to explain fully why the rates of heme ejection and probably of unfolding level off at identical rates in the two species at low pH. Experiments that measure unfolding directly, *e.g.*, further work on changes in Cotton effect, or in fluorescence quenching, must be resorted to, and are in progress.

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The Reductive Conversion of N-Terminal Pyroglutamyl into Prolyl Residues in Polypeptides and Proteins*

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ABSTRACT: The pyrrolidone ring of an N-terminal pyroglutamyl residue in a polypeptide or protein may be converted, by reduction with diborane in tetrahydrofuran or tetramethylurea, into a pyrrolidine ring. Thus, the originally nonbasic N-terminal residue is identified by its conversion into proline. Although the reduction shows only limited selectivity (carboxyl and peptide groups are partially reduced), yields of proline up to 46% have been obtained in the reduction of an octapeptide analog of gastrin. The generation of N-terminal proline by reduction of native or performic acid oxidized bovine γ-globulin is also observed. Diborane reduction offers a rapid and facile means of demonstrating the presence of an N-terminal residue of pyroglutamic acid and of initiating sequential analysis of the polypeptide chain.

A sizeable number of natural polypeptides and proteins are now known to possess N-terminal pyroglutamic acid (pyrrolidonecarboxylic acid) residues. The lack of basicity of the pyrrolidone nitrogen prevents the formation of derivatives which might be useful for N-terminal identification or for sequential cleavage of the polypeptide chain. Since a search for nucleophilic agents which could attack the pyrrolidone carbonyl selectively proved fruitless,[1] alternative approaches to the selective chemical modification of the cyclic lactam were explored.

We have found that diborane, which has already been

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[1] L. A. Cohen, unpublished data.

used for the selective reduction of free carboxyl groups in proteins (Atassi and Rosenthal, 1967), is also partially selective for the pyrrolidone ring, effecting a reductive conversion into proline. The method has been applied to several simple pyroglutamyl peptides, to an octapeptide analog of gastrin, and to bovine γ-globulin. Conversions of pyroglutamic acid into proline of up to 46% have been realized (see p 869 for a typical reaction).

During the course of this work, a report appeared on the isolation and utilization of a specific pyrrolidonyl peptidase (Doolittle and Armentraut, 1968). While the chemical method may not offer the degree of specificity attainable by enzymatic hydrolysis, the facility and rapidity of the technique invite its consideration, both for qualitative N-terminal residue identification and for sequential analysis.

Materials and Methods

Materials. Diborane was obtained as a solution in tetrahydrofuran, 1.0 M in BH_3 (Ventron Chemicals). Manometric determination of the hydrogen gas evolved upon addition of dilute HCl to an aliquot of the reagent showed the BH_3 content to be approximately 0.9 M. Reagent tetrahydrofuran was used directly. Tetramethylurea (Aldrich Chemical Co.) was purified by fractional distillation (bp 32° (1.5 mm)). The stability of diborane in tetramethylurea was demonstrated by the constancy of its manometric titer over a 1-hr period.

Substrates and Reference Compounds. L-Pyroglutamamide (L-2-oxopyrrolidine-5-carboxamide) was synthesized from diethyl L-glutamate and aqueous ammonia, according to Angier *et al.* (1950). The compound was recrystallized from ethanol (mp 168–168°). L-Prolinamide was prepared from ethyl L-prolinate and aqueous ammonia (Putochin, 1926) and the product recrystallized from ethanol (mp 96–98°). L-2-Aminomethylpyrrolidine was prepared by lithium aluminum hydride reduction of L-prolinamide (Schnell and Karrer, 1955) and isolated as the dihydrochloride (mp 122–124°). The free amine (bp 71–72° (18 mm)) was used in an attempt at further reduction by diborane. Ethyl L-pyroglutamylglycinate was synthesized from L-pyroglutamylhydrazide (Angier *et al.*, 1950) and ethyl glycinate by the method of LeQuesne and Young (1952). The compound was recrystallized from ethyl acetate (mp 118–120°). Saponification of the ethyl ester afforded L-pyroglutamylglycine (LeQuesne and Young, 1952), mp 166–169°, from ethanol–ethyl acetate–ether. L-α-Amino-δ-hydroxyvaleric acid was prepared according to Thompson *et al.* (1964) by lithium borohydride reduction of γ-ethyl-L-glutamate and recrystallized from ethanol–water (mp 225–230° dec).

Ethyl L-Pyroglutamylglycylglycinate. L-Pyroglutamyl azide was coupled with ethyl glycylglycinate, according to the general method of LeQuesne and Young (1952). The reaction mixture was extracted with chloroform, the extract was evaporated, and the residue was chromatographed on a silicic acid column. Elution of the column with chloroform–methanol (4:1, v/v) afforded crystals of the tripeptide ester, which was recrystallized from ethanol (mp 157–159°).

Anal. Calcd for $C_{11}H_{17}N_3O_5$: C, 48.70; H, 6.32; N, 15.49. Found: C, 48.82; H, 6.42; N, 15.28.

PyroGlu-Ala-Tyr-Gly-Trp-Met-Asp-PheNH$_2$. The synthetic octapeptide was the generous gift of Dr. J. S. Morley, Imperial Chemical Industries, Ltd., Macclesfield, England. Amino acid analysis of the material showed a slight excess of glutamic acid. Since there was no evidence of free amino groups and since chromatography failed to alter the analytical results, it was assumed that an additional peptide was present containing a glutamic acid residue adjacent to the pyroglutamyl N terminal. As obtained, the crystalline peptide was insoluble in tetrahydrofuran and somewhat soluble in tetramethylurea. A sample (1–3 mg) was first dissolved in dimethylformamide and the solution evaporated *in vacuo* to provide an amorphous film, which was then dissolved in 0.3 ml of acetic acid with warming; the solution was diluted with 50 ml of water and the material was lyophilized. The amorphous powder remaining was now almost completely soluble in tetramethylurea. Amino acid analysis showed no change in composition resulting from the several treatments.

N-Dinitrophenyl Derivatives. Dinitrophenylation was performed according to the general method of Fraenkel-Conrat *et al.* (1955). DNP-2-aminoethanol was recrystallized from ethanol–ether (mp 86–88°) (Oikawa *et al.*, 1961); ultraviolet max (C_2H_5OH) 345 mμ (ε 15,000). Bis-DNP-1,5-diaminopentane was recrystallized from dimethylformamide (mp 179–180°) (Oikawa *et al.*, 1961); ultraviolet max (1% dimethylformamide in CH_3OH) 348 mμ (ε 35,100). DNP-DL-norvaline was recrystallized from water or acetone–hexane (mp 138°);[2] ultraviolet max (1% $NaHCO_3$) 363 mμ (ε 16,300). DNP-δ-aminovaleric acid was recrystallized from ethanol or acetone–cyclohexane: mp 169–171° (Burmistrov and Plit, 1959), ultraviolet max (1% $NaHCO_3$) 364 mμ (ε 17,800).

Chromatographic data for some less common DNP derivatives is summarized in Table I.

DNP-L-prolinamide. The derivative was prepared in the usual manner and recrystallized from acetone–hexane: mp 130–133°, ultraviolet max (CH_3OH) 364 mμ (ε 15,400).

Anal. Calcd for $C_{11}H_{12}N_4O_5$: C, 47.14; H, 4.32; N, 20.00. Found: C, 47.37; H, 4.11; N, 19.72.

Bis-DNP-L-2-aminomethylpyrrolidine. The compound was prepared from L-2-aminomethylpyrrolidine and recrystallized from dimethylformamide–ether: mp 233–235°, ultraviolet max (2% dimethylformamide–CH_3OH) 355 mμ (ε 33,800).

Anal. Calcd for $C_{17}H_{16}N_6O_8$: C, 47.22; H, 3.73; N, 19.44. Found: C, 47.22; H, 3.85; N, 19.49.

DNP-L-α-amino-δ-hydroxyvaleric Acid. The derivative was recrystallized from water (mp 114–116°), resolidifying and remelting at 180° dec (lactone); ultraviolet max (1% $NaHCO_3$) 361 mμ (ε 16,400).

Anal. Calcd for $C_{11}H_{13}N_3O_7$: C, 44.15; H, 4.38; N, 14.04. Found: C, 44.22; H, 4.41; N, 14.28.

[2] DNP-L-norvaline (mp 58–60°) has been described by Rao and Sober (1954).

TABLE I: R_F Values of DNP Derivatives.[a]

DNP Derivative of	R_F Values[b] A	B	C	D	E
2-Aminoethanol		0.62	0.49	0.45	0.32
L-α-Amino-δ-hydroxy-valeric acid		0.20	0.25	0.09	0.05
δ-Aminovaleric acid	0.06	0.91	0.84	0.71	0.43
L-α-Amino-δ-valero-lactone		0.77	0.53	0.42	0.42
1,5-Diaminopentane	0.81	~1.0	0.90	0.59	0.76
L-2-Methylamino-pyrrolidine	0.78	~1.0	0.71	0.30	0.63
DL-Norvaline	0.16	0.83	0.78	0.40	0.35
L-Prolinamide	0.63	0.58	0.47	0.33	0.36

[a] Run on silica gel GF plates, 0.25 mm thickness. [b] Solvent systems were as follows: (A) upper phase of toluene–2-chloroethanol–0.8 N ammonium hydroxide–pyridine (10:6:6:3, v/v), (B) chloroform–benzyl alcohol–acetic acid (7:3:0.3, v/v), (C) chloroform–*t*-amyl alcohol–acetic acid (7:3:0.3, v/v), (D) benzene–pyridine–acetic acid (8:2:0.2, v/v), and (E) chloroform–methanol–acetic acid (95:5:1, v/v).

The *DNP derivative* of the *lactone* was easily formed by heating the solid acid *in vacuo* or by addition of dilute mineral acid to its solution in ethanol. The compound was recrystallized from acetone or DMF–ether (mp 180° dec).

Anal. Calcd for $C_{11}H_{11}N_3O_6$: C, 46.98; H, 3.94; N, 14.94. Found: C, 47.30; H, 4.51; N, 14.81.

General Procedure for Reduction with Diborane. In order to avoid losses due to transfer of poorly soluble materials, all reactions from reduction to total acid hydrolysis were performed in one vessel, generally a 25- or 50-ml ampoule. Ampoules were attached to a rotary evaporator by means of a heavy rubber sleeve. Because of their limited solubility or insolubility in the solvents used, the polypeptide subtrates should be in a very finely divided state, preferably as the residues of lyophilized solutions.

A sample of peptide (10–100 μmoles) was dissolved or suspended in 1 ml of solvent (tetrahydrofuran or tetramethylurea), the mixture was chilled, and 1–2 ml (1–2 mmoles) of diborane in tetrahydrofuran was added. The reaction mixture was kept in a closed container for several hours at the desired temperature, with occasional shaking, and then evaporated to dryness *in vacuo*. To the residue was added 10 ml of 2% trifluoroacetic acid in methanol and the resulting solution was evaporated to dryness. The process was repeated several times, followed by 5–10 repetitions with methanol alone. The trifluoroacetic acid served to hydrolyze borate esters or amides; boric acid was removed as the volatile methyl borate. For total acid hydrolysis, 3 ml of distilled 6 N HCl was added to the ampoule and the solution was frozen in a Dry Ice bath; the ampoule was evacuated to 0.5 mm and sealed. Hydrolysis was performed at 105–107° for 15–24 hr.

For the detection of unnatural amino acids in the diborane-treated materials, dinitrophenylation and work-up were performed according to Mills (1952), both before and after acid hydrolysis. Reaction mixtures were extracted in turn with ether and with ethyl acetate, and the extracts were examined by thin-layer chromatography. Water-soluble DNP derivatives were not investigated.

Dinitropyridyl Derivatives of Amino Acids. The reagent, 2-chloro-3,5-dinitropyridine, and 3,5-dinitropyridyl derivatives of the common amino acids were obtained from Nutritional Biochemicals Corp. The latter were checked for homogeneity by thin-layer chromatography on silica gel GF (solvent system chloroform–methanol (24:1 or 4:1)). Chromatographic data on 3,5-dinitropyridylamino acids are summarized in Table II. These derivatives are highly sensitive to light; adequate precautions should be taken in all chromatographic procedures.

TABLE II: R_F Values of 3,5-Dinitropyridylamino Acids.[a]

3,5-Dinitropyridyl Derivative of	R_F Values[b,c] A	B	C	D
Ala	0.48		0.28	0.36
Asp	0.06	0.20	0.04	0.05
Glu	0.13	0.46	0.06	0.05
Gly	0.16		0.24	0.23
Met			0.29	0.55
NVal			0.43	0.51
Phe			0.33	0.65
Pro	0.46		0.40	0.26
Ser	0.07	0.37		0.10
Val	0.51		0.46	0.49

[a] Run on silica gel GF plates, 0.25 mm thickness (fully protected from light). [b] Values given are the averages of several runs. [c] Solvent systems were as follows: (A) chloroform–methanol–acetic acid (24:1:0.1, v/v), (B) chloroform–methanol–acetic acid (4:1:0.1, v/v), (C) benzene–pyridine–acetic acid (8:2:0.2, v/v), (D) upper phase of toluene–2-chloroethanol–0.8 N ammonium hydroxide–pyridine (10:6:6:3, v/v); plates were equilibrated with vapor of lower phase of D for 1 day prior to use.

Stability of 3,5-Dinitropyridylproline to Acid Hydrolysis. A sample of 3,5-dinitropyridylprolylglycine, which had been prepared according to the general procedure of Signor *et al.* (1964), was dissolved in a mixture of 2 ml of 60% formic acid and 2 ml of concentrated hydrochloric acid and the solution maintained at 60° for 13 hr. Following removal of solvent and ethyl acetate extraction of the residue, 3,5-dinitropyridylproline was purified by thin-layer chromatography and assayed

spectroscopically at 360 mμ, a 56% yield being obtained. Under similar conditions, 3,5-dinitropyridylproline itself was recovered to the extent of 84%.

Analysis of End Groups in Bovine γ-Globulin. An 80-mg sample of lyophilized bovine γ-globulin (Cohn fraction II, Sigma Chemical Co.) was dissolved in 4 ml of water containing 100 mg of sodium bicarbonate. A solution of 0.1 mmole of 2-chloro-3,5-dinitropyridine in 3 ml of ethanol was added and the mixture was stirred 2 hr at room temperature (dark). The resulting suspension was acidified to pH 1–2 with dilute HCl and extracted with ethyl acetate (four 6-ml portions). The extracts were discarded and the aqueous phase was centrifuged, discarding the supernatant, which was almost colorless. The yellow precipitate was transferred to a test tube with 2 ml of 60% formic acid; 2 ml of concentrated HCl was added and the solution was maintained at 60° for 9 hr (dark). The solution was diluted to 50 ml with water and the 3,5-dinitropyridylamino acids were extracted with several portions of ethyl acetate. The combined extracts were washed with water, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was transferred to a thin-layer chromatography plate (silica gel GF, 0.25 mm) and developed in two dimensions, first with solvent system D and then with system C. Yellow spots were eluted with 1% sodium bicarbonate and the 3,5-dinitropyridylamino acids assayed at 340 mμ (3,5-dinitropyridylproline was assayed at 360 mμ). The results of end-group analysis are given in Table VI.

Performic Acid Oxidation of Bovine γ-Globulin. To a solution of 250 mg of γ-globulin in 7.5 ml of 99% formic acid was added dropwise, at 0°, a solution of 0.8 ml of 30% hydrogen peroxide in 14.3 ml of 99% formic acid (Hirs, 1967). The solution was kept at 0° for 2.5 hr, diluted with 800 ml of ice-cold water, and lyophilized. Lyophilization of an aqueous solution was repeated twice and the product was finally dried over phosphorus pentoxide.

Diborane Reduction of γ-Globulin. To a suspension of 80 mg of lyophilized γ-globulin in 4 ml of tetramethylurea was added 2 ml of diborane in tetrahydrofuran and the mixture was stirred at 0° for 8 hr. An additional milliliter of diborane reagent was added after 4 hr. Solvents were removed *in vacuo*, the residue was suspended in methanol (containing 2% trifluoroacetic acid), and the mixture was stirred for several hours. Solvent was removed *in vacuo* and the methanol treatment was repeated several times. The same procedure was applied to performic acid oxidized γ-glogulin. End-group analyses were performed as described above.

Results

Reduction of Pyrrolidone. As the simplest model' pyrrolidone was reduced by diborane in tetrahydrofuran to pyrrolidine in 80% yield (identified and assayed as the DNP derivative), the reduction being performed at 0° for 2–3 hr.

Reduction of L-Pyroglutamamide. Owing to the poor solubility of the crystalline amide in tetrahydrofuran, at least 60% was recovered after several hours' exposure to diborane. More extensive reduction was observed using lyophilized samples (Table III). Yields of glutamic acid and proline were determined by dinitrophenylation of acid hydrolysates and thin-layer chromatography purification of the DNP derivatives. Because of further reduction of the prolinamide formed, the maximum recovery of proline (after acid hydrolysis) was 27%. In separate experiments it was found that prolinamide is reduced by diborane to 2-methylaminopyrrolidine, 30–40% conversion occurring under the conditions of reduction of pyroglutamide. To check the possibility of hydrogenolytic ring opening, dinitrophenylated acid hydrolysates were examined for components other than DNP-glutamic acid and DNP-proline. A trace (<1%) of the DNP derivative of norvaline was detected by thin-layer chromatography; there was no evidence for the formation of other products of reductive ring opening or for the reduction of the primary amide prior to that of the pyrrolidone ring.

TABLE III: Diborane Reduction of Pyroglutamamide.[a]

Temp (°C)	Time (hr)	Solvent	Yield (%)[b] Glu	Pro
0	0.5	Tetrahydrofuran	65	6
0	1	Tetrahydrofuran	45	10
0	3	Tetramethylurea	15	27
25	0.5	Tetrahydrofuran	31	8

[a] Crystalline material was lyophilized prior to reduction. [b] Yields of glutamic acid and proline were determined by dinitrophenylation of acid hydrolysates and thin-layer chromatography purification of the DNP derivatives.

Reduction of Simple Pyroglutamyl Peptides. As shown in Table IV, the highest yield of proline obtained in the reduction of ethyl pyroglutamylglycinate was 32%. It is clear that both the ester and peptide bonds are reduced competitively with the pyrrolidone ring. Since the reduction of the proline peptide bond appears to follow reduction of the pyrrolidone ring, the yield of proline will depend upon the relative rates of the consecutive reduction steps. Undoubtedly, the optimum reaction conditions will differ according to the peptide being reduced. Reduction at temperatures above 0° or in the presence of lower molar ratios of diborane resulted in decreased yields of proline. The formation of proline in solvents such as pyridine, dioxane, or 1,2-dimethoxyethane was considerably reduced relative to tetrahydrofuran or tetramethylurea. Although use of the collidine salt of pyroglutamylglycine inhibited reduction of the terminal carboxyl group, the recovery of glycine was still low, indicating a significant degree of attack at the peptide bond. Results with ethyl pyroglutamylglycylglycinate were comparable with those for the shorter peptide.

Reduction of Gastrin Octapeptide. Because of the insolubility of the peptide in tetrahydrofuran, only a

TABLE IV: Diborane Reduction of Pyroglutamyl Peptides at 0°.

Compound	Time (hr)	Solvent	Yield (%)[a]			
			Glu	Pro	Gly	2-Aminoethanol
PyroGlu-GlyOEt	0.5	Tetrahydrofuran	59	14	43	23
PyroGlu-GlyOEt	1	Tetrahydrofuran	45	29	37	29
PyroGlu-GlyOEt	3	Tetrahydrofuran	17	32	11	31
PyroGlu-GlyOEt	3	Tetramethylurea	45	14	45	21
PyroGlu-GlyOEt	5	Pyridine	71	0	71	0
PyroGlu-Gly collidine salt	5	Tetrahydrofuran	18	18	25	0
PyroGlu-Gly-GlyOEt	1	Tetrahydrofuran	56	16	36[b]	23
PyroGlu-Gly-GlyOEt	2	Tetrahydrofuran	45	18	28	23
PyroGlu-Gly-GlyOEt	3	Tetrahydrofuran	24	25	32	21
PyroGlu-Gly-GlyOEt	2.5	Tetramethylurea	33	20	49	17

[a] Yields were determined by dinitrophenylation of acid hydrolysates and thin-layer chromatography purification of the DNP derivatives. [b] Based on 2 equiv of glycine.

TABLE V: Diborane Reduction of Gastrin Octapeptide.[a]

Time (hr)	Solvent	Yield (%)[b]								
		Asp	Glu	Gly	Ala	Met	Tyr	Phe	Pro	Homoser[c]
	d	100[e]	127	99	99	94	100	100		
3	Tetramethylurea[f]	100	127	100	100	96	100	98		
3	Tetramethylurea	40	62	100	100	76	0	83	8	26
6	Tetramethylurea	18	54	49	83	73	0	67	46	14
4	Tetramethylurea[g]	25	63	67	79	80	70	67	29	6
4	Tetrahydrofuran[g]	87	76	96	98	84	85	85	<1	8

[a] Lyophilized material, reduced at 0°. [b] No effort was made to determine tryptophan. [c] Values do not include homoserine lactone, which behaves as a basic amino acid chromatographically. [d] Analysis of original peptide. [e] The relative value for aspartic acid was taken as 100%. [f] Analysis of peptide which had been lyophilized and stored in tetramethylurea for 3 hr. [g] Crystalline peptide was used in these runs.

small degree of reduction was observed, primarily at the free carboxyl groups; for the same reason, presumably, the amount of proline formed by reduction was negligible (Table V). The lyophilized peptide was almost completely soluble in tetramethylurea and was unaffected by exposure to the solvent. Reduction in tetramethylurea for 6 hr produced 46% proline, extensive reduction of aspartic acid to homoserine occurring at the same time. Unexpectedly, the amino acid analysis showed total loss of tyrosine, as well as the appearance of a new peak in the position of methionine sulfone. Such extensive destruction of tyrosine did not occur upon reduction of the crystalline octapeptide, which was partially soluble in tetramethylurea, or upon substitution of trifluoroacetic acid for HCl in the hydrolytic step. Furthermore, simple tyrosine derivatives were unchanged when subjected to a similar sequence of steps (see below). That the loss of tyrosine occurred during acid hydrolysis was supported further by the fact that the ultraviolet spectrum of the octapeptide was essentially unchanged (both in neutral and alkaline media) following reduction and methanol treatment. These transformations of tyrosine and methionine, which are evidently oxidative in nature, will require further investigation for their clarification.

Dinitrophenylation of the reduced octapeptide, followed by acid hydrolysis, failed to reveal any ether or ethyl acetate extractable DNP derivatives, other than that of proline. Considerable yellow color remained in the aqueous phase, presumably due to DNP derivatives of polyamines resulting from reduction of peptide bonds. The reduced octapeptide showed, upon electrophoresis, several basic, ninhydrin-positive spots with mobilities (on paper) about twice that of lysine at pH 2.

Although small amounts of proline are generated by diborane reduction of native bovine γ-globulin, considerably greater yields are obtained following performic acid oxidation (Table VI). It is interesting to note that glycine appears as an N terminal only after performic acid oxidation. Whether glycine becomes

TABLE VI: Diborane Reduction of Bovine γ-Globulin.

State of Protein[a]	Recoveries of 3,5-Dinitropyridylamino Acids[b]				
	Val	Ala	Gly	Asp + Ser[c]	Pro[d]
Control	0.30	0.04	0.02	0.22	0
Diborane reduced	0.24	0.04	0.02	0.20	0.04
Oxidized control	0.34	0.05	0.16	0.20	Trace
Oxidized and reduced	0.24	0.05	0.16	0.20	0.32

[a] Lyophilized sample of bovine γ-globulin, Cohn fraction II. [b] Expressed as moles per mole of protein, molecular weight 160,000. [c] Reported as combined yield because of uncertainty in separation by thin-layer chromatography. [d] Corrected for destruction during hydrolysis.

exposed by unfolding or as the result of oxidative cleavage of peptide bonds following tryptophan or tyrosine (Witkop, 1961) has not been determined.

The use of 2-chloro-3,5-dinitropyridine as a reagent for identification and assay of N-terminal amino acids (Signor *et al.*, 1964) was explored as an alternative to the more common methods. Despite the sensitivity of 3,5-dinitropyridylamino acid to light (slightly greater than that of DNP-amino acids), the ease of manipulation, hydrolytic cleavage, and separation of products by thin-layer chromatography invite further consideration of the method. It should be noted that the loss of 3,5-dinitropyridylproline from a dipeptide was greater than that from the amino acid derivative itself, a factor which may be relevant in other estimates of destructive loss during acid hydrolysis.

Stability of Other Functional Groups toward Diborane. TRYPTOPHAN. Solutions of tryptophan, methyl tryptophanate, *N*-acetyltryptophanamide, and methyl bis(trifluoroacetyl)tryptophanate in tetramethylurea were exposed to diborane for 6 hr at 0°. After the usual work-up, the indole chromophore was regenerated almost quantitatively in each case. Prior to methanol treatment, the spectra showed additional peaks at 270 mμ, presumably due to borate complexes with the indole nitrogen.

HISTIDINE. A solution of *N*-benzoylhistidine in tetrahydrofuran was exposed to diborane for 5 hr at 0°. After work-up and methanol treatment, at least 98% of the imidazole ring was still present, on the basis of color yield in the diazo coupling reaction (Macpherson, 1946).

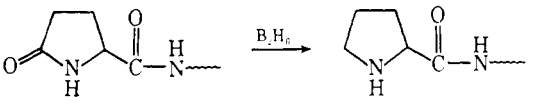
ARGININE. A solution of ethyl *N*-benzoylarginate in tetramethylurea was treated with diborane for 6 hr at 0°. Colorimetric analysis by the Sakaguchi method (Weber, 1930), following methanol treatment, showed 95% recovery of the guanidino function.

TYROSINE. Phenol, tyramine, tyrosylglycine, *N*-acetyltyrosinamide, and ethyl *N*-acetyltyrosinate, in tetramethylurea solution, were exposed to diborane for 6 hr at 0°. Following work-up, methanol treatment, and 24-hr hydrolysis with 6 N HCl, the phenolic chromophore was regenerated intact in each case.

Discussion

The rate of reduction of carboxyl derivatives by lithium aluminum hydride and related hydrides decreases in the order: ester > acid > amide. In remarkable contrast, diborane reduces acids and amides more rapidly than esters (House, 1965, p 43; Kornet *et al.*, 1968) and secondary and tertiary amides more rapidly than primary amides (Brown and Heim, 1964). The inversion in order may be the result of coordinate complexing of the electron-deficient boron with the more basic electron pair of amide nitrogen or carboxyl oxygen, followed by intramolecular hydride transfer to carbonyl. With this concept in mind, we reasoned that borane might complex selectively with the nitrogen atom of a pyrrolidone ring, which should be more accessible sterically than a peptide nitrogen. Furthermore, reduction might be selective by analogy with the fact that lactones are reduced considerably faster than esters by borohydride (Cocker and McMurry, 1956).

The selective reduction of carboxyl groups by diborane has been reported for peptides (Rosenthal and Atassi, 1967) and for proteins (Atassi and Rosenthal, 1967). Our results with the gastrin octapeptide indicate that, whereas the β-carboxyl group of aspartic acid is attacked most rapidly by diborane, peptide bonds are subject to somewhat slower reduction[3] and



the pyrrolidone ring occupies an intermediate position. Conversions of pyroglutamic acid into proline of 30–45% are ample to permit qualitative identification of a newly formed N-terminal proline residue. Furthermore, the concurrent loss of peptide bonds by reduction is not so great as to discourage sequence determination studies.

The reductive generation of N-terminal proline in bovine γ-globulin confirms the earlier demonstration, by indirect methods, of the presence of N-terminal pyrrolidonecarboxylic acid in the γ-globulins of other species (see, *e.g.*, Bennett, 1968; Wilkinson *et al.*, 1966). Furthermore, the conversion demonstrates the applicability of the method for high molecular weight substrates. The sizeable increase in proline yield following performic acid oxidation may be the result of unfolding or of reduction in unit molecular weight, or both.

[3] A similar lack of selectivity has been observed by Yonemitsu *et al.* (1968).

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