concentration, and buffer concentration similar to those used in the kinetic experiments. Since it was found that less than 3% of the total thiol present was oxidized during the time required for a kinetic run, no corrections for the oxidation of the thiols were applied in the calculation of the rate constants.

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Cleavage of Peptide Proline Bonds by Lithium Aluminum Hydride*

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The effect of lithium aluminum hydride on peptides containing proline has been studied. The major reaction was a reductive cleavage at the acyl proline linkage to give an aldehyde and amino-terminal proline. The method was applied to the following peptides where the yields in the cleavage reactions are given in the parentheses: glycyl-L-proline (20% at 25°), L-leucyl-L-prolylglycine (23% at 0°; 98% at 25°), *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine (100% at 25°), gramicidin S (42% at 25°), and tyrocidine B (30% at 0°). The peptides were treated with lithium aluminum hydride for 1 hour in anhydrous tetrahydrofuran. Under these conditions reduction of amides besides those at proline nitrogen was slight; the overall reactivity of amides in the peptides studied was found to be tertiary amide ≫ primary amide > secondary amide.

Lithium aluminum hydride reduction of primary and secondary amides usually requires vigorous reaction conditions to yield the corresponding amines. However, similar reduction of tertiary amides proceeds with great ease; as a result of reductive cleavage, aldehydes or alcohols and secondary amines are often the products (Micovic and Mihailovic, 1953; Mousseron *et al.*, 1952; Mosettig, 1954; Brown and Tsukamoto, 1961). Knowledge of these reactivity differences suggests the use of $LiAlH_4$ treatment as a possible procedure for the specific cleavage of acyl proline bonds, as these bonds are the only tertiary amide linkages in peptides. Therefore reduction studies were carried out with five proline-containing peptides, and in each case the expected specific cleavage occurred. Several side reactions also took place, but they could be controlled by the proper choice of reaction conditions. These are the findings to be reported in this paper.

Materials and Methods

Materials.—Tetrahydrofuran was refluxed for 2 days over a sodium-potassium amalgam and then distilled in an atmosphere of nitrogen. It was stored under nitrogen in an amber glass bottle. All other solvents used were redistilled. $LiAlH_4$ (Metal Hydrides, Inc., Beverly, Mass.) was suspended in tetrahydrofuran and the mixture was refluxed for 6 hours, cooled, then filtered through a sintered glass disk. The solution was stored under nitrogen in an automatic buret with an amber glass reservoir. It was assayed from time to time by measuring the amount of hydrogen evolved when water was added to an aliquot. The concentration of $LiAlH_4$ remained constant at 2.3 M for several months.

Glycyl-L-proline was purchased from Nutritional Biochemicals, Inc. L-Leucyl-L-prolylglycine and *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine were generous gifts from Dr. Charlotte Ressler of the Institute for Muscle Disease. Crude gramicidin S (Sharp and Dohme) was purified by countercurrent distribution in the system chloroform–methanol–0.1 N HCl (2:2:1) (Craig *et al.*, 1950). Tyrocidine B was obtained from crude tyrocidine (Wallerstein No. ON 13554) by countercurrent distribution as described by King and Craig (1955).

All the glassware used for the reactions was heated to 110° in an oven for 24 hours and then allowed to cool in a dessicator.

$LiAlH_4$ *Reduction.*—In a typical experiment, a weighed amount of peptide, which had been dried in a vacuum dessicator for 4 days at room temperature over P_2O_5, was placed in a three-necked flask fitted with a reflux condenser, a dropping funnel, and a nitrogen inlet. A magnetic stirring bar was introduced and an appropriate amount of tetrahydrofuran was added (usually 1 ml per mg of peptide). The $LiAlH_4$ solution was then added dropwise and the mixture was stirred for the duration of the reaction. To stop the reaction, the mixture was cooled in an ice

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TABLE I
SUMMARY OF RESULTS OBTAINED WITH $LiAlH_4$ REDUCTION OF FIVE PEPTIDES CONTAINING PROLINE

Peptide	Temperature	Reaction Time (hours)	Molar Excess Amount of $LiAlH_4$	Cleavage at Acyl-Proline Linkage (%)	Side Reactions
Tyrocidine B	0°	1	10	30[a]	Reduction of phenylalanyl-proline linkage to tertiary amine and reduction of asparagine and glutamine to primary amines amounting in total to 20%
	65°	8	50	96[b]	4% reduction of phenylalanyl-proline linkage to tertiary amine; 71% reduction of asparagine to α,γ-diaminobutyric acid; 82% reduction of glutamine to ornithine
Gramicidin S	25°	1	100	42[a]	20% reduction of phenylalanyl-proline linkage to tertiary amine
Gly-L-Pro	25°	1	10	20[b]	10% reduction of C-terminal proline to prolinol
L-Leu-L-Pro-Gly	0°	1	40	23[b]	None
	25°	1	40	98[b]	Complete reduction of C-terminal glycine to ethanolamine
S-Benzyl-L-Cys-L-Pro-L-Leu-Gly	25°	1	10	100[b]	Complete reduction of C-terminal glycine to ethanolamine

[a] Calculated on the basis of isolation. [b] Calculated on the basis of amino acid analysis.

bath and 6 N HCl was added dropwise until the evolution of hydrogen ceased and all of the inorganic precipitate was dissolved; this required approximately 0.2 ml of 6 N HCl per mmole of $LiAlH_4$. The material was then brought to dryness on a rotary evaporator.

Reactions of the Terminal Aldehyde Group Formed upon Reduction.—(1) Reduction to an Alcohol with $NaBH_4$.—Following evaporation of the tetrahydrofuran, the dry material, consisting of salts of lithium and aluminum together with the reduced peptide, was washed with four portions of methanol and filtered. For those peptides studied it was found that most of the reduced peptide could be extracted in this manner by the methanol. The filtrate was added to a 10-fold molar excess of $NaBH_4$ and the reduction was allowed to proceed for 3 hours at room temperature. At the end of this time the excess $NaBH_4$ was neutralized with acetic acid, the solvent was removed in a rotary evaporator, and the residue was dissolved in 50% acetic acid. The solution was desalted on a column of Sephadex G-25, 80–100 mesh (Pharmacia Co., Uppsala, Sweden) equilibrated with 50% acetic acid.

For loads of 2 μmoles of peptide or less the sample was applied in 2 ml of 50% acetic acid to a 150 × 0.9-cm column; for loads of 20–200 μmoles it was dissolved in 2–10 ml of 50% acetic acid and applied to a 200 × 2.5-cm column. For larger loads of up to 2 g a 210 × 4-cm column was employed which could accommodate a sample volume of up to 100 ml.

Samples of the desalted peptide were taken for amino acid analysis and Edman degradation. When enough material was present the peptide was further purified by countercurrent distribution.

(2) Catalytic Hydrogenation to an Alcohol.—The dry material from the $LiAlH_4$ reduction was dissolved in 50% acetic acid and reduced with hydrogen over PtO_2 for 3 hours at room temperature and atmospheric pressure. Although the use of a trace of ferrous ion has been shown to facilitate the reduction of aldehydes in the presence of PtO_2 (Carothers and Adams, 1924), we found that better yields were obtained using a relatively large amount of PtO_2 (10% by weight of the original peptide) alone. Following the reduction, the catalyst was removed by filtration and the filtrate was applied directly to an appropriate column of Sephadex G-25 (see procedure for desalting of $NaBH_4$ reduction product).

(3) Performic Acid Oxidation to a Carboxylic Acid.—The method used for performic acid oxidation was that described by Schram *et al.* (1954).

Edman Degradation.—The procedure for the Edman degradation was that described by Konigsberg and Hill (1962) with the exception that the coupling reaction with phenylisothiocyanate was performed in 90% pyridine instead of *N*-ethylmorpholine–acetic acid buffer.

Acid Hydrolysis and Amino Acid Analysis.—The aliquots for acid hydrolysis were transferred to 15 × 150–mm Pyrex test tubes. Constant-boiling HCl was added until the concentration of peptide was approximately 2 mg/ml. The tubes were repeatedly evacuated and flushed with nitrogen, then sealed under vacuum. Hydrolysis was carried out at 110° for 24 hours. Under these conditions recovery of the tryptophan present in tyrocidine B was nearly quantitative. Amino acid analyses were performed on a Spinco Model 120 automatic amino acid analyzer as described by Spackman *et al.* (1958), using loads of approximately 0.3 μmole of each constituent amino acid.

Phenylalaninol was determined by chromatography on a column (0.9 × 20 cm) of phosphocellulose (Whatman, Ltd.) at room temperature with a 0.2 M sodium citrate buffer, *p*H 3.25 (same buffer composition as that used for amino acid analysis). For quantitative estimation, the column effluent was channeled directly into the Spinco amino acid analyzer. Authentic phenylalaninol, prepared according to Karrer *et al.* (1948), emerged at 57 minutes, with a color constant one-sixth that of leucine.

Ethanolamine was determined by chromatography on the 15-cm column of the Spinco amino acid analyzer. It emerged at 130 minutes. The color value was one-tenth that of leucine.

RESULTS

The effect of $LiAlH_4$ on five proline-containing peptides was studied. These were tyrocidine B (Fig. 1), gramicidin S (Fig. 2), glycyl-L-proline, L-leucyl-L-prolylglycine, and *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine. A summary of the results is given in Table I.

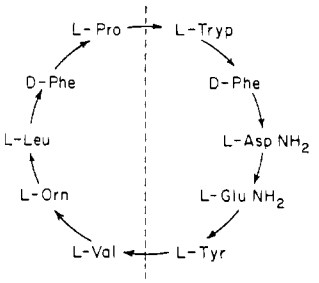


FIG. 1.—Amino acid sequence of tyrocidine B (King and Craig, 1955). The dotted line indicates the pentapeptide which is also present in gramicidin S.

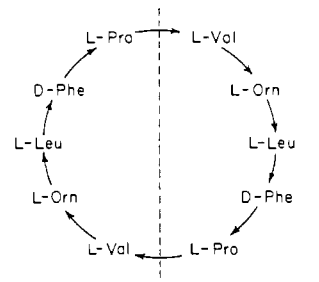


FIG. 2.—Amino acid sequence of gramicidin S (Consden *et al.*, 1947; Battersby and Craig, 1951).

Tyrocidine B.—Tyrocidine B (100 mg) was reduced with a 10-fold molar excess of $LiAlH_4$ for 1 hour at 0°. The product was subsequently treated with $NaBH_4$ and desalted on Sephadex G-25. The desalted peptide was then subjected to countercurrent distribution in the system *sec*-butanol–3% acetic acid (1:1). Analysis after 1245 transfers gave the pattern in Figure 3. Peak 1 contained the cleaved tyrocidine B in 30% yield. Peak 2 contained material with a low proline content in which, presumably, the phenylalanyl-proline bond had been reduced to the tertiary amine, and in addition contained small amounts of the reduction products of asparagine and glutamine. This material has not been characterized. Peak 3 was intact tyrocidine B which was 50% of the starting material.

Peak 1 was identified as the cleaved tyrocidine B, a linear peptide, from the following observations. By Edman degradation it was found to have an amino-terminal residue of proline. Acid hydrolysis of the material liberated 1 mole of phenylalanine and 1 mole of phenylalaninol. When $LiAlH_4$-treated tyrocidine B was hydrolyzed in acid without the additional reduction by $NaBH_4$, no phenylalaninol was obtained but, instead, an additional mole of ammonia was observed. These observations are consistent only with the interpretation that the phenylalanyl-proline linkage in tyrocidine B had been cleaved by $LiAlH_4$ and that the phenylalanine residue had been converted to 2-amino-3-phenylpropionaldehyde which was deaminated on acid hydrolysis. Treatment of the cleaved peptide with $NaBH_4$ converted the aldehyde to a residue of phenylalaninol. These reactions are illustrated in the structures; the deamination products of 2-amino-3-phenylpropionaldehyde were isolated in an analogous case to be described under gramicidin S. The analytical data for reduced tyrocidine B are given in Table II.

Gramicidin S.—Gramicidin S (200 mg) (Fig. 2) was reduced with a 100-fold excess of $LiAlH_4$ and the crude product was treated with hydrogen in the presence of platinum oxide. Chromatography on Sephadex G-25 gave the pattern in Figure 4. Peak 1 contained decapeptides consisting of incompletely cleaved gramicidin S together with material which had undergone complete reduction at proline to tertiary amine. Peak 2 was the pentapeptide resulting from cleavage of the two phenylalanyl-proline linkages. This pentapeptide with phenylalaninol as the C-terminal residue was obtained in 42% yield after chromatography of the material from peak 2 on phosphocellulose (Fig. 5). The presence of amino-terminal proline was demonstrated by Edman degradation.

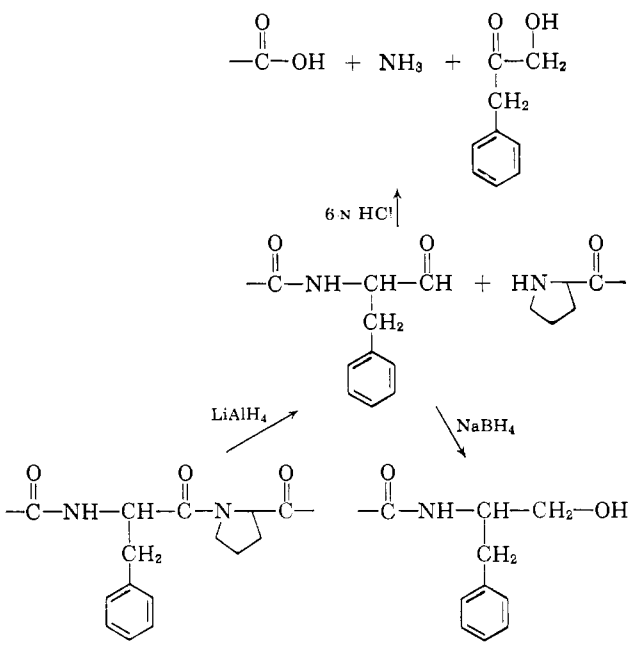
With gramicidin S, no difference in yield or in the nature of the product was observed when the reaction

TABLE II

AMINO ACID ANALYSIS OF PRODUCTS FROM REDUCTION OF TYROCIDINE B

	Number of Residues per Mole of Peptide		
	Untreated Peptide	Peptide Treated for 8 Hours at 65° with 50-fold Excess of $LiAlH_4$	Peak 1 of Fig. 5 Obtained from Reduction with 10-fold Excess of $LiAlH_4$ for 1 Hour at 0° and Subsequent Treatment with $NaBH_4$
Valine	1.02	1.03	1.02
Ornithine	1.00	1.82	1.04
Leucine	1.00	1.00	1.00
Phenylalanine	2.00	0.98	1.09
Proline	1.02	0.96	1.01
Tryptophan[a]	1.00	0.91	1.00
Aspartic acid	1.00	0.22	0.99
Glutamic acid	1.00	0.14	1.01
Tyrosine	0.98	1.01	1.03
Ammonia	2.03	1.38[b]	2.04
α,γ-Diaminobutyric acid	0.00	0.71	0.00
Phenylalaninol	0.00	0.00	0.92

[a] Estimated spectrophotometrically from known molar extinction for tyrocidine B in 50% acetic acid at 280 mμ of 7.4×10^3. [b] This ammonia is derived from the unreduced asparagine and glutamine and from the deamination of 2-amino-3-phenylpropionaldehyde formed by the cleavage at the phenylalanyl-proline linkage.



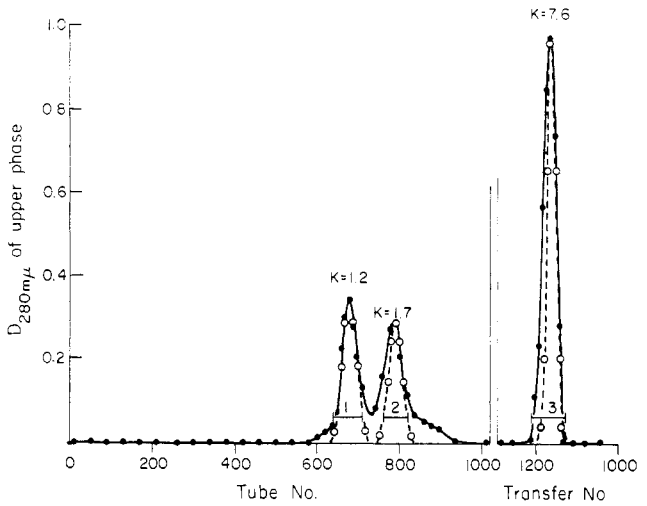


FIG. 3.—Countercurrent distribution of tyrocidine B reduced with $LiAlH_4$ and subsequently treated with $NaBH_4$. Analysis after 1245 transfers in the system *sec*-butanol–3% acetic acid (1:1) using 3 ml of each phase in a 1000-tube automatic apparatus. ●—●, experimental points; O---O, theoretical Gaussian distribution.

TABLE III

AMINO ACID ANALYSIS OF PRODUCTS FROM REDUCTION OF GRAMICIDIN S

	Number of Residues per Mole of Peptide			
	Untreated Peptide	Crude Reduced Peptide before Hydrogenation	Peak 1 (Fig. 4)	Peak 2 (Fig. 4) Following Chromatography on Phosphocellulose (Fig. 5)
Proline	2.00	1.62	1.18	1.00
Valine	2.02	2.02	2.04	1.02
Ornithine	2.04	2.08	2.04	0.98
Leucine	2.00	2.00	2.00	1.00
Phenylalanine	2.00	0.34	0.28	0.00
Phenylalaninol	0.00	0.00	0.86	0.97
Ammonia	0.00	1.20	0.00	0.00

was run for longer than 1 hour either at 65° or at room temperature. The analytical data are summarized in Table III.

In order to identify the deamination product of 2-amino-3-phenylpropionaldehyde, the hydrolysate of 120 mg of cleaved gramicidin S was extracted with ether and the compound obtained was purified by a micro distillation (bath temperature 96° at 2 mm). An infrared spectrum of the distillate compared with synthetic standards indicated a mixture of 3-hydroxy-1-phenyl-2-propanone and cinnamaldehyde. Reaction of the distillate with 2,4-dinitrophenylhydrazine gave 63 mg of orange needles, mp 245–249° (decomp). The melting point for the osazone of 3-hydroxy-1-phenyl-2-propanone according to Baltazzi and Robinson (1953) is 245–250° (decomp).

Anal. Calcd. for $C_{21}H_{16}N_8O_5$: C, 49.61; H, 3.17; N, 22.05. Found: C, 49.68; H, 3.17; N, 21.80.

The compound 3-hydroxy-1-phenyl-2-propanone was synthesized according to the method of Pfeil and Barth (1955). Reaction of this compound with 2,4-dinitrophenylhydrazine gave a compound which was identical by mixed melting point to the above osazone.

The most probable interpretation of these results is

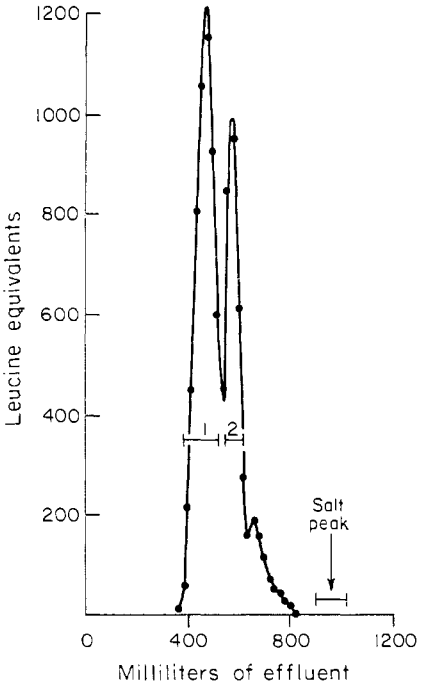


FIG. 4.—Separation on a 2.5 × 200-cm column of Sephadex G-25 with 50% acetic acid of gramicidin S reduced with $LiAlH_4$ and subsequently treated with hydrogen over PtO_2. Fractions (20 ml) were collected at 25° at a flow rate of 25 ml/hr.

that deamination proceeds through tautomerization of 2-amino-3-phenylpropionaldehyde to a ketimine which is then hydrolyzed to give 3-hydroxy-1-phenyl-2-propanone as the major product. This subsequently undergoes a certain amount of dehydration to form cinnamaldehyde. The tautomerization is analogous to that of 2-hydroxy-3-phenylpropionaldehyde observed by Danilow and Venus-Danilowa (1930).

Other Peptides.—Treatment of three other peptides with $LiAlH_4$ also led to the cleavage of the acyl-proline linkage. In these cases the products were not isolated and the cleavage reaction was monitored by measuring the loss of that amino acid preceding proline and the concomitant appearance of ammonia. The results are given in Tables IV, V, and VI.

With the exception of the dipeptide gly-pro, the reduction of the peptides resulted in practically quantitative cleavage. The accompanying reduction of terminal carboxyl group occurred also to a lesser extent with gly-pro than with the other two peptides. The low yields obtained with gly-pro as contrasted with those of the larger peptides might be caused by structural features of a dipeptide. Nevertheless, the results substantiate the view that the cleavage can be specific for the acyl-prolyl linkage.

DISCUSSION

Reduction of peptides with complex metal hydrides has been investigated by several authors (for a review, cf. Gaylord, 1956). Sodium borohydride ($NaBH_4$), the mildest of the reagents which have been extensively studied, has been used by Crestfield *et al.* (1963) to effect reduction of disulfide bonds. In addition to the desired reaction, these authors have observed some peptide bond cleavage in ribonuclease. They observed no peptide bond cleavage in the B chain of insulin. Lithium borohydride ($LiBH_4$), a reagent of reducing power intermediate between $NaBH_4$ and $LiAlH_4$, has been used by Chibnall and Rees (1954) as a method for

TABLE IV

AMINO ACID ANALYSIS OF PRODUCTS FROM REDUCTION OF GLYCYL-L-PROLINE WITH $LiAlH_4$[a]

	Number of Residues per Mole of Peptide[b]		
	Untreated Peptide	Reduced Peptide, before Acid Hydrolysis	Reduced Peptide, after Acid Hydrolysis
Proline	1.00	0.10	0.92
Glycine	1.00	0.00	0.78
Aminoacetaldehyde[c]	0.00	0.20	0.00
Prolinol[d]	0.00	0.11	0.10
Ammonia	0.03	0.03	0.24
Proline and prolinol	1.00	0.21	1.02

[a] Glycyl-L-proline was treated with a 10-fold excess of $LiAlH_4$ for 1 hour at 25°. [b] The yield in the cleavage reaction was 20% based upon the amount of aminoacetaldehyde observed before acid hydrolysis and the corresponding increase in ammonia and decrease in glycine values observed after acid hydrolysis. [c] Chromatography of the reduced peptide on a 15-cm column of Spinco type 15 A resin in 0.2 M citrate buffer at *p*H 5.28 on a Spinco Model 120 amino acid analyzer gave a peak at 74 minutes. This peak was not present in the hydrolysate of the reduced peptide but was replaced by an equivalent amount of ammonia. The sum of the residues indicates that this peak must have been aminoacetaldehyde which was deaminated under the conditions of acid hydrolysis to yield ammonia and glycolaldehyde. The conversion of aminoacetaldehyde to glycolaldehyde by acid hydrolysis was confirmed by the specific test for glycolaldehyde of Dische and Borenfreund (1949). The value for aminoacetaldehyde given in this analysis was calculated using the color value of glycine. [d] Calculated using the color value of proline. This peak emerged at 164 minutes from the 15-cm column of the amino acid analyzer.

TABLE V

AMINO ACID ANALYSIS OF PRODUCTS FROM REDUCTION OF L-LEUCYL-L-PROLYLGLYCINE WITH $LiAlH_4$

	Number of Residues per Mole of Peptide[a]			
	Untreated Peptide	Peptide Reduced for 1 hour at 0° with 40-fold Excess of $LiAlH_4$	Peptide Reduced for 1 hour at 25° with 40-fold Excess of $LiAlH_4$	Peptide Reduced at 25° then Oxidized with Performic Acid
Proline	1.02	1.00	1.00	0.88[b]
Glycine	1.00	1.00	0.00	0.02
Ethanolamine	0.00	0.00	0.95	0.91
Leucine	1.00	0.77	0.02	0.51
Ammonia	0.02	0.25	1.06	0.54
Leucine and ammonia	1.03	1.02	1.08	1.05

[a] Yield in cleavage reaction at 0° was 23% based on the decrease in the amount of leucine and corresponding increase in the amount of ammonia. The yield at 25° was 98%. [b] Decrease in proline value may be due to amine oxide formation followed by subsequent decomposition. Performic acid oxidation at 35° resulted in quantitative loss of proline.

the analysis of C-terminal amino acid residues by the reduction of peptide esters. This method has also been used to distinguish between α- and β-linked aspartyl and α- and γ-linked glutamyl residues. Meyer and Jutisz (1957) have reported that $LiBH_4$ causes peptide bond cleavage in dipeptides composed of

TABLE VI

AMINO ACID ANALYSIS OF PRODUCTS FROM REDUCTION OF S-BENZYL-L-CYSTEINYL-L-PROLYL-L-LEUCYLGLYCINE WITH $LiAlH_4$[a]

	Number of Residues per Mole of Peptide[b]		
	Untreated Peptide	Reduced Peptide	Reduced Peptide following Edman Degradation
Proline	1.00	1.00	0.22
Glycine	0.98	0.00	0.00
Leucine	1.00	1.00	1.00
S-Benzylcysteine	1.00	0.00	0.00
Ammonia	0.00	0.97	
Ethanolamine	0.00	0.96	

[a] Reduction was carried out at 25° for 1 hour using a 10-fold excess of $LiAlH_4$. [b] The yield in the cleavage reaction was 100% based upon the disappearance of S-benzylcysteine and corresponding appearance of ammonia. The yield in the Edman degradation was 78%.

glycine and alanine to give amino alcohols. Fromageot and Jutisz (1954) have avoided the necessity of esterification by using the more powerful $LiAlH_4$ to reduce carboxyl groups in peptides to alcohols. $LiAlH_4$ reduction of peptide bonds to secondary amines was reported to occur at elevated temperatures and with prolonged reaction times (Karrer and Nicolaus, 1952; Stoll *et al.*, 1951; Zahn and Heinz, 1962). However, the reductive cleavage of the acyl proline bond in a peptide has not been observed previously.

Several investigators have attempted to develop a specific hydrolytic cleavage for use at acyl proline bonds employing sodium-sodamide in liquid ammonia (Witkop, 1961; Hofmann, 1960). This reaction requires neighboring group assistance and is therefore dependent on the nature of the acyl substituent; attempts to extend the reaction from simple model compounds to peptides have been unsuccessful. In contrast, the cleavage presently described appears to be independent of neighboring group effects. Therefore this new procedure should serve as a useful adjunct to the growing list of specific chemical cleavages of peptide bonds. The practical application of this procedure is handicapped by: (1) the occurrence of side reactions and (2) the limited solubility of peptides in the nonaqueous solvents which must be used with $LiAlH_4$. However, complete solution of the peptide may not be necessary since of the peptides studied, only gramicidin S showed appreciable solubility in tetrahydrofuran.

The side reactions which were encountered in the present study were (a) the reduction of C-terminal carboxyl groups,[1] (b) the reduction of asparaginyl and glutaminyl side chains to primary amines, and (c) possibly the reduction of certain peptide bonds. The side reactions, however, can be minimized through the proper choice of reaction conditions. Racemization, another possible side reaction, was not observed in this study. This is in agreement with the results of Karrer *et al.* (1948) in their study of the reduction of optically active amino acid esters.

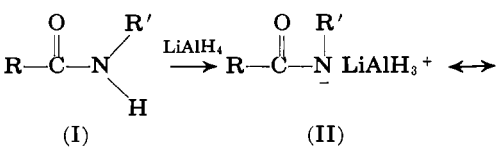
Of the three types of amide bonds in a peptide, we observed that their susceptibility to reduction by $LiAlH_4$ is in the following order: tertiary amide $\gg$ primary amide > secondary amide. These differences

[1] Jolles and Fromageot (1952) and Vogl and Pöhm (1952) have observed a low reactivity of C-terminal carboxyl groups in peptides to $LiAlH_4$.

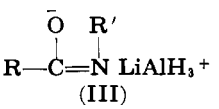
in reactivity are illustrated by the rapid reductive cleavage of the acyl proline bond as compared to the slower reduction of the asparaginyl and glutaminyl side chains and by the apparent resistance toward reduction of secondary peptide bonds.

These observed reactivities are in agreement with the action of $LiAlH_4$ on amides as reported by several investigators (Micovic and Mihailovic, 1953; Mousseron *et al.*, 1952). Newman and Fukunaga (1960) have presented evidence that the fastest reaction occurring between $LiAlH_4$ and a primary amide is the removal of an active hydrogen from the amide nitrogen. They have also demonstrated the presence of a nitrile as an intermediate in the reduction of primary amides to primary amines. These two observations may be used to rationalize our results as follows:

The removal of the active hydrogen from the nitrogen of a primary or secondary amide (I) results in the formation of an anion (II) which is stabilized by resonance (III).[2] It is reasonable to assume that this anion would tend to repel any negatively charged hydride species and would therefore be less susceptible to reduction. Whereas secondary amides would remain in this anionic form for the duration of the $LiAlH_4$ reduction, a primary amide (R′ = H) can undergo dehydration to form a nitrile (IV). The subsequent reduction of the nitrile to an amine is known to be a fast reaction, except in the case of hindered nitriles (Gaylord, 1956).

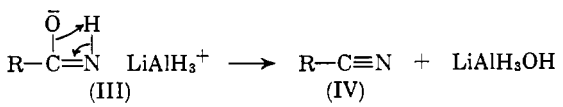


(I) (II)



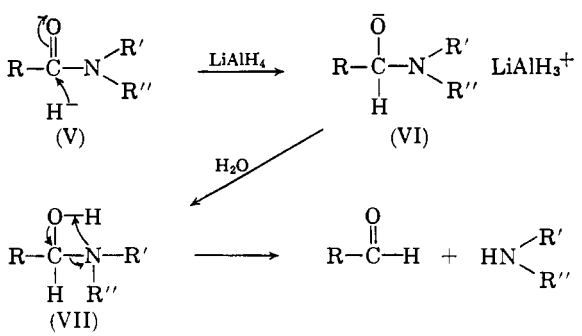
(III)

For R′ = H:



(III) (IV)

The special feature of the tertiary amide linkage (V) is its inability to form an anion with $LiAlH_4$. This fact makes possible an attack by a hydride ion to give the anion (VI) which, as in the case of the anions derived from primary and secondary amides, becomes less susceptible to further reduction. Following hydrolysis of the complex, (VI) would be converted to the amino alcohol (VII); this would subsequently decompose to give an aldehyde and a secondary amine.



(V) (VI) (VII)

Finally, if one proposes that under more drastic conditions the anionic intermediates (III) and (VI) can be further attacked by $LiAlH_4$, then this would explain the ability of secondary and tertiary amides to be reduced to secondary and tertiary amines when elevated temperature and prolonged reaction time are employed.

It is probable that the intermediate anion in all of these reductions is stabilized as a complex with aluminum (Mousseron *et al.*, 1952; Micovic and Mihailovic, 1953); however, there is no compelling evidence at this time to favor any particular structure for such complex, nor is the exact nature of the reducing species known.

[2] Removal of the hydrogen on the α-carbon apparently does not occur to any great extent as indicated by the absence of racemization.

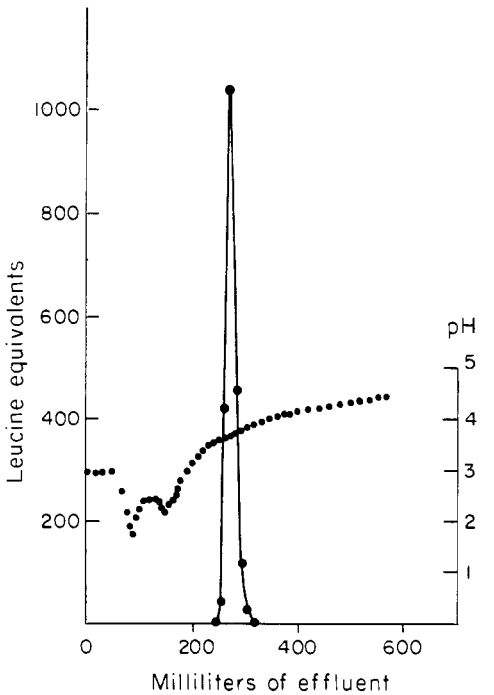


FIG. 5.—Chromatography on a 3 × 13-cm column of phosphocellulose of the pentapeptide obtained from reductive cleavage of both prolines in gramicidin S (Fig. 4, cut 2). The material was eluted using a linear gradient of pyridine formate buffer: buffer 1, 400 ml of 0.1 M pyridine-formate, *p*H 3.0; buffer 2, 400 ml of 2 M pyridine-formate, *p*H 4.5. Fractions (10 ml) were collected at 25° at a flow rate of 30 ml/hr. The change in *p*H of the effluent is indicated on the figure.

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Dialysis Studies. VII. The Behavior of Angiotensin, Oxytocin, Vasopressin, and Some of Their Analogs*

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The technique of thin-film dialysis has been used to study the conformations in aqueous solution of angiotensin II, oxytocin, vasopressin, and some of their analogs. By using membranes of high selectivity it was found that angiotensin II exists in a coiled form of low axial ratio. Oxytocin has a compact conformation with the tripeptide tail held close to the ring, whereas vasopressin has a more extended conformation because of the repulsion between the basic groups in the ring and the tail. Small differences in diffusion rate were detected among some of the analogs studied. The effect of ammonium acetate on some of the peptides was determined.

Now that many of the difficulties encountered in the isolation and precise characterization of a number of the smaller polypeptide hormones have been successfully overcome it seems of some interest to reconsider the particular properties that have contributed to these difficulties and also other properties of primary interest to the separation problem. These include properties which determine stability, adsorbability, diffusibility, partition behavior, solubility, association behavior, and detectability by quantitative biological assay. While much has been learned in the course of the isolation studies by trial and error it is immediately evident that much further information is needed before a precise correlation of properties and structure can be made. The properties of interest mentioned above are probably the same as those which will ultimately prove to be of the greatest interest in understanding the physiological role of the substances.

Thin-film dialysis (Craig and King, 1962) is a technique which can well give information about many of the properties in question. It offers direct information particularly about diffusibility in different solvent environments and at more than one temperature. This information can be interpreted in terms of the association of an individual molecule with others of its own species or association with the solvent molecules. When the chain length of polypeptides is sufficiently great intrachain association or interaction can also take place and a balance of interactions is involved. A study of diffusibility under different conditions can give information (Craig, 1962) about overall changes which take place in the conformation.

The structural formulas for angiotensin (Skeggs *et al.*, 1956; Elliot and Peart, 1956), oxytocin (Du Vigneaud *et al.*, 1953b, 1954), and 8-lysine vasopressin (Du Vigneaud *et al.*, 1953a, 1957) have been determined by degradation and synthesis (Fig. 1). In addition many analogs of these substances are now being synthesized and their physiological actions are being studied in an attempt to determine the features of the structure which are required for the physiological action. In view of these developments, it has appeared a particularly appropriate time to study a series of these peptides by the dialysis technique. Some of our experience gained thus far is reported in the present paper.

Experimental

The type of diffusion cell employed in this study was the type previously reported (Craig and Konigsberg, 1961) with the removable center tube. The cellophane membranes were all prepared from Visking dialysis casing.

The tetrapeptide, the hexapeptide, angiotensin II, angiotensinamide[1] and angiotensin I all were synthetic peptides (Schwyzer, 1958) obtained from Dr. R. Schwyzer of the Ciba Company. The heptapeptide was a synthetic preparation (Shields and Carpenter, 1961) obtained from Dr. F. H. Carpenter of the University of California. It is the peptide which includes residues 23–29 of the B chain in insulin. The bradykinin was a synthetic peptide obtained from Dr. M. Bodanszky of the Squibb Research Institute. Oxytocin, vasopressin and their analogs were all obtained

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[1] Abbreviations used in this work: angiotensinamide, β-aspartylamide of angiotensin II; ACTH, adrenocorticotropic hormone.