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Reductive Cleavage of Acylproline Peptide Bonds*

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ABSTRACT: To determine the utility of selective, reductive cleavage of proline-containing peptides as a degradative method in structural determinations of polypeptides and proteins, the cleavage of acylproline peptide bonds induced by sodium metal–liquid ammonia was studied in peptides and peptide derivatives. Attention was given to finding reaction conditions sufficient to obtain extensive cleavage, to identifying the nature of the new C-terminal groups produced by the reaction, and to determining the nature and degree of side reactions. The maximal extents of cleavage obtained ranged from 56 to 90%. Variation in the lability of various acylproline peptide bonds was suggested by a comparison of the conditions required for the cleavage of different peptides. Thus, glycylproline and the threonylproline peptide bond of insulin B chain were much more readily reduced than *N*-acetylproline, *N*-acetylglycylproline, or the acylproline peptide bonds in performic acid oxidized apoferredoxin. The extents of reduction of acetylproline in the presence and absence of methanol and sodium amide (a strong base) were essentially the same, indicating that the inclusion of proton donors in the reaction mixture is not necessary for cleavage. The reduction of *N*-acetylglycylproline resulted in the conversion of the glycine residue to both aminoacetaldehyde and ethanolamine residues, but these forms accounted for only about two-thirds of the reduced glycine residues. Using amino acid analysis and amino-terminal analysis of sodium–ammonia–reduced ferredoxin and insulin B-chain derivatives, nonspecific cleavage of internal peptide bonds was found to range from 0 to 4% per peptide bond. The cleavage of amino-terminal peptide bonds was more extensive than that of internal, nonproline peptide bonds. The recoveries of amino acids after acid hydrolysis of reduced polypeptides indicated that side reactions in general were minimal for amino acid residues which did not possess a free α-amino group and which were not amino terminal to prolyl residues.

Recent reports have appeared describing chemical methods for the reductive cleavage of polypeptides at acylproline peptide bonds. Various reducing agents have been employed in this reaction including lithium aluminum hydride (Ruttenberg *et al.*, 1964), lithium in methylamine (Patchornik *et al.*, 1964), and sodium in liquid ammonia (Benisek and Cole, 1965; Wilchek *et al.*, 1965; Ressler and Kashelikar, 1966). All of these techniques result in cleavage of acylproline peptide bonds, to various extents.

In this paper we present the results of a study of the reductive cleavage of proline-containing peptides using sodium metal in liquid ammonia as the reducing agent. This study was undertaken to obtain information which could aid in the design of structural studies of polypeptides. Therefore we have examined the reaction with regard to parameters which affect the extent of cleavage of various acylproline peptide bonds as well as the nature and degree of side reactions, including nonselective peptide-bond cleavage. Some experiments designed to determine the nature of the new C-terminal residues produced by the reductive cleavage are also described.

Materials and Methods

Glycyl-L-proline and acetyl-DL-proline were obtained from Mann Research Laboratories, Inc. Car-

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bobenzoxy-glycyl-L-prolyl-L-leucyl-glycyl-L-proline · H_2O · EtOAc was purchased from Sigma Chemical Co. Beef insulin A chain obtained from performic acid oxidized insulin was kindly provided by Dr. S. S. Wang. Beef insulin B chain was prepared from crystalline zinc beef insulin by the method of Craig *et al.* (1961).

N-Acetyl-glycyl-L-proline was synthesized from glycyl-L-proline and acetic anhydride essentially by the peptide acetylation procedure of Hofmann *et al.* (1960). The acidified reaction mixture was passed over a column of the hydrogen form of AG-50W-X8 resin (200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif.) using distilled water as the eluent; the acidic effluent was lyophilized. The very hygroscopic residue was dissolved in water and the resulting solution was stored at 2–4°. Aliquots of this stock solution were tested for solute heterogeneity by electrophoresis at pH 3.5 (pyridine–acetic acid–water, 1:10:289) and paper chromatography, using three solvent systems: pyridine–methanol–water (4:80:20), 1-butanol–acetic acid–water (65:10:25), and ethanol–water (77:23). In every case only one spot was detected; this spot was ninhydrin negative and chlorine–starch–KI (Rydon and Smith, 1952) positive. Acid hydrolysis followed by amino acid analysis showed only glycine and proline (mole ratio of amino acids = 1.01).

Performic acid oxidized apoferredoxin was prepared from the ferredoxin of *Clostridium pasteurianum* and was generously donated by Dr. W. A. Lovenberg. The bound iron was removed by dissolving 100 mg of ferredoxin in 8.4 ml of freshly deionized 8 M urea containing 161 mg of 1,10-phenanthroline followed by the addition of 1.7 ml of 4 M Tris-HCl buffer (pH 8.5) and 0.67 ml of 2-mercaptoethanol. The mixture was stirred under a nitrogen barrier for 6 hr. The pH was then adjusted to 2.5 by addition of 1 N HCl and the solution was dialyzed exhaustively against 0.1 N acetic acid. The colorless material remaining inside the dialysis bag was lyophilized to dryness. Oxidation of the apoprotein was performed according to Hirs (1956) at 0–5° for 8 hr.

Acid Hydrolysis of Peptides. Acid hydrolysis of peptides and peptide mixtures obtained by reduction of peptides was carried out as recommended by Moore and Stein (1963). In some hydrolysis tubes a small crystal of phenol was included since it was found that better recoveries of tyrosine were thereby obtained, especially when the hydrolysis was carried out in the presence of sodium chloride.

N-Terminal Residue Determination. Phenylthiohydantoins of N-terminal amino acids were obtained by one of two modifications of the Edman degradation. In the experiments with performic acid oxidized apoferredoxin the procedure of Konigsberg and Hill (1962) was followed. The experiments with insulin A chain utilized the Edman method essentially as described by Doolittle (1965). Phenylthiohydantoins of N-terminal amino acids were degraded to the free amino acids by alkaline hydrolysis as described by Africa and Carpenter (1966). The yields of free amino acids were greatly reduced if hydrolysis was performed in the presence of traces of air or oxygen. The resulting amino acids were determined using a Beckman–Spinco (Model B) amino acid analyzer.

Sodium–Liquid Ammonia Reduction. The procedure for reduction of peptides was that which we described previously (Benisek and Cole, 1965).

Determination of N-Acetylethanolamine and N Acetylaminoacetaldehyde. In one reduction of *N*-acetylglycyl-L-proline the formation of ethanolamine residues from glycyl residues was measured by chromatographing on the amino acid analyzer the free ethanolamine released by acid hydrolysis of a portion of the unfractionated reduced peptide. For this analysis a 0.9 × 55 cm column was employed rather than the 0.6 × 12 cm column normally used for the determination of basic amino acids. Care was taken to equilibrate the column thoroughly with buffer before each analysis in order to avoid the appearance of spurious peaks. The extent of conversion of glycyl residues to aminoacetaldehyde residues was estimated indirectly since it has been shown that α-aminoaldehydes are not stable to acid hydrolysis (Ruttenberg *et al.*, 1964). Siggia and Segal (1953) have shown that the silver oxide reagent of Tollens quantitatively oxidizes simple aliphatic aldehydes to the corresponding carboxylic acids. Thus, any *N*-acetylaminoacetaldehyde formed by the reduction of *N*-acetylglycylproline would be expected to be oxidized to *N*-acetylglycine with Tollens' reagent. Acid hydrolysis of the oxidized material followed by amino acid analysis for glycine would then give an estimate of the amount of *N*-acetylaminoacetaldehyde formed by the sodium–liquid ammonia reduction of the original peptide derivative. (*N*-Acetylethanolamine would not be expected to be oxidized; Morgan and Micklethwait, 1902.) The Tollens' reagent used was a nitrate-free modification of that described by Siggia and Segal and was prepared by the addition of 0.1 ml of 12 N NaOH to a suspension of 0.17 g of silver acetate in 5 ml of water. The resulting brown precipitate of silver oxide was just dissolved by the addition of 0.38 ml of concentrated ammonium hydroxide. To 1.0 ml of this reagent solution was added 1.0 ml of a solution of the unfractionated reduced peptide mixture in 0.1 N acetic acid containing 10 μmoles of reduced *N*-acetylglycylproline. Immediately a precipitate of metallic silver appeared. After 15 min at room temperature a 0.2-ml aliquot of the supernatant solution was removed, diluted with 0.8 ml of water, and 1.0 ml of concentrated HCl was added thereto. Hydrolysis was carried out at 120° for 12 hr in sealed evacuated tubes. Aliquots of the hydrolysate were analyzed for amino acids.

Since the reduction of acetylglycylproline was not complete as determined from either the amount of free proline formed or the amount of glycine recovered after acid hydrolysis of the reduced peptide, some of the glycine found in the determination of *N*-acetylaminoacetaldehyde must have been derived from unreduced acetylglycylproline. This amount was subtracted from the observed quantity of glycine in order

TABLE I: Sodium–Liquid Ammonia Reductions of Glycyl-L-proline and Related Compounds.[a]

Compound	Modification of Standard Conditions	Time (sec)	Yield of Proline (%)
Glycylproline	Sufficient NH_4OAc to destroy the sodium was present before addition of sodium	0	12.7
Glycylproline	None	2	19.1
Glycylproline	None	23	77.7
Glycylproline	None	83	91.5
Glycylproline	−78°	33	27.6
Acetylproline	−78°	180	6.1
Acetylproline	−78° + 1.0 mmole of methanol	180	7.5
Acetylproline	None	180	56.3
Acetylproline	+0.2 mmole of sodium amide	180	56.0
Acetylproline	None	900	69.0
Acetylproline	+0.2 mmole of sodium amide	900	63.1
Acetylglycylproline	None	90	54.9
Acetylglycylproline	None	900	75.2

[a] Unless otherwise noted reductions were performed using 0.1 mmole of peptide in 7–10 ml of anhydrous liquid ammonia at −33°. Sodium (1.0–1.2 mmoles) was added to initiate the reactions, which were terminated by the addition of excess solid ammonium acetate. The residues remaining after allowing the solvent to boil off were dissolved in 0.2 N sodium citrate buffer (pH 2.2) containing sufficient 1 N HCl to give a final pH of 2.2. Aliquots were analyzed in the amino acid analyzer.

to obtain the figure for glycine derived from *N*-acetylaminoacetaldehyde.

Peptide Fragments from Insulin B Chain. Peptide fragments were isolated from a reduced insulin B chain preparation as outlined earlier (Benisek and Cole, 1965). Insulin B chain (8.62 mg) was dissolved in 10 ml of liquid ammonia at −33° and the solution was cooled to −78° with a Dry-Ice bath. Reduction was carried out with 300 μmoles of sodium, allowing the blue color to persist for 40 sec before stopping the reaction by the addition of excess solid NH_4OAc. The excess ammonium acetate was removed by sublimation from the dry residue *in vacuo* over NaOH pellets and concentrated H_2SO_4 at room temperature. The residue was taken up in 2.5 ml of pyridine (0.1 M)–acetate (pH 3.51) and the pH was adjusted to 2.5 by the addition of 1 N HCl. Glacial acetic acid (*ca.* 0.5 ml) was added to dissolve some insoluble material and the solution was made up to 4.0 ml with pyridine (0.1 M)–acetate (pH 3.51) buffer. The concentration of reduced peptide in this solution was determined by amino acid analysis of an aliquot of this solution, following acid hydrolysis. The absolute amounts of aspartic acid, glutamic acid, glycine, alanine, leucine, and lysine and the known abundance of these amino acids in insulin B chain were used in this calculation.

A 2.0-ml aliquot of the reduced peptide stock solution was chromatographed on a 0.9 × 17 cm column of Beckman–Spinco resin (type 50A) using a 250-ml linear gradient elution from pyridine (0.1 M)–acetate (pH 3.51) to pyridine (2.0 M)–acetate (pH 6.24) at a flow rate of 30 ml/hr. Aliquots (0.25 ml) of the 1.25-ml fractions were analyzed with ninhydrin (Moore and Stein, 1954) in order to locate peptides. The tubes containing ninhydrin-positive material were pooled, evaporated to dryness, and taken up in known volumes of water, or 25% aqueous acetic acid when the peptides were insoluble in water. Aliquots of these stock peptide solutions were taken for acid hydrolysis and amino acid analysis in order to characterize each peptide fragment and determine its yield based on the amount of reduced insulin B chain applied to the column.

Results

Factors Affecting the Extents of Reduction of Small Peptides. Reductions of glycylproline, acetylproline, and acetylglycylproline were performed for various lengths of times in order to compare the labilities of the acylproline peptide bonds in these three molecules. The extent of cleavage in each case was determined by measurement of the amount of free proline present after reduction. The data obtained are summarized in Table I. These data suggest marked differences in the rates of reduction of these peptides, glycylproline being reduced much more rapidly than either acetylproline or acetylglycylproline.

As shown in Table I, the presence of excess sodium amide, which would be expected to neutralize any proton donors such as water that might be present during the reaction, was essentially without effect on the extents of reduction of acetylproline. Moreover (in another reduction under a different set of conditions) the inclusion of excess methanol in the reaction mixture

was also without marked effect on the extent of reduction.

A similar difference in labilities of acylproline peptide bonds was encountered in the reduction of carbobenzoxyglycylprolylleucylglycylproline. The extents of reduction at each of the two glycylproline peptide bonds were compared in the following manner. From the yield of free proline obtained after reduction of the peptide the extent of cleavage at the C-terminal peptide bond was calculated. The loss of glycine obtained (referred to the untreated peptide) after acid hydrolysis of an aliquot of the unfractionated reduced peptide was taken as a measure of the sum of the extents of reduction at both the N- and C-terminal peptide bonds. Thus, the extent of reduction at the N-terminal peptide bond could be calculated by difference. The data for this reduction are given in Table II. Although only 0.64 residue of free proline was formed upon reduction, a total of 1.55 residues of glycine was lost, having been converted in the reaction to a reduced form. Therefore, the extent of cleavage at the N-terminal peptide bond (it was assumed that the carbobenzoxy residue was split from the peptide in the earliest stages of the reaction) was 91% *vs.* 64% for the C-terminal peptide bond.

TABLE II: Reduction of Carbobenzoxyglycylprolylleucylglycylproline.[a]

Amino Acid	Untreated Peptide after Acid Hydrolysis[b]	Residues (per residue of leucine) Reduced (before acid hydrolysis)	Reduced (after acid hydrolysis)[c]
Glycine	2.01	0	0.45
Proline	1.97	0.64	1.90
Leucine	1.00	0	1.00

[a] The reduction was performed on 0.01 mmole of peptide in 10 ml of anhydrous ammonia using 2.2 m-moles of sodium at −33°. After 90 min the reaction was terminated with ammonium acetate. The residue remaining after evaporation of the ammonia was dissolved in a known volume of water. One aliquot of this solution was added to three volumes of pH 2.2, 0.2 N sodium citrate buffer and a portion of the resulting solution was analyzed in the amino acid analyzer (column 2). Another aliquot was added to one volume of concentrated HCl and the resulting solution was heated in a sealed tube *in vacuo* for 21 hr at 110°. The residue remaining after evaporation of the acid was dissolved in pH 2.2 citrate buffer and a known portion was analyzed for amino acids (column 3). [b] The absolute recovery of leucine based on the weight of peptide hydrolyzed was 98%. [c] The absolute recovery of leucine based on the weight of peptide reduced was 96%.

Fate of the Glycyl Residue in the Reduction of N-Acetylglycylproline. In one reduction of *N*-acetylglycylproline an attempt was made to measure the amounts of *N*-acetylaminoacetaldehyde and *N*-acetylethanolamine formed as described in Materials and Methods; Table III summarizes the data. From the amount of free proline found after reduction but before acid hydrolysis and from the amount of glycine found after reduction and acid hydrolysis, the extent of reductive cleavage of the glycylproline peptide bond was found to be 75 and 73%, respectively. The level of ethanolamine found in the hydrolysate of the reduced peptide corresponded to a 40% conversion of glycyl residues to ethanolamine residues. The increase in glycine obtained by oxidation of the reduced peptide by Tollens' reagent corresponded to a 26% conversion of glycyl residues to aminoacetaldehyde residues. The fate of the other 34% of the reduced glycyl residues is unknown if it is assumed that Tollens' reagent reacted quantitatively. As shown in the last line of Table III, the absolute recovery of material was satisfactory at each step of the analysis.

A similar kind of experiment was performed in connection with the reduction of glycylproline. Here only 14% of the reduced glycyl residues (extent of cleavage was 81%) could be recovered as ethanolamine. Treatment of the reduced material with 0.1 M sodium borohydride at room temperature for 3 hr resulted in an increase in ethanolamine which suggested that only 6% of the reduced glycyl residues was present as aminoacetaldehyde after the sodium–ammonia treatment. Reduced glycylproline was also treated with a suspension of silver oxide in order to oxidize any aminoacetaldehyde present to glycine (Mitchell and Smith, 1950). The observed increase in glycine again accounted for only 6% of the reduced glycyl residues as aminoacetaldehyde.

Effect of Sodium–Liquid Ammonia Treatment on the Amino Acid Compositions of Large Peptides. The effect of treatment with sodium in liquid ammonia on the amino acid compositions of three large polypeptides was examined in order to measure the extents of cleavage of the acylproline peptide bonds in each polypeptide and to detect side reactions that would result in the chemical modification of amino acid residues. Since the reductive cleavage of acylproline peptide bonds results in reduction of the acyl group to either an alcohol or aldehyde or both (Ressler and Kashelikar, 1966; Birch *et al.*, 1955), one would expect that amino acid residues adjacent and N terminal to proline residues would be destroyed in proportion to the extent of reductive cleavage of the acylproline peptide bonds. For example, the extent of cleavage of acetylglycylproline calculated from glycine destruction agreed very well with the extent of cleavage calculated from proline release (Table III). On this basis we determined extents of cleavage of the acylproline peptide bonds in the large peptides from the losses (after acid hydrolysis of the unfractionated reduced material) of those amino acids known to be adjacent and N terminal to proline residues. Thus the reduction of insulin B chain

TABLE III: Reduction of *N*-Acetylglycyl-L-proline Followed by Oxidation with Tollens' Reagent.[a]

Amino Acid	Amino Acid Analyses of 1-μmole Portions			
	Before Reduction after Hydrolysis (μmoles)	After Reduction before Hydrolysis (μmoles)	After Reduction and Hydrolysis (μmoles)	After Reduction, Oxidation, and Hydrolysis (μmoles)
Glycine	1.01	0.00	0.265[c]	0.450
Proline	1.00	0.75[b]	0.975	1.00
Ethanolamine	0.00		0.292	
% recovery	100[d]		97.5[e]	98.0[e]

[a] Reduction was performed as described in the legend of Table I. The reaction was allowed to proceed for 15 min. The residue remaining after evaporation of the ammonia was dissolved in 1.0 ml of 1 N acetic acid and made up to 10 ml with water. Aliquots were taken for amino acid analysis (after dilution with 0.2 N (pH 2.2) sodium citrate buffer), acid hydrolysis with an equal volume of concentrated HCl followed by amino acid analysis, and oxidation with Tollens' reagent as described in Materials and Methods followed by acid hydrolysis and amino acid analysis. [b] Corresponding per cent cleavage is 75%. [c] Corresponding per cent cleavage is 73%. [d] Taken as 100% and used as the basis for calculation of per cent recoveries for reduced peptide and reduced–oxidized peptide. [e] Calculated from the absolute values for proline. For the reduced–oxidized analysis the actual amount taken for analysis was a volume equivalent to 0.2 μmole of the initially unreduced peptide. The absolute value for proline in this case was 0.196 μmole.

TABLE IV: Amino Acid Composition of Insulin B Chain and Reduced Insulin B Chain.[a]

Amino Acid	Molar Ratio[b]				
	Before Reduction	After Ammonia Treatment	Mild Reduction	Vigorous Reduction	Theory
Aspartic acid	1.02	1.04	1.03	1.00	1
Threonine	0.91[c]	0.93[c]	0.094[c]	0.034[c]	1
Serine	0.82[c]	0.86[c]	0.83[c]	0.87[c]	1
Glutamic acid	2.97	3.04	3.14	3.09	3
Proline	1.10	1.02	1.01	0.97	1
Glycine	3.01	3.04	3.05	2.89	3
Alanine	2.00	2.00	2.00	2.00	2
Valine	3.07	3.06	3.14	3.22	3
Leucine	4.10	4.00	4.23	3.99	4
Tyrosine	1.98	1.69	1.93	1.89	2
Phenylalanine	2.97	2.51	2.60	1.30	3
Lysine	1.02	0.96	1.02	1.01	1
Histidine	1.98	2.05	2.12	1.99	2
Arginine	1.01	0.80	1.07	0.96	1

[a] The reductions were performed at −78° on 2.2–2.3-μmole portions of insulin B chain in 7 ml of liquid ammonia. Mild reduction was carried out by adding 250 μmoles of sodium to the solution and stopping the reaction with excess ammonium acetate 40 sec after the appearance of the blue color due to dissolved sodium. Vigorous reduction was performed in the same fashion using 2100 μmoles of sodium and stopping the reaction after 180 sec. The ammonia treatment consisted of adding the insulin B chain *after* a solution of 2100 μmoles of sodium had been oxidized by the addition of excess ammonium acetate and allowing the solution to stir for 180 sec at −78° before allowing the ammonia to boil. The solid residues remaining in the reaction flask after allowing the solvent to boil and room temperature sublimation of excess ammonium acetate were suspended in 0.25-ml portions of 1 N HCl and made up to 5 ml with 33% aqueous acetic acid yielding clear solutions. Aliquots were diluted with equal volumes of concentrated HCl for hydrolysis. A small crystal of phenol was added to the hydrolysis mixtures before sealing. [b] Molar ratio relative to alanine. The absolute recoveries of alanine based on the weight of peptide treated were 93–99%. [c] Not corrected for hydrolytic destruction.

TABLE V: Amino Acid Composition of Performic Acid Oxidized Apoferredoxin before and after Reduction.[a]

Amino Acid	Molar Ratios[b]			
	Before Reduction	Reduction for 1 min	Reduction for 15 min	Theory
Cysteic acid	6.70	5.22	5.08[d]	8
Aspartic acid	8.00	8.00	8.00	8
Threonine	1.05[c]	1.11[c]	1.08[c]	1
Serine	3.77[c]	3.91[c]	3.96[c]	4
Glutamic acid	3.94	4.09	4.11	4
Proline	2.86	2.85	2.76	3
Glycine	4.05	4.18	4.16	4
Alanine	7.00	6.23	6.22	8
Valine	5.35	5.71	5.67	6
Isoleucine	4.27	4.40	4.44	5
Leucine	0.16	Trace	0.19	0
Tyrosine	0.91	1.02	0.99	1
Phenylalanine	1.02	0.90	0.97	1
Lysine	1.20		1.22	1

[a] The reductions were performed in 10 ml of liquid ammonia at −33° using 700 μmoles of sodium for the times indicated in the column headings. [b] Molar ratios relative to aspartic acid. [c] Not corrected for hydrolytic destruction. [d] When insulin B chain was reduced under identical conditions the recovery of cysteic acid was 102% and the recovery of alanine was 98%.

which contains a single proline residue adjacent to a threonine residue ought to be accompanied by the loss of threonine. Similarly, performic acid oxidized ferredoxin contains three proline residues, two of which are next to cysteic acid residues and one next to an alanine residue (Tanaka *et al.*, 1964), and therefore its reduction should result in destruction of cysteic acid and alanine. In contrast, insulin A chain contains no proline and one would expect, in the absence of side reactions, that its amino acid composition would be unaffected by treatment with sodium in liquid ammonia.

The amino acid analysis of insulin B chain before and after treatments with liquid ammonia and with sodium in liquid ammonia are given in Table IV. Reduction under the "mild" conditions described in the legend of Table IV resulted in about a 90% loss in threonine indicating 90% reductive cleavage of the threonylproline peptide bond. Significantly, no appreciable changes were observed in the recoveries of the other amino acids present in this polypeptide under these conditions. However, when the reduction was performed under "vigorous" conditions (2100 μmoles of sodium at −78° for 180 sec) in addition to the expected loss of threonine the destruction of about 1.5 residues of phenylalanine occurred indicating that some kind of side reaction had taken place involving phenylalanine residues of this polypeptide.

The results of a similar series of experiments using performic acid oxidized apoferredoxin are shown in Table V. The only amino acids that were observed to undergo destruction were cysteic acid and alanine, the only amino acids adjacent and N terminal to proline in this protein. From the per cent losses of cysteic acid and alanine relative to the measured amounts of these amino acids in the untreated polypeptide it can be calculated that the average cleavages of the cysteicyl-proline peptide bonds were 88% for a 1-min reduction and 97% for a 15-min reaction while the corresponding cleavages of the alanine–proline peptide bond were 88 and 89%. Notably, such vigorous reductive treatment appeared to have no significant effect on phenylalanine in this polypeptide in contrast to the results obtained with insulin B chain. The acylproline peptide bonds in performic acid oxidized ferredoxin seemed to be less labile to sodium in liquid ammonia than the threonyl-proline peptide bond in insulin B chain, since when the ferredoxin derivative was reduced under the "mild" conditions that resulted in 90% cleavage of the threonyl-proline bond in B chain (see column 3 of Table IV), no losses of cysteic acid or alanine were observed.

The analogous experiments using insulin A chain were particularly revealing since, as shown in Table VI, a loss of glycine was observed upon treatment of the polypeptide with sodium in liquid ammonia. This result is in contrast to the apparent stability of glycine in insulin B chain (Table IV) and performic acid oxidized ferredoxin (Table V) to the reductive procedure.

Nonspecific Cleavage during Reduction of Large Polypeptides. In order to obtain a rough estimate of the extent of cleavage at peptide bonds other than acylproline peptide bonds, the changes in amino-

TABLE VI: Amino Acid Composition of Insulin A Chain before and after Reduction.[a]

Amino Acid	Molar Ratios[b]			
	Before Reduction	Mild Reduction	Vigorous Reduction	Theory
Aspartic acid	1.92	1.85	1.86	2
Serine	1.74[c]	1.48[c]	1.76[c]	2
Glutamic acid	3.92	3.71	4.03	4
Glycine	1.01	0.93	0.75	1
Alanine	1.04	1.06	1.09	1
Valine	1.71	1.56	1.75	2
Isoleucine	0.66	0.67	0.66	1
Leucine	2.00	2.00	2.00	2
Tyrosine	1.66	1.61	1.59	2

[a] Reductions were carried out on 1.76-μmole portions of insulin A chain in 10 ml of liquid ammonia under mild conditions (380 μmoles of sodium, −78°, 40 sec) and vigorous conditions (1200 μmoles of sodium, −33°, 15 min). The residues remaining after evaporation of the solvent and sublimation of excess ammonium acetate were taken up in 5 ml of 0.3 N HCl. Aliquots were diluted with equal volumes of concentrated HCl for hydrolysis. [b] Molar ratios relative to leucine. The per cent recovery of reduced A chain after mild reduction was 99% and after vigorous reduction was 96% based on the absolute values of Asp, Glu, Ala, and Leu. [c] Not corrected for hydrolytic destruction.

TABLE VII: Amino-Terminal Analysis of Performic Acid Oxidized Apoferredoxin before and after Reduction.[a]

Amino Acid	Residues/Mole of Performic Acid Oxidized Ferredoxin[b]			
	Before Reduction	After Reduction	Change due to Reduction	% Cleavage[c]
Aspartic acid	0.036	0.052	0.016	0.20
Threonine	0	Trace	Trace	Trace
Serine	0.014	0.015	0.001	0.025
Glutamic acid	0.010	0.027	0.017	0.43
Proline	0	1.07	0.07	36
Glycine	0.047	0.096	0.049	1.2
Alanine	0.530	0.152	−0.378	
Valine	0.011	0.044	0.034	0.56
Isoleucine	Trace	0.218	0.218	4.3
Tyrosine	0	0.084	0.084	8.4
Phenylalanine	0	0	0	0

[a] Reduction was carried out on 0.5 μmole of performic acid oxidized ferredoxin in 10 ml of liquid ammonia at −33° for 15 min using 690 μmoles of sodium. Amino-terminal analyses were performed as described in Materials and Methods. [b] The figures are not corrected for any destruction during alkaline hydrolysis which may have occurred or for nonquantitative removal of N-terminal groups as the phenylthiohydantoins. [c] Per cent cleavage was calculated as the average per cent cleavage of peptide bonds involving each particular amino acid as the amino donor using the increase of each N terminal due to reduction and the known amino acid composition of the protein.

terminal residues which occurred as a result of treatment of polypeptides with sodium in liquid ammonia were determined as outlined in Materials and Methods.

In Table VII are tabulated the apparent number of N-terminal residues of amino acid per mole of performic acid oxidized ferredoxin before (column 1) and after (column 2) vigorous treatment with sodium in liquid ammonia. The data of column 3 were calculated from the observed increase of each amino acid as an N terminus and the known abundance of each amino acid in the protein. The most striking fact apparent in these data is that N-terminal alanine, the true N-terminal residue of ferredoxin (Tanaka *et al.*, 1964), decreased markedly upon reductive treatment

TABLE VIII: Amino-Terminal Analyses of Insulin A Chain before and after Treatments with Sodium–Liquid Ammonia.[a]

Amino Acid	Residues/Moles of Polypeptide[b]				% Cleavage Mild Reduction[c]	% Cleavage Vigorous Reduction[c]
	Untreated A Chain	Ammonia-Treated A Chain	Mild Reduction	Vigorous Reduction		
Aspartic acid	Trace	Trace	Trace	Trace		
Serine	Trace	0.014	Trace	Trace		
Glutamic acid	0.045	0.059	0.038	0.039		
Glycine	0.51	0.52	0.43	0.31		
Alanine	0	Trace	Trace	Trace	Trace	Trace
Valine	0	Trace	Trace	0.018	Trace	1.8
Isoleucine	0	Trace	0.049	0.166	4.9	16.6
Leucine	0.018	0.050	0.020	0.039	0.1	2.1
Tyrosine	Trace	0.021	Trace	0.047		4.7

[a] The reductions were performed as indicated in the legend to Table VI. Ammonia treatment consisted of allowing 1.76 μmoles of insulin A chain to stand in 10 ml of ammonia at −33° for 15 min. Amino-terminal analyses were carried out on lyophilized portions of the residue remaining after reduction as noted in Materials and Methods. [b] The figures are not corrected for any destruction during alkaline hydrolysis which may have occurred or for non-quantitative removal of N-terminal groups as the phenylthiohydantoins. [c] Per cent cleavage was calculated as the average per cent cleavage of peptide bonds involving each particular amino acid as the amino donor using the increase of each N terminal due to reduction and the known amino acid composition of the protein.

of the polypeptide. N-terminal proline increased substantially indicating cleavage at acylproline bonds. Increases, though much smaller, were observed for most of the other amino acids, and so it is apparent that some random cleavage of the polypeptide occurred. Noteworthy is the relatively large increase in tyrosine as a new N-terminal residue. There is only one tyrosine in native ferredoxin and it is known to be adjacent to the N-terminal alanine.

The results of N-terminal analysis of insulin A chain before and after reductive treatment under two different sets of conditions are given in Table VIII. Behavior analogous to that of ferredoxin was observed, the N-terminal glycine decreasing upon reductive treatment of the polypeptide. Concomitant with the decrease in N-terminal glycine was a corresponding increase in N-terminal isoleucine. Again, isoleucine is known to be penultimate to glycine in the A chain. Nonspecific cleavage at residues other than isoleucine appeared to be somewhat lower in both mild and vigorous reductions than was the case with performic acid oxidized apoferredoxin.

Peptide Fragments from Insulin B Chain. As a further check on the nature of the products of sodium–ammonia treatment the peptides from reduced insulin B chain were isolated. The ion-exchange chromatogram of the peptide mixture is shown in Figure 1. Five ninhydrin-positive peaks were observed and the amino acid composition of the material from each is given in Table IX. The material from peak 5 showed only a single ninhydrin-positive spot on paper electrophoresis in pH 3.5 pyridine acetate buffer with a cathodic electrophoretic mobility 0.84 times that of concurrently run lysine and 6.6 times that of concurrently run leucine The material from peak 4 showed no ninhydrin-positive spot except for traces of the peak 5 material. The material from peaks 1 to 3 was not tested electrophoretically. The amino acid compositions of material from peaks 1 to 3 indicate that all of these species are derived from residues 1 to 27 of the polypeptides and that peak 5 is the tripeptide prolyllysylalanine, derived from residues 28 to 30. The amino acid analysis of the material from peak 4 showed only ammonia in significant quantities. We conclude that peak 4 is due

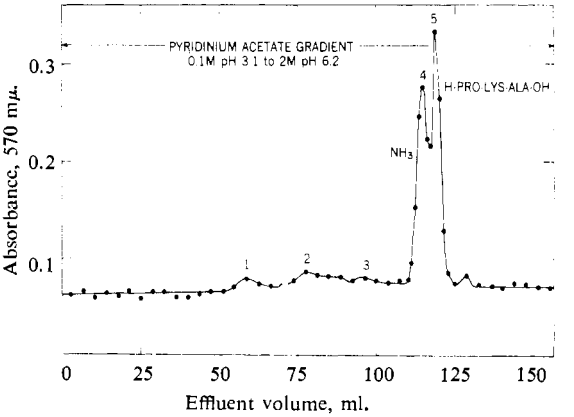

FIGURE 1: Dowex 50 chromatography of oxidized insulin B chain after mild reduction using sodium in liquid ammonia. Experimental conditions given in Materials and Methods.

TABLE IX: Amino Acid Compositions of Products of the Reduction of Insulin B Chain.[a]

Amino Acid	Molar Ratios[b]				
	Unfractionated Reduced B Chain	Peak 1	Peak 2	Peak 3	Peak 5
Aspartic acid	0.98	1.31	1.05	1.06	
Threonine	0.09[c]	0	0.10[c]	0	
Serine	0.79[c]	1.11[c]	0.87[c]	0.91[c]	
Glutamic acid	2.96	3.25	3.06	2.81	
Proline	0.95	0	0	0	1.07
Glycine	2.88	2.85	3.10	2.91	
Alanine	1.89	1.19	1.13	1.07	1.00
Valine	2.97	3.42	3.17	3.16	
Isoleucine	0	0	0	0	
Leucine	4.00	4.00	4.00	4.00	
Tyrosine	1.82	1.11	1.84	1.97	
Phenylalanine	2.46	1.70	2.66	2.66	
Lysine	0.96	0	0.25	0	1.23
Histidine	2.00	1.97	1.96	2.18	
Arginine	1.01	0.89	0.96	1.12	
% yield[d]		16	31.5	5.8	80

[a] Reduction of insulin B chain and fractionation of the resulting peptide mixture is described in Materials and Methods. As mentioned in Results the material from peak 4 gave only ammonia in significant quantities after acid hydrolysis. [b] Leucine was taken as the base for calculation of molar ratios. [c] Not corrected for hydrolytic destruction. [d] Average value calculated from the absolute values for Asp, Glu, Gly, and Leu found.

to ammonia which was not removed from the peptide mixture by sublimation.

Discussion

Reduction of acylproline peptide bonds by sodium–liquid ammonia has been demonstrated for a variety of proline-containing peptides examined in this work. However, the lability of these different acylproline peptide bonds extends over a wide range as indicated by the varying conditions required to obtain extensive cleavage. Illustrative of the extreme cases are the easily reduced threonylproline peptide bond of insulin B chain and the more difficultly reduced acylproline peptide bonds in performic acid oxidized apoferredoxin or acetylproline. It would seem from our experiments that the cleavage of a general acylproline peptide bond should occur over at least the range of conditions, −78°, 0.025 N sodium, 40 sec to −33°, 0.1 M sodium, 900 sec.

The more facile reduction of glycylproline *vs.* acetylproline and acetylglycylproline is somewhat surprising. The greater electron-withdrawing ability of the acetamido function of acetylglycylproline compared to the unprotonated amino group of glycylproline or the hydrogen atom of acetylproline would be expected to favor addition of electrons to the carbonyl group of the acylproline peptide bond, thereby reducing it. That glycylproline was the most rapidly reduced of the three suggests that some other effect predominates.

It is tempting to suggest that the anomalously rapid reduction of glycylproline is due to the presence of a free α-amino group adjacent to the peptide bond reduced. Since sodium–liquid ammonia rapidly removes *N*-carbobenzoxy groups from blocked peptides liberating free amino groups (Sifferd and du Vigneaud, 1935), this hypothesis would explain the preferential reduction of the N-terminal glycylproline peptide bond in carbobenzoxyglycylprolylleucylglycylproline compared to the C-terminal glycylproline peptide bond (Table II).

A possible mechanistic explanation for the rate enhancement by a neighboring α-amino group might be based on a postulated ability of the amino group to bind the solvated electrons present in solutions of sodium in ammonia (Smith, 1963) by replacement of one of the solvating ammonia molecules. Alternatively one might envisage proton abstraction from the uncharged α-amino group followed by cyclization of the amide salt to an α-lactam, thus liberating a polypeptide with a new N-terminal residue. The α-lactam might then be reduced to the corresponding α-aminoaldehyde or alcohol by sodium.

In considering the mechanism of this reduction, a hydrogen source is of importance. Wilchek *et al.* (1965) and Ressler and Kashelikar (1966) have also observed the reductive cleavage of acylproline peptide

bonds by sodium–ammonia solutions in a wide variety of small peptides. Both of these groups have included methanol in their reaction mixtures, presumably as a source of hydrogen for the reduction. We have not included any hydrogen donors (except the peptides themselves) in our reaction mixtures but have still obtained reductive cleavage. This might be due to several factors. (1) Acidic groups on the peptides themselves might provide protons for the reduction. (2) In spite of measures taken to secure anhydrous conditions, adventitious water may have been introduced with the sodium metal or peptide into the reaction mixture and this water could serve as the proton donor. (3) Under the conditions reported here perhaps no reduction took place until ammonium acetate was added to destroy the sodium; ammonium ions then might have functioned as the hydrogen source.

Our finding that added methanol does not markedly affect the extent of reduction of acetylproline (Table I) would indicate that if proton donors are required for reductive cleavage, they are already present in sufficient quantity under the conditions for reduction we have employed. The further observation that the extent of reduction of acetylproline was only slightly, if at all, reduced by carrying out the reaction in the presence of a large excess of the strong base, sodium amide, provides evidence that the presence of adventitious proton donors (moisture or the acidic groups on the peptide itself) is also not required for any rate-limiting step in the reductive cleavage. Proton donation could possibly occur in a relatively fast reaction between a preformed intermediate and ammonium acetate or between an intermediate and water (when the sample is dissolved for analysis and further study), but in any case it is unlikely that reduction takes place only upon destruction of the sodium with ammonium acetate since the extent of reduction of glycylproline correlates with the contact time of the peptide with sodium in liquid ammonia (Table I). If the reduction took place only on addition of ammonium acetate, the extents of reduction would have been the same for all contact times. Ressler and Kashelikar (1966) were able to demonstrate that under their conditions the fate of the acyl portion of several acylproline peptides is principally reduction to the amino alcohol. These reductions were carried out in the presence of methanol. We found only low yields (14%) of ethanolamine when glycylproline was reduced under our conditions. Perhaps the presence of methanol influences the nature of the reduced acyl group rather than the extent of reductive fission of the peptide bond.

Under our conditions, the formation of multiple forms of new C-terminal residues was demonstrated by the results of C-terminal analysis of partially reduced acetylglycylproline which shows that the glycyl residue can be reduced to both an aminoacetaldehyde residue and a residue of ethanolamine. The analysis accounted for only 66% of the reduced glycyl residues in these two forms. This might represent a failure to complete the oxidation of the aldehyde group of *N*-acetylaminoacetaldehyde to a carboxyl group by the Tollens' reagent or to the existence of other forms of the reduced glycyl residues. The latter possibility must be considered since it is known that aldehydes with hydrogen on the α-carbon atom spontaneously undergo aldol condensation in liquid ammonia (Strain, 1932). This multiplicity of C-terminal forms obviously would complicate the use of reductive cleavage in structural studies of peptides, at least under the conditions we used. The application of sodium–ammonia treatment to peptides would be further complicated by certain side reactions encountered during the reductions. One such side reaction is the small amount of cleavage which occurred at other peptide bonds as revealed by the N-terminal analyses of reduced performic acid oxidized ferredoxin and insulin A chain. The extent of this nonspecific cleavage was 0–4% per peptide bond (Tables VII and VIII).

Another side reaction is revealed in the losses of phenylalanine in insulin B chain and of glycine in insulin A chain that occurred during treatment with sodium and liquid ammonia, at least under the more vigorous conditions examined. These facts when considered with the observations that N-terminal glycine decreased in the reductive treatment of insulin A chain and N-terminal alanine decreased in the reductive treatment of performic acid oxidized apoferredoxin would suggest that sodium–liquid ammonia treatment of polypeptides can induce appreciable reductive cleavage of N-terminal peptide bonds in addition to acylproline peptide bonds. This hypothesis is supported by the observation that isoleucine appears as the predominant new N-terminal amino acid residue in insulin A chain after treatment with $Na–NH_3$ and that anomalously large amounts of N-terminal tyrosine are present after reduction of the ferredoxin derivative. In these two polypeptides isoleucine and tyrosine occur (respectively) only once and they are the residues adjacent to the N-terminal residues, glycine and alanine. Thus the appearance of isoleucine and tyrosine as N-terminal residues can be uniquely ascribed to cleavage of the N-terminal peptide bonds of these polypeptides. The enhancement of the cleavage of N-terminal nonproline peptide bonds relative to internal, nonproline bonds may well be analogous to the cleavage of N-terminal acylproline bonds relative to internal acylproline bonds.

The partial destruction of phenylalanine in insulin B chain can therefore be explained in two ways. Sodium in liquid ammonia may reduce the aromatic ring of phenylalanine (Wilchek and Patchornik, 1962) or it may reduce the N-terminal peptide bond of this polypeptide. The latter possibility is favored by the finding that the phenylalanine in the ferredoxin derivative was essentially unaffected by sodium–ammonia treatment.

It becomes clear that in attempts to apply sodium–ammonia reductions to structural investigations of peptides the chances for success diminish rapidly as the number of proline residues in the peptide increase, and to some extent as the size of the peptide itself increases. While the partially specific cleavage of N-terminal, nonproline peptide bonds might be controlled by acetylation of the amino group, the nonspecific cleav-

age of general peptide bonds, and the multiplicity of forms in the newly created C termini in residues originally adjacent to proline, combine to give a multiplicity of products. This multiplicity not only compounds the problem of isolating peptides, but it lowers the yield of each of them. Even in the favorable case of insulin B chain these problems are significant, although they are not overwhelming. In this case, a mild reduction gave a good (80%) yield of the tripeptide prolyllysylalanine. However, the yields of peptides corresponding to residues 1–27 of insulin B chain were only fair, since we could recover peptides accounting in total for only about 50% of the expected amount. Furthermore, the portion of the polypeptide corresponding to residues 1–27 appeared distributed among at least three chromatographically different forms. Although such yields are workable, it seems likely that treatment with sodium in liquid ammonia will have its best application in the selective cleavage of slightly smaller peptides containing perhaps one or two prolines. Such peptides are frequently encountered in tryptic digests, and bonds between proline and either lysine or arginine resist tryptic action. If the facility of splitting the threonylproline bond in insulin B chain is due to the adjacent ϵ-amino group of lysine, then lysyl and perhaps arginylproline bonds might be reduced especially easily.

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