considered for evolidine; this structure is not inconsistent with the sequences of the peptides found on partial acid hydrolysis.

L-Ser-L-Phe-L-Leu-L-Pro-L-Val-L-Asp-L-Leu(NH_2)

XII

Moreover, the nature of the aspartylleucine linkage in evolidine had been assigned on indirect evidence. Both α- and β-aspartylleucine were present in partial acid hydrolysates which suggests that in the native polypeptides the aspartic acid residue could be linked to leucine through either the α- or β-carboxyl group. The α linkage had been assigned because α-aspartylleucine appeared sooner during hydrolysis, and, on the basis of analogy with the behavior in acid of synthetic

Table II. Identification of Asparaginyl Residue in Evolidine. Chromatographic Ninhydrin Analyses[a]

Peptide	Theoretical, untreated[b]	Untreated (1)	Dehydrated[c] (2)	Dehydrated, reduced, desalted[c-e] (3)
Evolidine wt, mg		0.99	1.20	2.07
Recovery, %[f]		100	78[g]	54[h]
Constituent		**Molar ratios**		
Aspartic acid	1	1.06	0.85	0.05
2,4-Diaminobutyric acid		0	...[i]	0.89
Proline	1	1.11	0.98	0.92
Valine	1	1.06	1.08	1.10
Leucine	2	2.00	2.00	1.00[j]
Serine	1	0.98	0.59[k]	0.16
Phenylalanine	1	1.06	0.95	0.22
Ammonia	1	1.63	...[i]	...
Leucinol		0	...[i]	0.45

[a] Chromatographic systems A and B; (1) and (3) analyzed also in system C. [b] Data of ref 18. [c] Dehydrated with 50 μl of ethylene chlorophosphite (ECP) in 200 μl of triethyl phosphite (TEP). [d] Reduced in presence of 100 μl of methanol. [e] Also present: trace amounts of glycine and alanine and unidentified materials eluted in system B, 52 ml before 2,4-DAB and 27 ml after NH_3, in the molar ratios 0.06, 0.07, 0.07, and 0.23, respectively. [f] Based on weight of evolidine. [g] Average recovery, except for serine. [h] Based on proline and valine recovery. [i] Not determined. [j] Loss of one residue of leucine confirmed in triplicate dehydration–reduction reactions. [k] Also present, a peak close to the position of phosphoserine in the molar ratio 0.22.

(18) H. D. Law, I. T. Millar, and H. D. Springall, *Proc. Chem. Soc.*, 198 (1958); *J. Chem. Soc.*, 279 (1961).

Table III. Formation of Amino Alcohols on Reductive Cleavage of Model Proline-Containing Peptides with Sodium-Ammonia-Methanol. Chromatographic Ninhydrin Analyses[f]

Compound	Wt, mg	μmoles	CH_3OH, μl	Cleavage[a]	System	Identified products[b] and yields, %
L-Pro-Gly·H_2O	4.2	22	300	−		
Cbz-L-Pro-L-Leu	3.6	10	250	−		
Cbz-L-Pro-Gly	3.6	11.7	150	−		
Gly-L-Pro	3.1	18.1	300	+	A, B, B-1	Ethanolamine, 94; [c] Pro, 108
Cbz-L-Ala-L-Pro	3.9	12.2	250	+	A, B-1	Alaninol, 74; [c] Ala, 5.7; Pro, 108
L-Leu-L-Pro-Gly[d]	0.5	1.75	100	+	C	Leucinol, 82[c]
Reduction, desalting, hydrolysis	1.6	5.61	150	+	A, C	Leucinol, 44 Molar ratios: Leu:Pro[e]: Gly:leucinol, 0.05:0.49: 1.00:0.42

[a] Inferred from liberation of proline or formation of amino alcohol. [b] Not further investigated in absence of cleavage. [c] Cochromatography with the authentic amino alcohol resulted in a single peak with the expected recovery. [d] A hydrolysate contained leucine, proline, and glycine, all in molar ratio of 1. [e] Also present: material, possibly prolinol, eluted at 142 ml with a high 440:570 mμ ninhydrin product absorption ratio, in molar ratio 0.16 calculated as proline. [f] Reactions carried out under anhydrous conditions in liquid ammonia redistilled over sodium.

α-aspartylleucine and β-aspartylvaline, because only the former rearranged to a mixture of isomeric dipeptides.

The results obtained on application of the dehydration–reduction reaction to evolidine are shown in Table II, column 3. The formation of 0.9 mole of 2,4-diaminobutyric acid and the virtual disappearance of the residue of aspartic acid indicate the presence of an asparaginyl residue in evolidine. Thus, the ammonia is linked to the β-carboxyl group of the aspartic acid residue which must be linked to leucine through its α-carboxyl group. A structure such as XII should yield no 2,4-diaminobutyric acid and would be expected to allow the residue of aspartic acid to be recovered. The results of the dehydration–reduction reaction thus confirm the *endo*-asparaginylleucine moiety of structure XI assigned previously to evolidine.[19]

The low recovery of phenylalanine after the reaction is traced to the reduction step. This side reaction probably involves partial reduction of the phenyl ring as this reagent is capable of reducing aromatic systems.[20,21]

Formation of Amino Alcohols on Reductive Cleavage of Proline Peptides with Sodium–Ammonia–Methanol. In view of structure XI for evolidine, it was unexpected to find after dehydration–reduction only one of the two leucine residues. Comparison of the compositions of dehydrated and dehydrated-reduced evolidine indicated that the residue of leucine had been lost in the reduction step (Table II, columns 2 and 3). Side reactions have been encountered during reduction of peptides and proteins by mixed metallohydrides, chiefly reduction of primary amides to amines and of peptide bonds to imines, and reductive cleavage of the peptide bond.[22] Recently, there have been several reports of rather specific cleavage at the site of the iminopeptide bond of certain proline-containing peptides on treatment with the reducing agents lithium in methylamine,[23] lithium aluminum hydride in tetrahydrofuran,[24] or sodium in liquid ammonia.[25] Since one of the two leucine residues in structure XI is bound through its carboxyl group to the imino group of a proline residue, the loss of a leucine residue on reduction of dehydrated evolidine might be associated with a reductive cleavage of the polypeptide at this acylproline bond. To examine this possibility, the products of reductive cleavage were investigated. In the cleavage of tyrocidin B and gramicidin S with lithium aluminum hydride, phenylalanine residue(s) acylating proline residue(s) was converted initially to residue(s) of 2-amino-3-phenylpropionaldehyde.[24a] Reductive cleavage with lithium in methylamine similarly converts the amino acid acylating proline to the corresponding aldehyde.[26] However, information has been lacking on the fate of the amino acid bound in acylpeptide linkage to the proline residue in the few reductive cleavages reported with sodium in liquid ammonia. In the early studies of Birch and co-workers on the production of aldehydes from amides and related compounds by dissolving metals, very low to moderate yields of benzaldehyde or hexanoicaldehyde were obtained from various N-disubstituted benzamides and hexanoamides on treatment with sodium–ammonia–ethanol. With amides giving little, if any, aldehyde, it was noted that the products obtained from the acyl portion of the molecule were nitrogen free.[15] Some model proline-containing compounds were therefore treated here with sodium–ammonia–methanol and the crude mixtures were examined by paper chromatography and electrophoresis. When evidence suggested cleavage, the products were analyzed quantitatively on the amino acid analyzer, with the results shown in Table III. As expected, prolylglycine, carbobenzoxyprolylglycine, and carbobenzoxyprolylleucine each showed only a single substance with no indication of cleavage. Free proline (XVa) was liberated quanti-

(19) When this manuscript was near completion, further degradative studies of natural evolidine, as well as its synthesis, were reported that confirmed the structure originally proposed: R. O. Studer and W. Lergier, *Helv. Chim. Acta*, **48**, 460 (1965).

(20) A. J. Birch, P. Hextall, and S. Sternhell, *Australian J. Chem.*, **7**, 256 (1954).

(21) Lithium in methylamine has been reported to reduce phenylalanine residues in peptides to a mixture of cyclohexene and cyclohexadiene derivatives: M. Wilchek and A. Patchornik, *J. Am. Chem. Soc.*, **84**, 4613 (1962).

(22) For a discussion of this subject, see J. C. Crawhall and D. F. Elliot, *Biochem. J.*, **61**, 264 (1955).

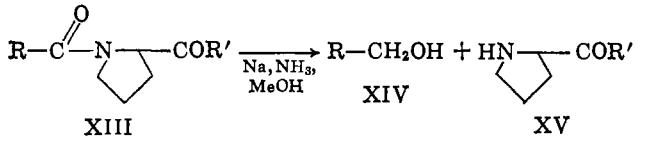
(23) A. Patchornik, M. Wilchek, and S. Sarid, *J. Am. Chem. Soc.*, **86**, 1457 (1964).

(24) (a) M. A. Ruttenberg, T. P. King, and L. C. Craig, *Biochemistry*, **3**, 758 (1964); (b) *ibid.*, **4**, 11 (1965).

(25) (a) K. Hofmann and H. Yajima, *J. Am. Chem. Soc.*, **83**, 2289 (1961); (b) St. Guttmann in "Peptides," G. T. Young, Ed., The Macmillan Co., New York, N. Y., 1963, p 41; (c) J. Rudinger, unpublished observations of cleavage during the synthesis of certain analogs of oxytocin.

(26) A. Pathchornik, Abstracts, 6th International Congress of Biochemistry, New York, N. Y., July 1964.

tatively from carbobenzoxyalanylproline (XIIIa) and glycylproline (XIIIb) having proline in C-terminal position. The alanyl and glycyl residues acylating proline in these were converted to the corresponding amino alcohols, alaninol (XIVd) and ethanolamine (XIVb). These were identified by comparison with authentic material of their behavior on chromatography on the amino acid analyzer, on cochromatography, and on electrophoresis at two pH conditions. Yields of the amino alcohols were 74 and 94%, respectively.



XIII XIV XV

a, R = CbzNHC(CH3)H; R′ = OH
b, R = NH2CH2; R′ = OH
c, R = NH2C((CH3)2CHCH2)H; R′ = NHCH2COOH
d, R = NH2C(CH3)H

The tripeptide leucylprolylglycine (XIIIc), having the leucylprolyl sequence of evolidine, on reduction, desalting, and hydrolysis showed almost quantitative loss of the leucine residue in agreement with the findings with evolidine. With synthetic leucinol,[27] it was found that system B originally used for analysis of the treated evolidine is inadequate to detect this amino alcohol. Chromatography in system C established the presence of 0.44 mole of leucinol (XIVc) in the hydrolysate of the reduced, desalted leucylprolylglycine and 0.45 mole of leucinol in evolidine subjected to dehydration, reduction, desalting, and hydrolysis. A crude reduction mixture of leucylprolylglycine when analyzed directly afforded 82% leucinol, which suggests some loss of the amino alcohol on desalting. The absence of a residue of leucine in the treated evolidine is therefore attributed largely to reductive cleavage of the leucylprolyl bond and the formation of bound leucinol.

Reductive fission[23,24] is expected to be particularly applicable as a degradative reaction for structural studies of peptide antibiotics that are not readily susceptible to proteolysis and that have residues of D-amino acids and secondary amino acids such as pipecolic acid, N-alkylamino acids, or proline. Cleavage of various synthetic proline-containing peptides with sodium in liquid ammonia has seemed somewhat variable, and a dependence on the presence of moisture or other proton sources has been pointed out.[25b,28] It may therefore be advantageous to standardize conditions for cleavage by carrying out this reaction in the presence of methanol.[29] The ready conversion of the residue acylating proline to an amino alcohol observed under these conditions and the ease with which an amino alcohol can generally be identified should increase the usefulness of the reductive fission reaction.

(27) P. Karrer, P. Portmann, and M. Suter, *Helv. Chim. Acta*, **31**, 1617 (1948).
(28) J. Ramachandran, D. Chung, and C. H. Li, *J. Am. Chem. Soc.*, **87**, 2696 (1965).
(29) When this manuscript was near completion, effective cleavage of certain proline peptides with sodium in ammonia containing methanol was noted independently: M. Wilchek, S. Sarid, and A. Patchornik, *Biochim. Biophys. Acta*, 104, 618 (1965)].

Tyrocidin

tyrocidin A: cyclo[L-Val-L-Orn-L-Leu-D-Phe-L-Pro-L-Phe-D-Phe-L-Asp(NH_2)-L-Glu(NH_2)-L-Tyr]
XVI

tyrocidin B: cyclo[L-Val-L-Orn-L-Leu-D-Phe-L-Pro-L-Tryp-D-Phe-L-Asp(NH_2)-L-Glu(NH_2)-L-Tyr]
XVII

tyrocidin C: cyclo[L-Val-L-Orn-L-Leu-D-Phe-L-Pro-L-Tryp-D-Tryp-L-Asp(NH_2)-L-Glu(NH_2)-L-Tyr]
XVIII

In early studies on the structure of tyrocidin A, the presence of an asparaginyl and a glutaminyl residue had been assumed on the basis of the presence in hydrolysates of 2 moles of ammonia, single residues of aspartic acid and glutamic acid, and the absence of C- and N-terminal groups.[30] The results of the dehydration reaction with commercial tyrocidin are given in Table IV, column 3. The formation of significant amounts of 2,4-diaminobutyric acid and ornithine together with the almost complete disappearance of aspartic acid and glutamic acid confirms the previous assignment of the aspartic acid and glutamic acid residues as residues of asparagine and glutamine. These results are in general agreement with a recent observation of Ruttenberg, King, and Craig, studying the cleavage of peptide proline bonds, of the formation of 2,4-diaminobutyric acid and ornithine as a side reaction on treatment of tyrocidin B with lithium aluminum hydride at elevated temperature.[24a]

Table IV. Identification of Asparaginyl and Glutaminyl Residues in Commercial Tyrocidin. Chromatographic Ninhydrin Analyses[a,b]

	—Theoretical,— tyrocidin[c]					Dehydrated, reduced, desalted[d]
Peptide	A	B	C	Untreated (1)	Dehydrated (2)	(3)
Wt, mg, of tyrocidin, except for column 2				13.2	1.39[e]	6.88
Recovery, %				92	64[f]	36[g]
Constituent				Molar Ratios		
Aspartic acid	1	1	1	1.02	0.92	0.09
2,4-Diaminobutyric acid				0	0.02	0.50
Glutamic acid	1	1	1	1.00	0.84	0.04
Ornithine	1	1	1	1.01	1.01	1.83
Leucine	1	1	1	1.00	1.00	1.00
Proline	1	1	1	1.09	1.08	0.52
Valine	1	1	1	0.92	0.85	0.91
Tyrosine	1	1	1	0.89	0.93	0.68
Phenylalanine	3	2	1	2.13	2.09	0.23
Tryptophan		1	2	0.86	...	...
Ammonia	2	2	2	1.96	2.50	...

[a] The content of phenylalanine and tryptophan in (1) indicates the presence of some tyrocidin B or C, or both, in addition to A. [b] Chromatographic systems A and B for (1); systems A and C for (2) and (3). [c] Tyrocidin A and C data of ref 30 and 24a; tyrocidin B: T. P. King and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 6624 (1955). [d] Dehydrated with 100 μl of ECP in 300 μl of TEP; reduced in presence of 100 μl of methanol. [e] Part of the 22 mg of product isolated after treating 25 mg of tyrocidin, suspended in 1 ml of TEP, with 1 ml of ECP. [f] Based on weight of dehydrated tyrocidin. [g] Based on weight of tyrocidin and on valine and leucine recoveries.

(30) A. R. Battersby and L. C. Craig, *J. Am. Chem. Soc.*, 74, 4019 (1952).

Oxytocin and Oxytocin Isomers

Cys-Tyr-Ileu-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Leu-Gly(NH_2)

oxytocin, XIX

Cys-Tyr-Ileu-Glu(NH_2)-Isoasp(NH_2)-Cys-Pro-Leu-Gly(NH_2)

isoasparagine-5-oxytocin, XX

Cys-Tyr-Ileu-Isoglu(NH_2)-Asp(NH_2)-Cys-Pro-LeuGly(NH_2)

isoglutamine-4-oxytocin, XXI

In proposing the structure of oxytocin (XIX) it had been assumed that the aspartic acid and glutamic acid and 2 of the 3 moles of ammonia found in acid hydrolysates were present in the hormone as asparaginyl and glutaminyl residues.[31] The assumptions on the location of the amide groups and on the nature of the linkages of the aspartic acid and glutamic acid residues were substantiated through the synthesis of oxytocin.[32] Further confirmatory evidence for the asparaginyl and glutaminyl residues was subsequently afforded by finding asparagine and glutamine in papain hydrolysate of oxytocin.[33] Isoasparagine-5-oxytocin (XX) and isoglutamine-4-oxytocin (XXI) had been synthesized from appropriate preformed isoglutamine- and isoasparagine-containing intermediates.[34,35]

Table V presents the results of applying the dehydration–reduction reaction to natural oxytocin and to the two synthetic isomers. In oxytocin and isoglutamine-4-oxytocin essentially complete loss of the asparaginyl residue resulted with formation of 0.6 mole of 2,4-diaminobutyric acid. In oxytocin and isoasparagine-oxytocin, the glutaminyl residue was similarly lost, and 0.6 and 0.9 mole of ornithine, respectively, were formed. The isoglutamine residue of XXI was recovered as 0.42 mole of γ-aminobutyric acid and 0.49 mole of glutamic acid. The isoasparagine residue of XX was recovered as 0.53 mole of β-alanine and 0.2 mole of aspartic acid. No evidence was obtained for an asparaginyl or glutaminyl residue affording β-alanine or γ-aminobutyric acid, or the isoasparaginyl or isoglutaminyl residue affording 2,4-diaminobutyric acid or ornithine in significant amount, which indicates the virtual absence of isomerization in both the synthetic and analytic (dehydration–reduction) reactions. The recovery of glycine from each of the treated oxytocins was less than 0.2 mole; this indicates reaction also of the C-terminal glycinamide residue. Extension of this technique to the identification of C-terminal amino acid amides in peptides would seem feasible.

Table V. Identification of Asparaginyl, Isoasparaginyl, Glutaminyl, or Isoglutaminyl Residues in Oxytocin and Oxytocin Isomers.[a] Chromatographic Ninhydrin Analyses

Peptide	Theoretical, untreated[b]	Oxytocin Dehydrated, reduced[c] (1)	Isoglutamine-oxytocin Dehydrated, reduced, desalted[d] (2)	Isoasparagine-oxytocin Dehydrated, reduced[e] (3)
Wt, mg		1.47	1.90	1.73
Recovery, %[f]		50	51	60
System		A, C	A, B	A, B, C
Constituent		Molar ratios		
Proline	1	0.70	0.98	0.95
Leucine	1	1.00	1.00	1.00
Isoleucine	1	1.15[g]	0.76[g]	1.00[g]
Tyrosine	1	0.61	0.53	0.28
Cystine	1	0	0	0
Glycine	1	0.13	0.16	0.19
Aspartic acid	1	0.08	0.05	0.20
Glutamic acid	1	0.04	0.49	0.04
Ammonia	3	...	...	...
2,4-Diaminobutyric acid		0.60	0.59	0[h]
β-Alanine			0.03	0.53[h]
Ornithine		0.59	0.04	0.89[h]
γ-Aminobutyric acid			0.42	0.04

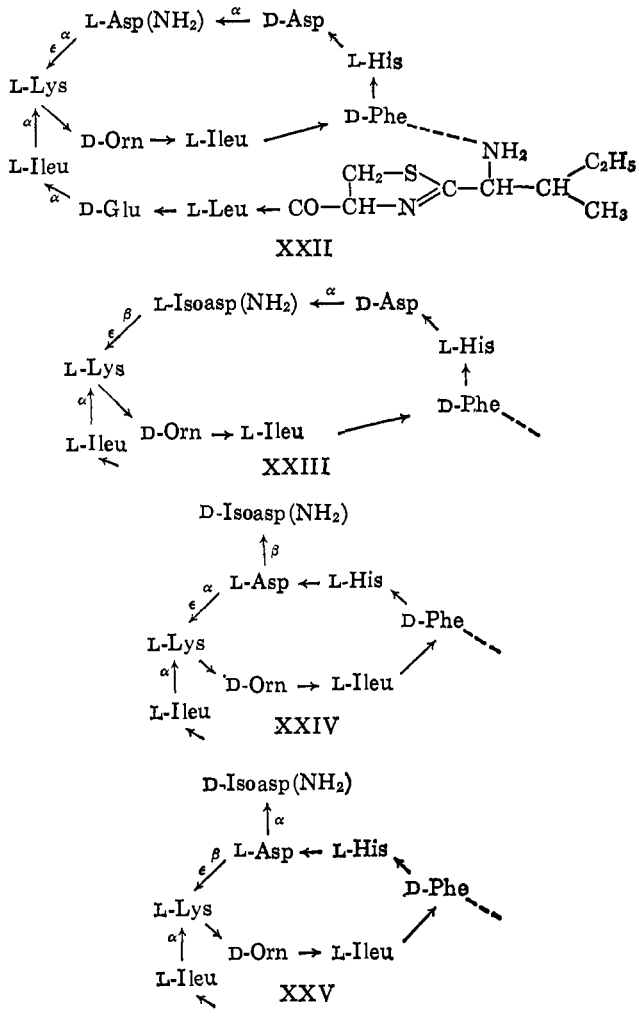
[a] Peptides treated with 100 μl of ECP in TEP: (1) and (3) in 200 μl; (2) in 300 μl. Dehydrated peptides reduced in presence of methanol: (1) in 50 μl; (2) and (3) in 100 μl. [b] Data of ref 34, 35, and 42. Reanalysis showed the expected composition except that *meso*-cystine formed part of the total cystine and in starting (2) and (3) allisoleucine formed part of the total isoleucine. [c] Also present: material eluted as alanine and cysteic acid in the molar ratios 0.09 and 0.16, respectively. [d] Also present: material eluted as alanine, cysteic acid, and unidentified material eluted in system B 31 ml after NH_3 in the molar ratios 0.13, 0.09, and 0.16, respectively. [e] Also present: material eluted as alanine, cysteic acid, and unidentified materials eluted in system B 15 ml before and 29 ml after NH_3 in the molar ratios 0.14, 0.32, 0.13, and 0.28, respectively. [f] Based on proline and leucine recoveries and corrected for 5% moisture and 1 mole of acetic acid in starting peptide. [g] Composite of isoleucine and alloisoleucine. [h] Average of two analyses.

Bacitracin

Bacitracin A contains 1 residue each of D-glutamic acid, L-aspartic acid, and D-aspartic acid, and 1 mole of ammonia, with the amino acid composition given in Table VI. Results of studies of partial hydrolysis in two separate laboratories in agreement allowed two possible sequences for this polypeptide.[36,37a] On the basis of indirect evidence obtained in a detailed study of the amide and carboxyl groups, the amide group was assigned to a residue of aspartic acid.[38a] This allowed the four possible structures for bacitracin A, XXII–XXV.[38a,b,39] Remaining to be established was whether the amide group was located on the L- or D-aspartic acid residue, whether it was present as a residue of asparagine or isoasparagine, whether the linkage to the ϵ-amino group of lysine involved the α- or β-carboxyl group of L-aspartic acid, the arrangement of the aspartic acid residues relative to each other, and the consequent size of the ring moiety of the molecule.

The results obtained on applying the dehydration–reduction reaction to commercial bacitracin are presented in Table VI. The formation of 2,4-diaminobutyric acid in the molar ratio of 0.7 indicates that the

(31) V. du Vigneaud, C. Ressler, and S. Trippett, *J. Biol. Chem.*, **205**, 949 (1953).
(32) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *ibid.*, **75**, 4879 (1953).
(33) H. C. Lawler, S. P. Taylor, A. M. Swan, and V. du Vigneaud, *Proc. Soc. Exptl. Biol. Med.*, **87**, 550 (1954).
(34) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **79**, 4511 (1957).
(35) W. B. Lutz, C. Ressler, D. E. Nettleton, Jr., and V. du Vigneaud, *ibid.*, **81**, 167 (1959).
(36) I. M. Lockhart and E. P. Abraham, *Biochem. J.*, **58**, 633 (1954).
(37) (a) W. Hausmann, J. R. Weisiger, and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 723 (1955); (b) *ibid.*, **77**, 721 (1955).
(38) (a) D. L. Swallow and E. P. Abraham, *Biochem. J.*, **72**, 326 (1959); (b) Structure XXII corresponds to Formula VI of ref 38a; structure XXV corresponds to their formula VII.
(39) E. P. Abraham, "Biochemistry of Some Peptide and Steroid Antibiotics," John Wiley and Sons, Inc., New York, N. Y., 1957, p 13.



single amide-bearing residue in bacitracin is present as an asparaginyl residue. Of the four presented structures this would exclude all except XXII. The *endo*-isoasparaginyl residue of XXIII would be expected to be recovered as β-alanine or aspartic acid or as a mixture of them.

The hydrolysate of the treated bacitracin contained very little glutamic acid and aspartic acid but over 2 moles of newly formed acidic material calculated as cysteic acid. The latter chemical and chromatographic behavior is consistent with that of the model aspartic acid derivatives having a free carboxyl group when the free carboxyl groups appeared to react with ethylene chlorophosphite to form derivatives that were not appreciably converted in acid to aspartic acid. The virtual disappearance of aspartic acid and glutamic acid from bacitracin and the formation of 2,4-diaminobutyric acid thus support structure XXII having one residue of aspartic acid as an *endo*-asparaginyl residue with the other residue of aspartic acid and the residue of glutamic acid each bearing a free carboxyl group. In contrast, after such treatment structures XXIV and XXV would be expected to have in hydrolysates a mole of aspartic acid derived from the protected L-aspartic acid residue.

Since the β-carboxyl group of the D-aspartic acid residue is thought to be free,[38a] the amide group would be located on the L-aspartic acid residue established[39] as being adjacent to the lysine residue. This would leave the L-aspartic acid residue to be linked as an L-asparaginyl residue through the α-carboxyl group to the ε-amino group of lysine. Barring rearrangement in the dehydration–reduction reaction affecting the position of the amide group, of the structures suggested by the studies of Abraham and Craig and their co-workers, structure XXII would seem the preferred one for bacitracin.[40] Its cyclopeptide moiety would be composed of seven rather than six amino acid residues with all the acylpeptide linkages within it of the classical peptide type except for the α→ε L-asparaginyllysine linkage.

Table VI. Identification of Asparaginyl Residue in Commercial Bacitracin. Chromatographic Ninhydrin Analyses[a]

Peptide	Theoretical, untreated[b]	Untreated[c] (1)	Dehydrated, reduced[d,e] (2)
Bacitracin wt, mg		3.81	2.26
Recovery, %[f]		84	33[g]
Constituent[b]	**Molar ratios**		
Leucine	1	1.00	1.00
Isoleucine	3	2.69[h]	2.98[h]
Phenylalanine	1	0.92	0.61
Glutamic acid	1	1.05	0.04
Aspartic acid	2	2.00	0.13
Half-cystine	1	0.48[i]	0
Histidine	1	1.01	0.11
Lysine	1	1.16	0.88[j]
Ornithine	1	1.09	0.97[j]
Ammonia	1	1.56	...
2,4-Diaminobutyric acid		0	0.68[j]

[a] Chromatographic systems A and B. [b] Data of ref 37b for bacitracin A. [c] Also present: 0.17 mole of valine, which suggests the presence of bacitracin B (ref 39), and 0.11 mole of unidentified material eluted in system B 176 ml after NH_3. [d] Dehydrated with 100 μl of ECP in 300 μl of TEP; reduced in presence of 50 μl of methanol. [e] Also present: valine and unidentified material eluted preceding aspartic acid and proline in the molar ratios 0.15, 0.07, and 0.10, and in the acidic region at 55 ml, 2.31, calculated as cysteic acid. [f] Based on calculation of starting material as free bacitracin A, mol wt 1422. [g] Based on leucine, lysine, and ornithine recoveries. A run desalted before hydrolysis resulted in 48% recovery. [h] Composite of isoleucine and alloisoleucine. [i] Composite of cystine and material eluted in position of mesocystine (0.35 + 0.13). [j] Average analysis of three dehydration–reduction experiments.

Experimental Section[41]

Materials. Tyrocidin·HCl was a commercial preparation obtained from Mann Research Laboratories, Lot No. C1403, potency designated as 114% tyrocidin·HCl standard. It probably is a mixture of tyrocidin A, B, and C (see Table IV, footnotes *a* and *c*). Bacitracin was also a commercial preparation from the same source, Lot No. D3904, U.S.P. grade, potency 65 units/mg. Evolidine was a crystallized sample generously supplied by Dr. H. D. Law.

(40) It has been pointed out that the only data that would be inconsistent with structures such as XXII or XXIII would be the composition of two peptides isolated from partial hydrolysates of bacitracin A and commercial bacitracin, His-Asp-Lys[37a] and (His-Asp-Lys-Orn-Ileu[36]), respectively. Those authors noted that the presence in these peptides of more than one residue of aspartic acid could not be rigorously excluded by the procedures used.[37a,39]

(41) Melting points were taken in capillaries and are corrected. Optical rotations were taken with a Rudolph polarimeter, Model 80. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. Evaporations were carried out under reduced pressure. Electrophoresis was carried out on Whatman No. 1 paper at 9 v/cm for 3 hr in pyridinium acetate buffer of pH 5.7 or sodium barbital buffer of pH 8.5. Unidentified materials on amino acid analysis were calculated as leucine unless specified otherwise. Molar ratios were expressed when possible with the leucine value chosen as integer.

It melted at 276–279° (lit[18] mp 277–279°). Isoglutamine-4-oxytocin and isoasparagine-5-oxytocin were purified synthetic peptides;[34,35] oxytocin was a sample of highly purified natural material isolated from beef pituitary glands;[42] all had been previously prepared in the laboratory of Dr. V. du Vigneaud.

Mann Research Laboratories supplied L-prolylglycine·H_2O and glycylproline, analyzed grade; ethanolamine and DL-alaninol, purified grade. L-Leucinol was prepared by Miss M. L. Snow from L-leucine methyl ester by reduction with lithium aluminum hydride as described for the ethyl ester.[27] Cyclo Chemical Corp., Los Angeles, Calif., supplied carbobenzoxy-L-isoleucyl-L-alanine benzyl ester, carbobenzoxy-L-prolyl-L-leucine, carbobenzoxy-L-prolylglycine, and carbobenzoxy-L-alanyl-L-proline, all analyzed grade. L-Leucyl-L-prolylglycine was a gift from American Cyanamid Co.

Dehydration of Carbobenzoxy-L-Asparagine Methyl Ester (V). A. With Ethylene Chlorophosphite (ECP). Carbobenzoxy-L-asparagine methyl ester[43] (1.12 g, 4 mmoles) was dissolved in 5 ml of dry, redistilled pyridine, and to this ethylene chlorophosphite[44] (2.02 g, 1.42 ml, 16 mmoles) was added. The solution was heated in an oil bath held at 100–102° for 30 min. The solution, which had become turbid and dark orange, was cooled and then diluted with 30 ml of water. After 3 hr in the cold the product, which had deposited in the form of fine white needles, was filtered off, washed thoroughly with cold water, and dried overnight under vacuum over P_2O_5. Additional product was isolated from the mother liquors. The combined crude carbobenzoxy-β-cyano-L-alanine methyl ester weighed 870 mg (83%) and melted at 92–94°. It was recrystallized from ethylene dichloride (Norit)–hexane, mp 93–94° (lit[7] mp 93–94°). The infrared spectrum (KBr) showed a characteristic C≡N absorption band at 4.4 μ.

Use of an equimolar amount of ECP led to a 47% yield of product. Reaction with 1 mole of ECP at 50° for 7 hr yielded material melting near the starting ester V. Reaction with 2 moles of ECP at 100° for 1 hr with triethyl phosphite in place of pyridine gave only recovered V. Dehydration of V (140 mg, 0.5 mmole) with ECP (0.4 ml, 4.5 mmoles) in triethyl phosphite (0.7 ml) at 100° for 3 hr gave 61 mg (47%) of product melting at 89–90°; after recrystallization, at 92–93°. The reaction mixture remained colorless.

B, With Dimethylformamidochloride. A solution of 140 mg of V in 0.3 ml of dry dimethylformamide in a 5-ml 19/38 ⅋ test tube protected by a drying tube was cooled to 5°. Thionyl chloride (50 μl, 0.7 mmole) was then added. After 5 min in the cold, the reaction mixture was stirred magnetically at room temperature for 3 hr. The yellow-brown solution was concentrated under vacuum with the water aspirator and then at 0.2 mm. To the syrup 5 ml of water was added. The oily product crystallized to a solid mass after standing overnight in the cold. The light yellow solid was filtered, washed thoroughly with cold water, and dried over P_2O_5, wt 100 mg (76%), mp 87–89°. The product was recrystallized from ethylene dichloride–hexane, wt 82 mg (63%), mp 92–93°.

C. With Tetraethyl Pyrophosphite (TEPP). A suspension of 140 mg of V in TEPP[13] (1 ml, 10.8 mmoles) was heated at 95–100°. A clear solution resulted after 10 min. Heating was continued for 35 min, when the mixture was cooled and diluted with 5 ml of water. A white solid precipitated after cooling to 0°. After 2 hr this was collected on the filter, washed with water, and dried over P_2O_5, wt 67 mg (51%), mp 91–93°. Recrystallization from ethylene dichloride–hexane raised the melting point to 92–93°.

A solution of 140 mg of V dissolved in 1 ml of dry pyridine was treated with TEPP (0.142 g, 0.55 mmole) at 100° for 3 hr. The solution was then concentrated under high vacuum. Addition of 5 ml of cold water and cooling yielded 91 mg of solid, melting at 148–150°, which suggests no appreciable dehydration.

General Procedure for Identification of Asparagine and Glutamine Residues in *endo* Position in Peptides. Dehydration of Peptide. Ethylene chlorophosphite (ECP, 50 μl) is added to a suspension of the predried peptide (1–6 μmoles) in triethyl phosphite (TEP, 0.1–0.4 ml) in a dry, 5-ml 19/38 ⅋ test tube. The reaction mixture is closed tightly with a glass stopper and heated in an oil bath held at 98–100° for 3–24 hr, depending upon the complexity of the peptide. Peptides containing 7–12 residues are treated for 24 hr. The mixture is then cooled, and absolute ethanol (0.2–0.4 ml) is added to decompose excess reagent. After 15–30 min at room temperature, liquids are removed on a rotary evaporator under high vacuum with gentle warming (50–70°) for 15–45 min. A colorless or slightly yellow, thick syrup generally remains. Alternatively, ethanol can be added after concentration of the reaction mixture.

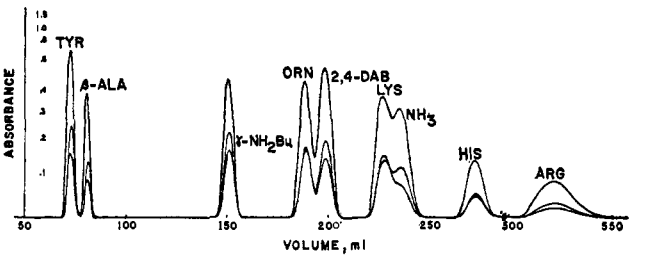


Figure 1. Chromatogram of reference amino acids and ammonia in system B, used for analysis of products formed from *endo*-asparaginyl, -glutaminyl, -isoasparaginyl, and -isoglutaminyl residues in peptides by dehydration–reduction (see Analysis of Residues).

Reduction of Dehydrated Peptide with Sodium–Ammonia–Methanol. The test tube containing the dehydrated peptide is fitted with a Teflon-covered microstirring bar (1/8 × 1/2 in.)[45] and a 19/38 ⅋ gas addition tube, the outlet of which is protected by a drying tube of standard size, loosely filled with Ascarite. Methanol (50–100 μl) is added. The test tube then is partly immersed in a cooling bath of a Dry Ice–Cellosolve mixture in a 250-ml beaker. Ammonia gas is allowed to distil from the tank into the cooled test tube until several milliliters of liquid is collected. The gas addition tube and drying tube are then removed as a unit and replaced by a rubber stopper fitted with a drying tube loosely filled with Ascarite. The test tube is removed from the cooling bath, and the solution is stirred magnetically and allowed to come to the boiling point. A glass capillary (3-mm i.d.) containing sodium is introduced briefly several times, or very small pieces are added, until a blue color appearing throughout the solution persists for at least 0.5 min. Further excesses of sodium are avoided. The ammonia is then allowed to evaporate spontaneously at room temperature. Residual traces of free ammonia and methanol are removed by evacuating the test tube by connecting it through a 19/38 ⅋ Kjeldahl bulb to a water aspirator for 1 hr.

Desalting and Hydrolysis of the Dehydrated, Reduced Peptide. The dry residue is dissolved in 2 ml of cold water, and the solution is adjusted to pH 2 with 4 *N* HCl and applied to a resin column (0.2 × 6.5 cm) of Dowex 50W-X2 (H^+) (200–400 mesh, analytical grade, obtained from Bio-Rad Laboratory). The sample is transferred quantitatively with three 0.2-ml washings of water. The column is washed with water until the eluent is free of chloride. The material is eluted with a three-column volume of 3 *N* NH_4OH, and the eluent is concentrated under vacuum to dryness. The residue is dissolved in 1 ml of constant boiling, glass-redistilled HCl and transferred quantitatively to a glass tube (6-mm i.d.) for hydrolysis. The contents are sealed under nitrogen and the tube heated in an oil bath at 115–120° for 12–18 hr. (Hydrolysis tubes frequently develop some pressure and should be opened with caution.) The hydrolysate is transferred to a small beaker and concentrated to dryness in an evacuated desiccator in the presence of KOH pellets and P_2O_5. The residue is dissolved in 1 ml of water for analysis.

Analysis of Residues. Acidic and neutral amino acids in the hydrolysate are analyzed on the Beckman–Spinco Model 120 amino acid analyzer in system A as recommended for physiological fluids:[14] the 150-cm resin column at pH 3.25 at 30° followed by pH 4.25 at 50°. The change in buffer and temperature is made at 12.7 hr. Basic amino acids are determined preferably in system B, the 50-cm column at pH 4.26 and 50°. Figure 1 shows a typical chromatogram of reference amino acids and ammonia in system B. This is the system recommended for physiological fluids,[14] system B-1, except that operation is at 50° throughout instead of at 30–50°. This modification allows ornithine (elution volume 189 ml) to be separated from 2,4-diaminobutyric acid (199 ml), and tyrosine (75 ml) to be separated from phenylalanine (83 ml). Other elution volumes are: γ-aminobutyric acid, 153 ml; ethanolamine, 219 ml; lysine, 226 ml; NH_3, 236 ml; δ-aminovaleric acid, 260 ml; methylamine, 264 ml; histidine, 273 ml; and 3-methylhistidine,

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295 ml. As β-alanine emerges together with phenylalanine, their concentrations in samples containing both are obtained from the analysis in system A. Alaninol (245 ml, constant 1.7) is determined in system B-1. System C, the 15-cm resin column at pH 5.28 and 50°,[14] is used for leucinol which is eluted as a somewhat skewed peak at 211 ml, constant 8.8.

Yields and Side Reactions in the Dehydration–Reduction Reaction. Average recoveries were calculated from the amino acid analyses of hydrolysates. With polypeptides of mol wt 771 to 1422, recoveries were 64–84% after dehydration and 35–60% after dehydration, reduction, and desalting. Recent developments in desalting peptides by gel filtration might lead to improved yields in the latter step. Results are best expressed as molar ratios of the amino acid residues in the products. Values for ammonia are not significant after reduction.

Those amino acids generally somewhat unstable usually suffered loss. Dehydrated tyrocidin after hydrolysis showed complete loss of tryptophan. Partial loss of tyrosine from tyrocidin occurred in the reduction step and was seen also in the dehydrated–reduced oxytocins. Recoveries of serine from evolidine and cystine and histidine from bacitracin were low. The loss of phenylalanine from bacitracin, as in evolidine, is probably the result of reactions involving reduction of the phenyl ring. Some of the cystine loss from the oxytocins[26c] and phenylalanine loss from tyrocidin may have been associated with reductive fission of the acylproline bonds involving these amino acids in analogy with the loss of a residue of leucine from leucylprolylglycine and dehydrated evolidine. Proline in leucylprolylglycine and in dehydrated tyrocidin showed some loss after reduction. In tyrocidin, the oxytocins, or bacitracin, no selective loss of leucine, which is not bound to proline, of valine, or of isoleucine occurred except for some conversion to alloisoleucine. The presence of 0.24 residue of alloisoleucine in treated natural oxytocin indicates that some racemization can occur in the dehydration–reduction reaction. However, similar treatment of carbobenzoxy-L-isoleucyl-L-alanine benzyl ester resulted in no appreciable formation of alloisoleucine. Lysine completely protected in bacitracin and ornithine having a free ω-amino group in tyrocidin and bacitracin were fully recovered. The stability of the ornithine residue was of interest in view of the almost complete loss of aspartic acid from derivatives in which it bore a free carboxyl group and the known reactivity of diester chlorophosphites with the α-amino and carboxyl groups of amino acid derivatives.[46]

Behavior of C-Terminal Aspartic Acid Derivtives in the Dehydration–Reduction Reaction. (a) Carbobenzoxy-L-asparagine,[4,43] (b) carbobenzoxy-L-aspartic-α-benzyl ester,[47] and (c) carbobenzoxy-L-aspartic-β-benzyl ester[44] were treated with ECP, reduced, and hydrolyzed as described in the general procedure for analysis of peptide ω-amide (5–7 μmoles in 200 μl of TEP with 50 μl of ECP at 100° for 24 hr; 100 μl of methanol for reduction). In each case amino acid analysis showed less than 5% aspartic acid with 82–93% of the recovered material in a series of unidentified peaks eluted in system A at the column breakthrough volume or closely after. The number of peaks, their elution volumes, position of the main peak, and total recovery were: (a) five peaks, 53–86 ml, 69 ml, 61%; (b) six peaks, 51–86 ml, 56 ml, 93%; (c) four peaks, 53–76 ml, 70 and 76 ml, 31%. The yield of 2,4-diaminobutyric acid in (a) was 4.1%. O-Phosphoserine and O-phosphoethanolamine are eluted at 55 and 70 ml, respectively.

Carbobenzoxy-L-prolyl-L-leucylglycine under the same conditions yielded Pro,Leu,Gly,$X_{45-59\ ml}$ in the molar ratios 1.08:1.00:0.17:0.25. Reduction and hydrolysis gave Pro,Leu,Gly in molar ratio of 1.

Carbobenzoxy-β-cyano-L-alanylglycine Benzyl Ester. Glycine benzyl ester *p*-tosylate[48] was converted to the hydrochloride, mp 139°, in 94% yield by passage of an aqueous solution of 16 g through a column of Amberlite IR-45 (Cl^-) resin (21 × 8 in.) followed by recrystallization from methanol–ether. A suspension of 2.61 g (0.013 mole) of glycine benzyl ester hydrochloride, in 20 ml of distilled peroxide-free tetrahydrofuran (THF) containing 4.0 ml (0.029 mole) of triethylamine, was stirred for 2.5 hr. The precipitate was filtered off and washed well with THF, and the filtrate was concentrated to dryness. The residue was taken up in 30 ml of THF and to this was added 3 g (0.012 mole) of carbobenzoxy-β-cyano-L-alanine.[4] As it dissolved, copious precipitation started, presumably of the glycine benzyl ester salt of the latter. The mixture was diluted with 30 ml of THF and was stirred mechanically. A solution of 2.6 g (0.013 mole) of N,N′-dicyclohexylcarbodiimide[49] in 6 ml of THF was added dropwise over 15 min at room temperature, during which period the character of the precipitate changed to a fine, crystalline solid. The mixture was stirred in the cold for 2 days. The precipitate was then filtered off and washed with THF, and the light yellow filtrate was concentrated. The oily residue was taken up in 40 ml of ethyl acetate, which was extracted twice with 4% $NaHCO_3$, water, twice with 0.1 *N* acetic acid and water, and then dried over $MgSO_4$ in the cold. After removal of the solvent, the residue was taken up in 50 ml of acetonitrile which was also removed. The residue was dissolved in 5 ml of acetonitrile and allowed to stand at room temperature overnight. A small amount of solid was removed by filtration and washed. The filtrate (14 ml) was diluted with 15 ml of ether and 12 ml of hexane (bp 65–69°). After standing at room temperature overnight the crystalline product was collected and washed with small portions of ether, wt 1.8 g, mp 79.5–81.5°. The mother liquor was concentrated and the residue solidified by trituration with ether, 1.4 g, mp 64–72°. Recrystallizing the first crop from acetonitrile–ether–hexane at room temperature, then cooling, yielded 1.6 g of soft needles melting at 81–82.5°. The second crop after two recrystallizations yielded 0.66 g melting at 80–82.5° (48.5%). A sample recrystallized for analysis melted at 81–82°, $[\alpha]^{23}D$ −19.4° (*c* 2, 95% ethanol).

Anal. Calcd for $C_{21}H_{21}N_3O_5$ (395.4): C, 63.8; H, 5.35; N, 10.6. Found: C, 64.1; H, 5.26; N, 11.2.

The amino acid composition and the results obtained on reduction with sodium–ammonia–methanol are given in Table I.

Carbobenzoxy-L-asparaginylglycine Benzyl Ester (VI). A solution in 45 ml of THF of carbobenzoxy-L-asparagine *p*-nitrophenyl ester[50] (2.32 g, 0.006 mole), and glycine benzyl ester from 1.31 g (0.0065 mole) of the hydrochloride was stirred at room temperature for 45 min and then in the cold for 2 days. The precipitate was collected on the filter, wt 1.83 g, mp 185.5–188°. A second crop of 0.48 g melted at 184–187°. The product was suspended and washed several times with cold ethanol, mp 186–188° (lit[51] 181–183°). A small sample was recrystallized from water with no change in melting point. Hydrogenolysis of 12 mg in 15 ml of methanol with palladium black, followed by paper electrophoresis at pH 5.7 and 8.5, showed a single ninhydrin-positive material.

Carbobenzoxy-β-cyano-β-L-alanine (VIIIa). To a solution of 5.0 g (0.019 mole) of carbobenzoxy-L-isoasparagine[52a] in 25 ml of dry pyridine maintained at 16–18° was added with magnetic stirring a solution of 4.07 g (0.02 mole) of N,N′-dicyclohexylcarbodiimide in 13 ml of pyridine, in portions over 20 min. After 3 hr, the mixture was cooled to 0°, and the dicyclohexylurea was filtered off and washed on the filter with a little pyridine. The clear, colorless filtrate and washings were concentrated to a small volume under high vacuum at a bath temperature of 25–30°. The residue was diluted with 15 ml of water and kept in the cold for 1 hr. The aqueous mixture was filtered and the filtrate was cooled and acidified to pH 2 with cold 6 *N* HCl. The thick, oily product which separated was extracted four times with 50-ml portions of ethyl acetate. The combined extracts were washed once with 50 ml of water and then dried over anhydrous magnesium sulfate and concentrated under vacuum to dryness. The syrupy residue was treated with 10 ml of dry benzene, and the solvent was removed under vacuum. The crystalline residue was triturated with petroleum ether (bp 30–60°) and collected by filtration, wt 4.74 g, mp 65–80°. The crude solid product was then extracted three times with 50-ml portions of anhydrous ether. To the combined ethereal extracts a little petroleum ether was cautiously added. Some amorphous solid melting at 140–155° separated and was filtered off after 1 hr in the cold. Additional petroleum ether was added slowly to the filtrate along the wall of the flask. The oily product which separated crystallized in the cold. Additional petroleum ether (total volume 230–240 ml) was then added. The white, crystalline product was

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collected on the filter. A small additional crop was obtained from the mother liquors, total wt 2.7 g (57.9%), mp 84–86°.

A sample melting at 82–86° was recrystallized three times from ethyl acetate–ether–petroleum ether (1:1:5). The soft, white needles melted at 87.5–89°, $[\alpha]^{28.5}D$ −42.4° (*c* 5, dimethylformamide).

Anal. Calcd for $C_{12}H_{12}N_2O_4$ (248.23): C, 58.1; H, 4.87; N, 11.3. Found: C, 58.0; H, 4.77; N, 11.1.

A small sample was treated for 30 min with an anhydrous solution of ethylene dichloride containing 20 equiv of hydrogen bromide. The reaction mixture was then diluted with anhydrous ether. The white solid which separated was collected and crystallized from methanol–ether. Paper electrophoresis in barbital buffer at pH 8.6 and amino acid analysis both indicated isoasparagine in good yield.[12b] Its composition and reaction with sodium–ammonia–methanol are given in Table I.

Acknowledgment. We thank Miss Audrey L. Hughes, Mrs. Harriet R. Levie, and Mr. Arnold Benjamin for amino acid analyses and valuable assistance.

The Synthesis of Glycyl-L-prolylglycyl and Glycyl-L-prolyl-L-alanyl Oligopeptides and Sequential Polypeptides[1]

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Abstract: The syntheses of the sequential oligomers, H(-Gly-L-Pro-Gly-)$_n$OH and H(-Gly-L-Pro-L-Ala-)$_n$OH (n = 1, 2, and 4) are reported. Poly(glycyl-L-prolylglycine) and poly(glycyl-L-prolyl-L-alanine) were prepared by the polymerization of the *p*-nitrophenyl esters of the appropriate tripeptides. Weight-average molecular weights of up to 6000 for poly(glycyl-L-prolylglycine) and 15,000 for poly(glycyl-L-prolyl-L-alanine) were obtained.

Collagen, the most abundant structural protein, has recently been shown to have a unique triple helical structure and a gross amino acid composition strikingly different from other proteins. The triple helical structure was originally postulated by Ramachandran[3] on the basis of X-ray diffraction analysis in 1954 and subsequently modified by Rich and Crick[4] and by him.[5] Chemical analyses of several collagens reveal that approximately a third of the amino acid residues are glycine and that about one-fourth of the remaining amino acid residues are L-proline and hydroxyl-L-proline.[6] The isolation of comparatively large amounts of glycyl-L-proline[7] and the later isolation of glycyl-L-prolylhydroxy-L-proline[8] suggested a specific role for glycine and the pyrrolidine-containing amino acids. When Grassman and co-workers[9] reported data which strongly supported the idea that every third residue in collagen is a glycine, several investigators concluded that the "collagen fold" was caused by the repeating unit, glycyl-L-prolyl-X. The unusual optical rotatory behavior of poly-L-proline[10] and of the random copolymers of sarcosine and L-proline[11] suggested the study of ordered peptides containing the (glycyl-L-prolyl-X) unit. Kitaoka, Sakakibara, and Tani were first to attempt a synthesis of an ordered polymer containing the unit, L-prolyl-X-glycyl. L-Prolyl-L-leucylglycine was polymerized employing tetraethyl pyrophosphite, but the isolated polymer was not amenable to study in aqueous media.[12]

In this paper we shall describe the stepwise synthesis and physical properties of two dodecamers, glycyl-L-prolylglycylglycyl-L-prolylglycylglycyl-L-prolylglycylglycyl-L-prolylglycine and glycyl-L-prolyl-L-alanylglycyl-L-prolyl-L-alanylglycyl-L-prolyl-L-alanylglycyl-L-prolyl-L-alanine and the synthesis and certain physical properties of poly(glycyl-L-prolylglycine) and poly(glycyl-L-prolyl-L-alanine). While our work was in progress Debabov, Andreeva, and co-workers reported their studies of poly(glycyl-L-prolylhydroxyl-L-proline).[13]

The synthesis of the dodecapeptide, H(-Gly-L-Pro-Gly-)$_4$OH is summarized in Chart I.

The synthesis of glycyl-L-prolylglycine has been reported by Davis and Smith.[14] Our synthesis differed. Carbobenzyloxyglycine was converted in high yield to carbobenzyloxyglycine thiophenyl ester with

(1) (a) This is Polypeptides L. For the preceding paper in this series see E. R. Simons and E. R. Blout, *Biochim. Biophys. Acta*, **92**, 197 (1964). (b) We are pleased to acknowledge support of this work in part by the Office of the Surgeon General, Department of the Army.

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