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Specific Reduction of Carboxyl Groups in Peptides and Proteins by Diborane

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1. The reaction of several peptides and proteins with diborane was studied under different conditions to determine those most suitable for the specific reduction of carboxyl groups. 2. In the reaction of model peptides and the cyclic peptides bacitracin and tyrocidin, reduction at 0° was entirely specific for the carboxyl groups without affecting the peptide bonds. Acid amide residues were not reduced. Some tripeptides showed anomalous results in that the *C*-terminal residue was quite resistant to reduction. 3. Specific reduction of carboxyl groups was achieved in each of the following proteins: human serum albumin, egg albumin, adult human haemoglobin, sperm-whale apomyoglobin, horse heart cytochrome *c* and egg-white lysozyme. The *C*-terminal amino acid was usually reduced. 4. Conditions for specific reduction of all available carboxyl groups are not easily found and may vary from one substance to another. Specific reduction of a limited number of available carboxyl groups may be generally accomplished by reactions at $-10°$. 5. It is suggested that this chemical modification, which has the advantage of permanence, may be useful in studying the role of carboxyl groups in the conformation of proteins and in the biological properties of peptides and proteins.

Chemical modification of proteins at a variety of specific amino acid sites can now be performed by various procedures. Specific modification of carboxyl groups, however, has not always been satisfactorily accomplished. Information about carboxyl groups in proteins has largely been based on methods with esterified derivatives. Conditions of esterification, however, are too drastic when methanol–hydrochloric acid is used, or suffer from non-specificity with diazomethane or poor yields with diazoacetoglycine amide (Riehm & Scheraga, 1965). Water-soluble carbodi-imides have been employed, but the derivatives obtained are not easily characterized (Sheehan & Hlavka, 1956; Franzblau, Gallop & Seifter, 1963; Goodfriend, Levine & Fasman, 1964; Riehm & Scheraga, 1966). Carboxyl-group activation with carbodi-imides followed by reaction with glycine methyl ester (Hoare & Koshland, 1966), glycine *N*-phthalimidomethyl ester or alanylglycine *N*-phthalimidomethyl ester (Wilcheck, Frensdorff & Sela, 1967) seems to present a substantial improvement in yield and ease of characterization.

The main problem with all these procedures is that the foreign group introduced is cleaved off on acid or alkaline hydrolysis and cannot, therefore, be used for identification of the *C*-terminus or wherever a permanent modification is desirable.

Diborane can reduce carboxyl groups to the corresponding alcohols (Brown & Subba Rao, 1960; Brown & Korytnyk, 1960; Brown & Heim, 1964). We showed (Rosenthal & Atassi, 1967) that this reaction can be adapted for the reduction of carboxyl groups in peptides and that under appropriate conditions the peptide bond is not attacked by the reagent. In the present work, a few more model peptides containing other *C*-terminal amino acids were studied to determine the reducibility of these residues. Reduction of bacitracin and tyrocidin provided further evidence of the specificity for carboxyl groups and demonstrated lack of reaction of acid amide groups. The reaction was also studied on several model proteins under different conditions to determine those most suitable for specificity, and to identify amino acids most likely to be modified when side reactions occurred. In all the proteins examined specificity for the reduction of carboxyl groups was achieved.

MATERIALS AND METHODS

Peptides and proteins. Bacitracin, tyrocidin hydrochloride and chromatographically pure dipeptides were from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.).

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Phenylalanylarginine and 2-amino-5-hydroxyvaleric acid were from Cyclo Chemical Corp. (Los Angeles, Calif., U.S.A.). Tri- and tetra-peptides were from Miles Laboratories Inc. (Elkhart, Ind., U.S.A.). Twice-crystallized hen's-egg albumin was prepared and generously supplied by Dr Rapier McMenamy. Crystalline horse heart cytochrome *c* (type III) and crystalline hen's-egg-white lysozyme (grade III) were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Crystalline human serum albumin was purchased from Calbiochem (Los Angeles, Calif., U.S.A.). Human adult haemoglobin and sperm-whale myoglobin were the major fractions (HbA_0 and MbX) isolated from the crystalline proteins as described by Atassi (1964*a*,*b*). Apomyoglobin was prepared from fraction MbX by the procedure of Theorell & Akeson (1955).

Reduction with diborane. Peptide trifluoroacetates were reduced with diborane by the procedure described by Rosenthal & Atassi (1967). Protein (3–4 mg.) was dissolved in 0·5 ml. of water at 0°; trifluoroacetic acid (2 drops) was added, and after agitation for 10–20 sec. the solution was immediately freeze-dried. After further drying over P_2O_5 *in vacuo* for 16–20 hr., the protein trifluoroacetates were reduced with different volumes of 1 M-diborane (see Tables 4–9) in tetrahydrofuran (Ventron Metal Hydrides Division, Beverly, Mass., U.S.A.). For each protein, reaction was carried out at various temperatures and durations to find conditions most suitable for specific reduction of carboxyl groups in that protein. After reaction, the solvent and excess of diborane were removed in a stream of N_2, and 1 ml. of a solution of 10% (v/v) glycerol in 0·15 M-potassium phosphate buffer, pH 7·5, was then added. The mixtures were kept for 2 hr., after which they were dialysed once against 100 vol. of the same buffer and extensively against water, and then freeze-dried.

Analytical methods. Electrophoresis was done in starch gel and in acrylamide gel at room temperature. Electrophoresis in starch gel was by the discontinuous buffer system of Poulik (1957). When acrylamide was employed, electrophoresis was in 6% (w/v) acrylamide either in 0·05 M-veronal buffer, pH 8·6, or in 0·015 M-sodium phosphate buffer, pH 7·0, with a potential gradient of 20 v/cm. The gels were stained with Amido Black. Absorption spectra were measured in a Cary model 14 spectrophotometer. Nitrogen was determined in a micro-Kjeldahl apparatus similar to that described by Markham (1942). Alkaline hydrolysis of proteins was carried out in saturated aq. $Ba(OH)_2$ solution by the procedure of Ray & Koshland (1962). Acid hydrolysis was performed at 110° for 22 or 72 hr. in constant-boiling HCl (double-distilled) in N_2-flushed evacuated sealed tubes. Amino acid analyses were done on a Spinco model 120 C amino acid analyser. Titration of total thiol groups with *p*-hydroxymercuribenzoate was done in 5 M-urea–0·05 M-sodium phosphate buffer, pH 5·2, by the procedure described by Atassi (1964*a*). *N*-Terminal determination was carried out by the subtractive Edman degradation procedure described by Konigsberg & Hill (1962).

RESULTS

Reduction of peptides. Small peptides containing different *C*-terminal amino acids were reduced to investigate the reducibility of various amino acids at the *C*-terminus. Table 1 summarizes the results of the reduction of ten different peptides containing the following amino acids at the *C*-terminus: proline, threonine, serine, isoleucine, arginine, tryptophan, lysine, phenylalanine and phenylalanine amide. The carboxyl group of a proline residue at the *C*-terminus was only slightly reduced (22%), whereas the reduction of the other *C*-terminal amino acids with a free carboxyl group was between 60 and 100%. The finding that phenylalanine was completely reduced (100%) whereas phenylalanine amide was only partially reduced (36%) suggests that acid amide residues are not reducible. The limited reduction obtained with phenylalanine amide might have been due to some removal of the amide group during trifluoroacetic acid treatment and manipulation. Rosenthal & Atassi (1967) showed that the amino acids aspartic acid, asparagine, glutamic acid, glycine, tyrosine and phenylalanine at the *C*-terminus in model peptides were also reducible with diborane. The extent of reduction varied, glycine being the most resistant, and there was also evidence that it was influenced by the neighbouring residue. Free amino acids were never observed by amino acid analysis in the diborane-reduced products before hydrolysis. On the other hand, quantitative recovery of the *N*-terminal amino acids after hydrolysis (Table 1) provided strong evidence that the peptide bond of the *N*-terminal amino acid

Table 1. *Reduction of some peptide trifluoroacetates with diborane*

The peptide (3–5 mg.) was reduced in its trifluoroacetate form with 0·5 ml. of 1 M-diborane in tetrahydrofuran at 0° for 2 hr. For the procedure and results of the reduction of other model peptides see Rosenthal & Atassi (1967). Yields were based on determination of nitrogen in a portion of the acid hydrolysate and the recovery of unchanged amino acids on the analyser.

Peptide	Reduction of *C*-terminus (%)	Recovery of *N*-terminus (%)
Gly-Pro	21·9	98·3
Gly-Phe amide	36·0	96·5
Gly-Thr	85·3	98·6
Gly-Ile	94·6	97·2
Phe-Arg	98·8	96·4
Leu-Trp	99·9	93·1
Gly-Phe	100·0	94·6
Gly-Asp-Ser	60·4	*
Pro-Phe-Gly-Lys	87·5	*
Val-Ala-Ala-Phe	100·0	*

* The reduced peptides had the following compositions (moles/mole): from Gly-Asp-Ser, Gly, 0·980; Asp, 0·296; Ser, 0·396; from Pro-Phe-Gly-Lys, Pro, 1·20; Phe, 1·00; Gly, 0·738; Lys, 0·125; from Val-Ala-Ala-Phe, Val, 1·00; Ala, 1·82; Phe, 0.

was not reduced with diborane under these conditions.

Several tripeptides showed anomalous results on reduction with diborane. The *C*-terminus of Val-Tyr-Val, Leu-Gly-Leu and Met-Gly-Met did not undergo any reduction under the conditions used in Table 1 (0·5ml. of 1M-diborane at 0° for 2hr.). Reduction of the peptide trifluoroacetates under more vigorous conditions (room temperature for 16hr.) resulted surprisingly in a hydrolytic reaction leading virtually to complete elimination only of the central amino acid. This was probably due to reduction of the carbonyl group of the central amino acid to the primary alcohol with bond cleavage. At any rate, these vigorous conditions were not suitable for use with proteins owing to their lack of specificity (see Tables 4 and 6).

The reduced *C*-terminal amino acids are accounted for in amino acid analyses by a shoulder on the ammonia peak. The resolution of these amino alcohols on the analyser has not yet been accomplished.

The results of reduction of bacitracin are shown in Table 2. The reaction resulted in the reduction of only dicarboxylic amino acids. After reduction, there was a decrease of 1 mole of aspartic acid/mole and 0·64 mole of glutamic acid/mole. The results suggested that the asparagine residue in bacitracin was not reduced, indicating the probable resistance of acid amide residues to reduction with diborane. This was well confirmed by the results of reduction of tyrocidin. Tyrocidin has one asparagine residue and one glutamine residue and, since it is a cyclic peptide, no terminal carboxyl group. Therefore if reduction with diborane were indeed specific for carboxyl groups no modification should be observed on reduction of tyrocidin. The results in Table 3 show that this was true to a large extent. Reduction of the glutamine residue was obtained to a small extent; this was most likely to be due to some removal of the amide group during manipulation and trifluoroacetic acid treatment. It is well known that glutamine is deaminated more readily than asparagine. Determination of *N*-terminal amino acids by the subtractive Edman procedure revealed that in reduced bacitracin and reduced tyrocidin no amino acids were *N*-terminal. This confirmed the integrity of the cyclic structure in the two reduced peptides and thus excluded the possibility of peptide-bond cleavage on reduction.

The specificity of diborane for carboxyl groups on model peptides having been demonstrated, it was desirable to study the application of this procedure to proteins.

Reduction of human serum albumin. The effect of diborane on human serum albumin under various conditions is shown in Table 4. If the protein is not first protonated no reduction of any amino acid is observed. When reaction was carried out for an extended duration (16hr.) at room temperature, extensive reduction of aspartic acid and glutamic acid was obtained, but the reaction was not specific for these amino acids. In addition, appreciable reduction of prolyl, histidyl and arginyl residues occurred. However, when reaction was carried out at room temperature for 30min. or at 0° for 4hr., the reduction was entirely specific for aspartic acid and glutamic acid. The number of total thiol groups in albumin (0·82 mole/mole of protein) increased to

Table 2. *Amino acid composition of bacitracin and diborane-reduced bacitracin*

Bacitracin (4mg.) was reduced with 0·5ml. of 1M-diborane in tetrahydrofuran at 0° for 2hr. The results are the means of four hydrolyses (two at 22hr. and two at 72hr.). The 'expected' values for the composition are from Hausmann, Weisiger & Craig (1955).

	Amino acid composition (moles/mole of peptide)		
Amino acid	Expected	Found (bacitracin)	Found (reduced bacitracin)
Asp	1	1·98	1·03
Asn	1		
Glu	1	1·01	0·360
Gln	0	—	—
CyS	1	0·240	0·231
Ile	3	2·59	2·62
Leu	1	0·965	1·02
Phe	1	1·02	0·979
Orn	1	1·05	1·08
Lys	1	1·06	1·06
His	1	0·810	0·905

Table 3. *Amino acid composition of tyrocidin and diborane-reduced tyrocidin*

Tyrocidin (4mg.) was reduced with 0·5ml. of 1M-diborane in tetrahydrofuran at 0° for 2hr. The results are the means of four hydrolyses (two at 22hr. and two at 72hr.). The 'expected' values for the composition are from Battersby & Craig (1952).

	Amino acid composition (moles/mole of peptide)		
Amino acid	Expected	Found (tyrocidin)	Found (reduced tyrocidin)
Asp	0	0·895	0·950
Asn	1		
Glu	0	0·981	0·635
Gln	1		
Pro	1	0·873	0·916
Val	1	1·23	1·12
Leu	1	1·17	1·31
Tyr	1	0·811	0·976
Phe	3	2·63	2·47
Orn	1	0·993	1·00

Table 4. *Reduction of human serum albumin with diborane*

The volume of 1M-diborane in tetrahydrofuran shown is that used per 3–4 mg. of protein. The values are expressed in moles of amino acid/mole of albumin (mol.wt. 69000). (*a*) The values represent the average of ten analyses; (*b*) the values represent the average of two analyses; (*c*) the values were obtained from one 22 hr. hydrolysis; n.d., not determined. The thiol groups were determined by titration with *p*-hydroxymercuribenzoate (see the text). Only the amino acids suffering change on reduction are given. All other amino acids remained unaltered.

		Amino acid composition (moles/mole)				
	Protein ...	Albumin		Albumin trifluoroacetate		
Amino acid	Treatment ...	None (*a*)	0·5ml. of diborane, 22°, 30min. (*b*)	3ml. of diborane, 22°, 16hr. (*c*)	0·5ml. of diborane, 22°, 30min. (*b*)	0·5ml. of diborane, 0°, 4hr. (*b*)
Asp		53·4	56·6	2·83	29·8	34·8
Glu		90·0	90·9	11·3	58·2	63·1
Pro		29·0	26·3	12·2	27·7	27·9
Gly		13·1	13·8	11·7	13·5	13·1
Ala		65·1	64·0	59·8	62·2	62·9
His		15·9	15·4	8·09	16·3	15·3
Arg		23·3	23·0	14·9	23·4	23·7
Thiol		0·82	30·2	30·8	n.d.	31·1

Table 5. *Reduction of hen's-egg albumin with diborane*

The volume of 1M-diborane in tetrahydrofuran shown was that used per 3–4mg. of protein. The values are expressed in moles of amino acid/mole of protein (mol.wt. 46000). Tryptophan was determined from a single alkaline hydrolysis. The thiol groups were determined by titration of the intact protein with *p*-hydroxymercuribenzoate (see the text). (*a*) Mean of ten analyses (six 22hr. and four 72hr. hydrolyses); (*b*) obtained from a single 22 hr. hydrolysis; (*c*) obtained from two analyses (one 22 hr. and one 72 hr. hydrolysis); n.d., not determined.

		Amino acid composition (moles/mole)			
	Protein ...	Egg albumin		Albumin trifluoroacetate	
Amino acid	Treatment ...	None (*a*)	0·5ml. of diborane, 22°, 30min. (*b*)	0·5ml. of diborane, 22°, 30min. (*c*)	0·5ml. of diborane, 0°, 4hr. (*c*)
Asp		33·1	32·9	23·5	28·7
Thr		16·0*	15·1	15·8*	15·9*
Ser		38·3*	38·1	37·7*	37·9*
Glu		51·7	52·6	35·5	42·1
Pro		13·6	14·2	14·6	13·8
Gly		21·1	23·5	19·9	20·8
Ala		35·6	36·1	36·0	36·5
CyS		5·24	4·65	5·41	6·2
Val		33·7	32·7	33·3	33·4
Met		16·3	17·1	16·6	16·4
Ile		24·7	23·0	23·5	24·2
Leu		30·8	31·0	31·4	31·3
Tyr		9·57	9·21	10·0	9·76
Phe		21·5	19·6	21·4	21·2
Lys		20·1	21·3	21·1	20·8
His		7·16	7·11	5·48	6·56
Arg		14·9	14·2	14·3	14·1
Trp		2·81	n.d.	2·72	2·79
Thiol		5·18	7·24	n.d.	6·70

* Extrapolated to zero hydrolysis time.

30·8 moles/mole after reduction at room temperature for 16 hr. and to 31·1 moles/mole after reduction at 0° for 4 hr., suggesting, as expected, a ready reductive cleavage of the disulphide bonds in albumin.

The reduced proteins were all positively charged on electrophoresis, but migrated as single bands to the cathode at pH 8·6 in starch or acrylamide gels and at pH 7·0 in acrylamide. Migration in acrylamide gel was greater than that in starch gel. In no case was any unmodified protein detected. No spectral changes were observed in the ultraviolet region on reduction.

Reduction of egg albumin. Reduction of egg albumin with diborane under different conditions is shown in Table 5. Reduction was obtained only when the protein had been protonated with trifluoroacetic acid. Reduction of the protein trifluoroacetate at room temperature for 30 min. showed that, in addition to an extensive reduction of the dicarboxylic amino acids, 1–2 moles of histidine/mole were also reduced. However, reduction at 0° for 4 hr. was specific for the carboxyl groups with concomitant reduction of the disulphide bond.

Reduction of haemoglobin. Table 6 shows the reduction of haemoglobin with diborane. Values are expressed in moles per mole of the half-molecule (i.e. of the $\alpha\beta$ unit). Prolonged reaction at room temperature for 22 hr. resulted in an extensive reduction of the carboxyl groups. The total aspartate reduced was greater than the number of aspartic acid residues present in haemoglobin. However, our work on model peptides has shown that acid amide groups are not normally reduced and only react when they are hydrolysed to carboxyl groups. Therefore the excess of aspartate reduced in haemoglobin might have been caused by the hydrolysis of asparagine residues during manipulation of the protein. Extensive destruction of histidine was observed. Also, 1 mole of arginine/mole was reduced, which might have been the arginine at the *C*-terminus of the α-chain.

Reduction at room temperature for 30 min. was less extensive for the carboxyl groups and in addition 1 mole of histidine/mole was modified, which may have been the *C*-terminal histidine of the β-chain. Reduction at 0° for 4 hr. was completely specific for some of the dicarboxylic amino acids. The *C*-terminal residues did not apparently react, suggesting their inaccessibility under these conditions. This derivative reduced at 0° had two *N*-terminal valine residues per $\alpha\beta$ unit. Valine occupies the *N*-terminal positions in both the α- and the β-chain (Braunitzer *et al.* 1961; Hill & Konigsberg, 1962; Goldstein, Konigsberg & Hill, 1963). No other *N*-terminal residues were revealed, suggesting the absence of peptide-bond cleavage.

Reduction of sperm-whale apomyoglobin. Table 7 shows the effect of diborane on apomyoglobin

Table 6. *Reduction of adult human haemoglobin with diborane*

Haemoglobin A_0 was reduced with 1 M-diborane in tetrahydrofuran, the volumes shown being used per 3–4 mg. of protein. The values are expressed in moles of constituent amino acids/mole of $\alpha\beta$ unit, and were obtained each from two hydrolyses (one 22 hr. and one 72 hr.). Tryptophan was obtained from duplicate alkaline hydrolyses. The thiol groups were determined by titration of the intact protein with *p*-hydroxymercuribenzoate (see the text). Only values for the amino acids that showed change on reduction are given. All other amino acids remained unaltered. The theoretical values are from Konigsberg & Hill (1962). Histidine is *C*-terminal in the β-chain (Braunitzer *et al.* 1961; Goldstein *et al.* 1963) and arginine in the α-chain (Braunitzer *et al.* 1961; Hill & Konigsberg, 1962). n.d., Not determined.

	Amino acid composition (moles/mole)						
Protein ...	Haemoglobin				Haemoglobin trifluoroacetate		
Amino acid	Theoretical	Treatment ...	None	0·5 ml. of diborane, 22°, 30 min.	3 ml. of diborane, 22°, 22 hr.	0·5 ml. of diborane, 22°, 30 min.	0·5 ml. of diborane, 0°, 4 hr.
Asp	15		25·0	24·9	4·27	16·7	16·6
Asn	10						
Glu	12		16·4	16·5	6·89	12·5	10·8
Gln	4						
Pro	14		14·5	14·1	9·48	11·7	13·9
CyS	3		2·83	2·55	2·55	n.d.	2·75
His	19		18·8	18·9	8·40	17·5	18·9
Arg	6		5·69	5·99	5·09	5·99	6·24
Trp	3		2·69	2·74	2·66	2·81	2·80
Thiol	3		2·70	2·63	2·85	2·72	n.d.

Table 7. *Reduction of sperm-whale apomyoglobin with diborane*

The apoprotein trifluoroacetate was reduced with 1M-diborane in tetrahydrofuran, 0·5ml. being used per 4–5mg. of protein. The extent of reduction was independent of the amount of diborane used in the range 0·25–3ml. per 4–5mg. The values are expressed in moles of the constituent amino acids/mole of protein and were obtained from four analyses (two 22hr. and two 72hr. hydrolyses). Tryptophan was determined by duplicate analyses of alkaline hydrolysates. Threonine and serine were extrapolated to zero hydrolysis times. Glycine is *C*-terminal (Edmundson, 1965).

	Amino acid composition (moles/mole)				
Protein ...	Untreated apomyoglobin			Reduced apomyoglobin	
Amino acid	Expected	Found	Treatment ...	0°, 4hr.	−10°, 4hr.
Asp Asn	6 2	7·84		4·73	4·54
Thr	5	4·85		5·22	4·60
Ser	6	5·93		5·78	5·75
Glu Gln	14 5	19·1		7·88	13·1
Pro	4	3·85		2·07	4·16
Gly	11	11·0		9·87	9·94
Ala	17	16·9		16·9	17·0
Val	8	7·74		7·65	7·54
Met	2	2·01		2·06	2·05
Ile	9	9·22		8·96	8·44
Leu	18	17·8		18·1	17·9
Tyr	3	3·11		3·19	3·03
Phe	6	6·03		6·00	5·91
Lys	19	19·0		18·8	19·2
His	12	12·0		9·92	11·9
Arg	4	3·96		3·07	4·03
Trp	2	1·86		1·88	1·79

trifluoroacetate. Reduction at 0° for 4hr. was not specific for carboxyl groups since, in addition, 2 moles of proline, 2 moles of histidine and 1 mole of arginine/mole were modified. The above results indicated that these conditions were completely specific for carboxyl groups in serum albumin, egg albumin and haemoglobin. Reduction with complete specificity for carboxyl groups was obtained when reaction was carried out at −10° for 4hr. Also 1 mole of glycine/mole was reduced. Glycine is *C*-terminal (Edmundson, 1965). In model peptides glycine residues at the *C*-terminus appeared to be resistant to reduction with diborane (Rosenthal & Atassi, 1967).

Myoglobin re-formed from apomyoglobin reduced with diborane at −10° for 4hr. and unmodified ferrihaem by the procedure of Atassi & Caruso (1968) was homogeneous on electrophoresis in starch gel at pH8·8 and in acrylamide gel at pH8·6. Under all these conditions the protein was positively charged and therefore migrated in the opposite direction to fraction MbX of myoglobin. The derivative in 10mM-sodium phosphate buffer, pH7·15, containing 0·01% of potassium cyanide gave spectra similar to those of fraction MbX, with maxima at 270, 360, 419 and 540mμ. In the same solvent, fraction MbX showed maxima at 278, 360, 423 and 540mμ. *N*-Terminus determination on apomyoglobin reduced with diborane at −10° for 4hr. revealed the presence of only one *N*-terminal amino acid, valine, which is at the *N*-terminus in native sperm-whale myoglobin (Edmundson, 1965).

Reduction of cytochrome c. The reaction of cytochrome *c* trifluoroacetate with diborane was carried out only under one set of conditions, namely at −10° for 2hr. with 1ml. of 1M-diborane. Under these conditions the reaction was completely specific for the dicarboxylic amino acids (Table 8); 2 moles of aspartic acid/mole out of a possible total of 3, but only 2 moles of glutamic acid/mole out of a possible total of 9, reacted. Glutamic acid is *C*-terminal in cytochrome *c* (Margoliash *et al.* 1961).

Reduction of lysozyme. Reduction of lysozyme trifluoroacetate with diborane is shown in Table 9. At room temperature the reaction was not specific for aspartyl or glutamyl residues: arginine and some histidine also suffered modification. Exactly 1 mole of leucine/mole was reduced. Leucine is *C*-terminal (Canfield & Liu, 1965), and it is likely that this is reduced to leucinol on reaction with diborane. There appeared to be a loss of 1 mole of glycine/mole, which cannot be explained.

Table 8. *Reduction of horse heart cytochrome c with diborane*

The reaction was carried out on the protein trifluoroacetate at −10° for 4hr. with 1ml. of 1M-diborane in tetrahydrofuran per 2mg. of protein. The values are expressed in moles of amino acid/mole of protein and were obtained from duplicate 22hr. and 72hr. hydrolyses. Tryptophan was determined from duplicate alkaline hydrolyses. Threonine was extrapolated to zero hydrolysis time. The 'expected' values are from Margoliash, Smith, Kreil & Tuppy (1961).

	Amino acid composition (moles/mole)		
Amino acid	Expected	Found (untreated cytochrome *c*)	Found (reduced cytochrome *c*)
Asp	3	7·96	6·21
Asn	5		
Thr	10	9·82	10·02
Ser	0	0·08	0·14
Glu	9	12·1	9·28
Gln	3		
Pro	4	3·88	4·29
Gly	12	12·0	11·5
Ala	6	5·93	6·23
CyS	2	1·80	1·88
Val	3	3·03	3·07
Met	2	2·00	2·04
Ile	6	6·30	6·51
Leu	6	6·11	6·31
Tyr	4	3·75	3·69
Phe	4	3·82	3·93
Lys	19	19·1	18·7
His	3	2·73	2·65
Arg	2	1·99	2·05
Trp	1	0·86	0·92

Reduction at −10° was specific for aspartic acid and glutamic acid residues, and the *C*-terminal leucine residue was again reduced. There was some loss of arginine, but the apparent increase in the value for glycine cannot be explained since there is no reaction product that is expected to (or has been observed to) appear in this position. The reduced protein showed only lysine at the *N*-terminus by subtractive Edman degradation. Lysine is *N*-terminal in native lysozyme (Canfield & Liu, 1965). This suggested that no cleavage of peptide bonds had taken place.

Products of reduction of aspartyl and glutamyl residues. Reduction of aspartyl and glutamyl residues involved in peptide linkage through their α-carboxyl groups should give rise to homoserine and 2-amino-5-hydroxyvaleric acid respectively. Indeed, in all the reduced proteins a new peak appeared on the analyser 2–3min. before glutamic acid at a position that coincided with that of homoserine and authentic 2-amino-5-hydroxyvaleric acid. The colour-yield constant of 2-amino-5-hydroxyvaleric acid was 0·971 ± 0·036 (mean ± S.D.) of that of aspartic acid. The recovery of homoserine or 2-amino-5-hydroxyvaleric acid or both did not account for the loss of aspartic acid and glutamic acid in a reduced protein. However, formation of γ- and δ-lactones can take place during acid hydrolysis and drying of the hydrolysates. In fact, a new peak always appeared 7min. after ammonia in the position where homoserine lactone emerged. It is likely that 2-amino-δ-valerolactone appears at the same position. The total recovery of lactones (obtained by using the colour-yield constant of homoserine lactone) and their corresponding hydroxy acids accounted for more than 90% of the loss in aspartic acid and glutamic acid contents on reduction of a protein.

DISCUSSION

The results reported here show for the first time that it is possible to achieve the reduction of carboxyl groups in proteins without affecting the peptide linkage. If the reagent were employed for complete reduction of all available carboxyl groups in a protein then, since the reaction would be carried out under relatively more vigorous conditions, non-specificity would probably be encountered. On the other hand, the conditions necessary to achieve the maximum possible reduction of carboxyl groups, with absolute specificity being maintained, are not easily found. These conditions vary from one substance to another and have to be determined for each protein independently. The results with apomyoglobin suggest that the extent of reduction is independent of the amount of diborane used (several-thousand-molar excesses are employed per mole of protein or peptide). Therefore only changes of specificity with temperature need be investigated. However, if the aim is to modify only a limited number of carboxyl groups to study their role in protein function, then the reaction should be carried out under very mild conditions, at a temperature of 0° or lower, and non-specificity can then be avoided. In fact, reaction at −10° was entirely specific for carboxyl groups, and it is suggested that it may be generally employed for protein reduction. It is not unusual that some of the carboxyl groups are not reducible under these mild conditions, since it has been well demonstrated (Beychok & Warner, 1959; Donovan, Laskowski & Scheraga, 1959, 1960; Hoare & Koshland, 1966; Hornishi, Nakaya, Tani & Shibata, 1968) that carboxyl groups in a protein differ in their accessibility.

The proteins studied varied in size, complexity, the number of sub-units, and the presence or absence of disulphide bonds and thiol groups. None

Table 9. *Reduction of lysozyme with diborane*

The protein trifluoroacetate was reduced with 1 M-diborane in tetrahydrofuran, the volumes indicated being used per 3–4 mg. of protein. The values are expressed in moles of the constituent amino acids/mole of protein and were obtained from duplicate analyses (one 22 hr. and one 72 hr. hydrolysis). Tryptophan was determined from duplicate alkaline hydrolysates. Thiol groups were determined by titration of the intact protein with *p*-hydroxymercuribenzoate (see the text). The values for threonine and serine were obtained by extrapolation to zero hydrolysis time. The 'expected' values are from Canfield & Liu (1965). Leucine is *C*-terminal (Canfield & Liu, 1965).

	Amino acid composition (moles/mole)			
Protein ...	Native lysozyme		Reduced lysozyme	
Amino acid	Expected	Found	Treatment ...	3 ml. of diborane, 22°, 16 hr.	0·5 ml. of diborane, −10°, 4 hr.
Asp	8	21·1		8·40	14·5
Asn	13				
Thr	7	6·70		7·11	7·04
Ser	10	9·89		10·2	9·62
Glu	2	5·28		4·20	4·24
Gln	3				
Pro	2	1·82		2·03	1·89
Gly	12	11·9		10·9	14·29
Ala	12	12·2		12·0	12·1
CyS	8	8·15		7·45	7·65
Val	6	5·66		6·15	6·17
Met	2	2·18		2·13	1·95
Ile	6	5·69		5·85	5·61
Leu	8	8·18		6·92	6·93
Tyr	3	2·85		2·75	2·91
Phe	3	2·96		2·80	3·16
Lys	6	6·25		5·99	5·83
His	1	0·925		0·315	0·918
Arg	11	10·62		5·86	10·3
Trp	6	5·86		5·74	5·81
Thiol	0	0		7·63	6·78

of these factors seemed to influence the specificity of the reaction that was achieved with all the proteins used. From these results it appears that the reaction is likely to be of general applicability. However, owing to the state of our knowledge of the reagent and the nature of side reactions, when reduction with diborane is applied to proteins or peptides of unknown constitution the results should be interpreted with caution and, as with all modification reactions, specificity ought to be confirmed for the protein under investigation.

The reductive cleavage of the disulphide bonds is, of course, to be expected, and perhaps diborane may in the future be applied to non-protonated proteins to accomplish just this, since, under these conditions, no reduction of any other amino acid is obtained. The reductive cleavage of disulphide bonds is not a disadvantage, since their correct re-formation by oxidation is not difficult and should yield the native protein (Anfinsen & Haber, 1960). There is some evidence to suggest that even in modified proteins reduced disulphides re-form in the correct manner on reoxidation (Anfinsen, Sela & Cooke, 1962; Epstein & Goldberger, 1964; Wilcheck, Frensdorff & Sela, 1966, 1967).

Amino acids that sometimes suffered modification when relatively vigorous conditions were used were histidine, arginine and proline. Elucidation of the mechanism of these 'undesirable' side reactions must await the characterization of the products of reduction. A reaction product usually appeared in amino acid analysis as a shoulder on the ammonia peak, another peak often appeared between lysine and histidine, and occasionally there was one between histidine and ammonia. Amino acids at the *C*-terminus are reduced to their corresponding amino alcohols and would be expected to appear with, or close to, ammonia, where ethanolamine appears. Appropriate resolution of the various amino alcohols on the analyser has not yet been achieved.

The chemical modification presented here has the advantage of permanence, since the original carboxyl moieties cannot be inadvertently regenerated by subsequent hydrolytic procedures. Also, the change in the size of the side chain is small on

conversion of the $-CO_2H$, group into $-CH_2 \cdot OH$, and this therefore affords an excellent procedure when it is desired that loss of hydrophilicity (esterification) or introduction of bulky side chains (Hoare & Koshland, 1966; Wilcheck *et al.* 1967) are to be avoided. This reaction should therefore prove useful in the study of the role of carboxyl groups in the conformation of proteins and in the biological properties of peptides and proteins.

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REFERENCES

Anfinsen, C. B. & Haber, E. (1960). *J. biol. Chem.* **236**, 1361.

Anfinsen, C. B., Sela, M. & Cooke, J. (1962). *J. biol. Chem.* **237**, 1825.

Atassi, M. Z. (1964*a*). *Biochem. J.* **93**, 189.

Atassi, M. Z. (1964*b*). *Nature, Lond.*, **202**, 496.

Atassi, M. Z. & Caruso, D. R. (1968). *Biochemistry*, **7**, 699.

Battersby, A. R. & Craig, L. C. (1952). *J. Amer. chem. Soc.* **74**, 4019.

Beychok, S. & Warner, R. C. (1959). *J. Amer. chem. Soc.* **81**, 1892.

Braunitzer, G., Gehring-Muller, R., Hilschmann, N., Hilse, K., Hobom, G., Rudloff, V. & Wittmann-Liebold, B. (1961). *Hoppe-Seyl. Z.* **325**, 283.

Brown, H. C. & Heim, P. (1964). *J. Amer. chem. Soc.* **86**, 3566.

Brown, H. C. & Korytnyk, W. (1960). *J. Amer. chem. Soc.* **82**, 3866.

Brown, H. C. & Subba Rao, B. C. (1960). *J. Amer. chem. Soc.* **82**, 681.

Canfield, R. & Liu, A. K. (1965). *J. biol. Chem.* **240**, 1997.

Donovan, J. W., Laskowski, M. & Scheraga, H. A. (1959). *J. molec. Biol.* **1**, 293.

Donovan, J. W., Laskowski, M. & Scheraga, H. A. (1960). *J. Amer. chem. Soc.* **82**, 2154.

Edmundson, A. B. (1965). *Nature, Lond.*, **205**, 883.

Epstein, C. J. & Goldberger, R. F. (1964). *J. biol. Chem.* **239**, 1089.

Franzblau, C., Gallop, P. M. & Seifter, S. (1963). *Biopolymers*, **1**, 79.

Goldstein, J., Konigsberg, W. & Hill, R. J. (1963). *J. biol. Chem.* **238**, 2016.

Goodfriend, T. L., Levine, L. & Fasman, G. D. (1964). *Science*, **144**, 1344.

Hausmann, W., Weisiger, J. R. & Craig, L. C. (1955). *J. Amer. chem. Soc.* **77**, 721.

Hill, R. J. & Konigsberg, W. (1962). *J. biol. Chem.* **237**, 3151.

Hoare, D. G. & Koshland, D. E. (1966). *J. Amer. chem. Soc.* **88**, 2057.

Hornishi, H., Nakaya, K., Tani, A. & Shibata, K. (1968). *J. Biochem., Tokyo*, **63**, 41.

Konigsberg, W. & Hill, R. J. (1962). *J. biol. Chem.* **237**, 2547.

Margoliash, E., Smith, E. L., Kreil, G. & Tuppy, H. (1961). *Nature, Lond.*, **192**, 1121.

Markham, R. (1942). *Biochem. J.* **36**, 790.

Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.

Ray, W. J. & Koshland, D. E. (1962). *J. biol. Chem.* **237**, 2493.

Riehm, J. P. & Scheraga, H. A. (1965). *Biochemistry*, **4**, 772.

Riehm, J. P. & Scheraga, H. A. (1966). *Biochemistry*, **5**, 99.

Rosenthal, A. F. & Atassi, M. Z. (1967). *Biochim. biophys. Acta*, **147**, 410.

Sheehan, J. G. & Hlavka, J. J. (1956). *J. org. Chem.* **21**, 43.

Theorell, H. & Akeson, A. (1955). *Ann. Acad. Sci. fenn.* **60**, 303.

Wilcheck, M., Frensdorff, A. & Sela, M. (1966). *Arch. Biochem. Biophys.* **113**, 742.

Wilcheck, M., Frensdorff, A. & Sela, M. (1967). *Biochemistry*, **6**, 247.