From the variation of mobility with pH, the principal surface-charge group of vegetative cells appeared to be carboxyl, possibly associated with a hexosamine-peptide substance. The behaviour of the protoplasts was consistent with their having a lipoprotein or protein surface.

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Studies on the Amide and *C*-Terminal Residues in Proteins

1. THE CHARACTERIZATION OF THE *C*-TERMINAL RESIDUE

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A normal open peptide chain carries an *N*-terminal and a *C*-terminal residue, so that it should be possible to determine the number of such chains in a protein by estimating one or other of these residues. Much of the information at present available has been obtained by applying the dinitrophenyl (DNP) method of Sanger (1945), but with certain proteins this method, like others designed for the same purpose, has so far failed to reveal the presence of any *N*-terminal residue at all, which has led to the suggestion that proteins may contain cyclopeptide chains. The need for complementary procedures dealing quantitatively with the opposite end of the peptide chain is thus apparent, and although carboxypeptidase and hydrazinolysis have indeed been fairly extensively used for the purpose in recent years, both are now known to have their limitations. (For review, see Bailey, 1954).

When the present research was started in 1951 it was realized at the outset that the identification and estimation of the *C*-terminal residues in a protein would not necessarily indicate the full number of peptide chains in the molecule, for in addition to the reservation on cyclic structures mentioned above, the issue might be further complicated by the presence of terminal amide residues. Such a possibility served to emphasize how little experimental evidence had been then adduced in favour of the so-called amide hypothesis, which was first adumbrated by Ritthausen (1872) and came into prominence early in the present century. According to this, the ammonia given on mild hydrolysis of proteins was derived exclusively from asparaginyl and glutaminyl residues in the molecule and, although direct evidence for the presence of these two residues in edestin and gliadin respectively was obtained many years ago, it still remains true that for lack of a suitable method for their quantitative estimation the question of whether an amide such as glycinamide, now known to be a constituent of oxytocin, might be present as a terminal residue has really been left open. With this particular point in view, the aim of the present investigation has been to characterize amide as well as *C*-terminal residues under conditions which would, if possible, be of general application in protein chemistry.

The procedure that has been under investigation is based on the observation of Nystrom, Chaiken & Brown (1949) that under the appropriate conditions lithium borohydride ($LiBH_4$) would reduce the ester group to the corresponding hydroxyl group, but was without action on the acid amide group. It was anticipated that if the free carboxyl groups in a protein could be first esterified and then reduced in this way, the various dicarboxylic acid and *C*-terminal residues originally present would become modified and after hydrolysis of the treated protein would be present in the hydrolysate as

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amino alcohols or hydroxyamino acids (Table 1). Quantitative analysis of this hydrolysate would then provide the data for characterizing the amide and *C*-terminal residues in the protein.

Our preliminary results were encouraging, and the distribution of the amide N in insulin between the asparaginyl, glutaminyl and *C*-terminal asparagine residues was resolved in apparent conformity with the contemporary work of Sanger and his colleagues. Attempts to characterize more completely the *C*-terminal residues, however, led to results which, in the light of the latter workers' findings, were confusing. For clarity in presentation we refrain from quoting in Table 2 the results obtained by the out-dated procedures of these early investigations and give, instead, those of a series of observations by the methods in current use (Rees, 1958). Alaninol, representing the *C*-terminal residue of the insulin *B* chain, was readily identified in full measure (Table 2, no. 3), but ethanolamine and small amounts of both leucinol and tyrosinol were also found (Chibnall & Rees, 1951, 1952). It seemed at the time that these latter products must either represent *C*-terminal residues of protein impurities, which the progress of Sanger's work with the same preparation of insulin was making less and less probable, or must indicate that we were dealing with a side reaction inimical to our purpose.

All the preparations of insulin we have handled, when finely ground and passed through the reduction procedure, gave a β-amino alcohol fraction of negligible magnitude (Table 2, no. 1). As we had already observed an increase in amino N (Van Slyke) on esterifying insulin with methanolic hydrochloric acid, we considered the possibility of an *N–O* acyl shift at serine and threonine residues (Chibnall & Rees, 1951, 1952; Chibnall, Mangan & Rees, 1958*c*), the *O*-peptidyl esters thus produced undergoing reduction to give the adventitious amino alcohols. Indeed the demonstration by Sanger & Tuppy (1951) of the sequence -glycyl-seryl- in the *B* chain made the hypothesis attractive. To probe more deeply we blocked the hydroxyl groups of the serine and threonine residues by preparing *O*-acetyl insulin. This was esterified with diazomethane, but the reduced ester gave the same array of amino alcohols as before. There remained the possibility that an acyl shift was conditioned by the suppression of the carboxyl groups on reduction (Chibnall & Rees, 1953).

The probable explanation of the enigma was provided by Crawhall & Elliott (1955) who showed that, under the conditions they employed, $LiBH_4$ could bring about a reductive cleavage of peptide bonds in lysozyme to produce amino alcohols. We had ourselves considered the possibility of such a direct action by the reagent, but had rejected it for reasons that seemed decisive at the time. As has already been mentioned, insulin itself appeared to undergo a negligible amount of reduction, and the same was true of β-lactoglobulin. In addition, Bailey (1955) had found that *N*-acetamidoethanol in tetrahydrofuran solution, when treated with $LiBH_4$ for 3 hr. under reflux, gave only 1·5% of *N*-ethylamino alcohol. Likewise we had found that glycylglycyl-α-aminomyristic acid, when submitted to our standard reduction treatment in tetrahydrofuran solution, exhibited peptide cleavage to the extent of only 1·3% of one group. These two substances, unlike the proteins mentioned, were soluble in the reaction mixture, yet in spite

Table 1. *Comparison between the various dicarboxylic acid, dicarboxylic acid amide, and* C*-terminal residues present in the intact protein with the corresponding products present in the hydrolysate of the reduced protein ester*

The following abbreviations have been used: γ-α-HAB, γ-hydroxy-α-aminobutyric acid; γ-β-HAB, γ-hydroxy-β-aminobutyric acid; δ-α-HAV, δ-hydroxy-α-aminovaleric acid; δ-γ-HAV, δ-hydroxy-γ-aminovaleric acid.

Residue in protein	Corresponding product in the hydrolysate of the reduced protein ester
Glutaminyl, *N*-terminal or in chain	Glutamic acid
Isoglutaminyl, *N*-terminal or in chain	Glutamic acid
Glutamine, *C*-terminal	δ-γ-HAV
Isoglutamine, *C*-terminal	δ-α-HAV
α-Glutamyl, *N*-terminal or in chain	δ-α-HAV
γ-Glutamyl, *N*-terminal or in chain	δ-γ-HAV
Glutamic acid, *C*-terminal	Glutamidiol
Asparaginyl, *N*-terminal or in chain	Aspartic acid
Isoasparaginyl, *N*-terminal or in chain	Aspartic acid
Asparagine, *C*-terminal	γ-β-HAB or lactone
Isoasparagine, *C*-terminal	γ-α-HAB or lactone
α-Aspartyl, *N*-terminal or in chain	γ-α-HAB or lactone
β-Aspartyl, *N*-terminal or in chain	γ-β-HAB or lactone
Aspartic acid, *C*-terminal	Aspartidiol
Monoamino or basic amino acid, *C*-terminal	β-Amino alcohol
Monoamino or basic amino acid amide	Monoamino or basic amino acid

of this they had undergone but little change. Nevertheless we prepared, by appropriate solution and precipitation, a sample of insulin which we hoped would be in a finer state of dispersion than we had formerly employed. On reduction this underwent reductive cleavage to a degree that we had not hitherto observed (Table 2, no. 2), confirming the results of Crawhall & Elliott and offering an explanation for the origin of the adventitious amino alcohols mentioned above. This means, in effect, that the changes listed in Table 1 need amplifying, because under conditions of reductive cleavage endopeptide residues will simulate *C*-terminal residues. The outcome, nevertheless, does not vitiate the procedure as we now apply it to proteins of low molecular weight like insulin where the overall interference is relatively small (Tables 2 and 3). With increase in molecular weight, however, the degree of uncertainty in the interpretation of the data becomes proportionately greater and our studies suggest that the profitable limit has already been reached in the multi-chain protein, β-lactoglobulin (Table 5).

As the work progressed and it was found that side reactions were interfering with the full exploitation of the procedure, it became necessary to devote considerable time *inter alia* to the quantitative analysis of certain β-amino alcohols and the four hydroxyamino acids listed in Table 1. β-Amino alcohols, having the amino and hydroxyl groups attached to contiguous carbon atoms, give an equivalent of formaldehyde on oxidation by periodate. After fractionation on a column of Dowex-2, followed by resolution on a column of buffered silica gel, they have been estimated quantitatively as periodate–formaldehyde (Rees, 1958). The four hydroxyamino acids, together with aspartic and glutamic acids, have been separated on a column of Dowex-50; the resolution of δ-hydroxy-α-aminovaleric acid (δ-α-HAV) from γ-hydroxy-β-aminobutyric acid (γ-β-HAB) is not at present satisfactory, but the latter hydroxyamino acid, as well as δ-hydroxy-γ-aminovaleric acid (δ-γ-HAV), can be readily estimated without interference as periodate–formaldehyde for the same reason as β-amino alcohols. In the present work δ-α-HAV and γ-hydroxy-α-aminobutyric acid (γ-α-HAB), which do not give formaldehyde on oxidation by periodate, have not been determined directly. It was necessary also to study more closely the efficiency of the procedures in current use for esterifying proteins and this, in its turn, forced us to inquire into the ammonia N as well as the amide N of the native protein and ester preparations with which we were dealing. We believe that our studies in these two fields have revealed observations of importance in general protein chemistry as well as in our own particular problem, and for this reason we have preferred to present them in detached form as separate papers in the present series.

EXPERIMENTAL

Materials

Glycylglycyl-α-aminomyristic acid. We have to thank Professor Damodaran for the gift of a sample prepared by Abderhalden & Damodaran (1930).

Proteins. The preparations of insulin, β-lactoglobulin and lysozyme used are described elsewhere (Chibnall, Mangan & Rees, 1958*b*). In our earlier work the samples taken for reduction had been ground as finely as possible in an agate mortar. The present procedure is as follows. The protein (100 mg.) is dissolved in 0·033 N-HCl (3 ml.) and treated immediately with acetone (30 ml.). The precipitated protein is centrifuged off, washed twice with acetone, three times with ether (A.R. dried over sodium) and the product dried *in vacuo* at room temperature. Occasionally, as noted in the text, the drying stage has been omitted and the ether-washed material, after being treated twice with tetrahydrofuran, was put through the reduction procedure forthwith.

Protein methyl esters. These were prepared with methanolic 0·1 N-HCl at 25° for 24 hr. unless otherwise stated (Chibnall *et al.* 1958*c*).

O-*Acetylinsulin methyl ester.* Insulin (100 mg.) was dissolved in acetic acid saturated with urea (4 ml.) and treated with acetyl chloride (1·4 ml.) at room temperature for 1 hr. Acetone (55 ml.) was then added and the separated product centrifuged. The material was given ten washings with acetone (55 ml.) to remove urea. The procedure, which has been successfully applied also to serine and β-lactoglobulin, brings about exclusive *O*-acetylation and has the advantage that the reaction products remain soluble. The methyl ester was prepared with diazomethane, the reaction mixture being kept below pH 5.

Tetrahydrofuran. The commercial product was purified by the procedure of Bailey (1955) and stored over sodium in the dark. It was redistilled before use.

Lithium borohydride ($LiBH_4$). In our earlier work a standard solution prepared by the method of Bailey (1955) was used. More recently we have found that the commercial product (New Metals and Chemicals Ltd., London) contains much inert material, the reducing power as assessed by titration of the lithium with standard acid being only 70% and by iodate titration (Lyttle, Jensen & Struck, 1952) only 65% of theory. Purification was readily effected as follows. The material (5 g.) in a 250 ml. centrifuge bottle, was stirred with 200 ml. of ether (A.R., dried over sodium) and the mixture centrifuged. The clear ethereal solution was poured off and the residue treated with two further lots of ether in the same way. The ethereal extracts were collected and on removal of most of the solvent by evaporation the material crystallized in long needles. The treatment was repeated. The final product (about 2·5 g.) dissolved easily in cold tetrahydrofuran to give a clear colourless solution. The reducing power and titration agreed and were within 2% of theoretical. A stock solution (about 1·2 M) slowly deposits white insoluble material when kept in the dark, and it is necessary to centrifuge and re-check the reducing power immediately before use.

Procedures and methods

Reduction with $LiBH_4$. Experience has shown that for products which are insoluble in tetrahydrofuran, such as protein methyl esters, an eightfold molar excess of $LiBH_4$ is required to effect full reduction of the ester groups. Provision must also be made for the bound HCl and disulphide linkages present. It is convenient to adjust the volume of tetrahydrofuran to make an approx. 0·3 M-solution of reducing agent. The reaction vessel (10 cm. × 2 cm.) is fitted at the top with a three-necked multiple adapter, and a mercury-sealed stirrer is inserted through the centre socket. One of the side sockets carries a condenser with a soda–lime trap, and the other is stoppered. The protein or ester (0·1 g.) was placed in the open reaction vessel and dried *in vacuo* for 2 hr. at 50°. The adapter with its furnishings was next placed in position, and through the third socket was introduced first a standard $LiBH_4$ solution containing 13 mg. of reagent and then sufficient tetrahydrofuran to bring the total volume to 2 ml. The reaction mixture was then boiled gently (oil bath, 90°) for the required time, generally 6 hr. It was cooled to room temperature, a slight excess of methanolic HCl was added dropwise and the mixture was stirred for 1 hr. to ensure decomposition of excess of reagent and any borane complexes present. Acetone–ether (1:1, v/v; 8 ml.) was then added and the reduced protein removed by centrifuging. It was washed twice with acetone, once with ether, and then dried *in vacuo* over P_2O_5.

Hydrolysis. The products were boiled under reflux with 50–70 parts (v/w) of 10 N-HCl–formic acid (1:1, v/v) for 24 hr. and the excess of acid was removed in the usual way. Evaporations *in vacuo* were conducted below 45°.

Separation of the β-amino alcohol fraction. A column (10 cm. × 1 cm.) of Dowex-2 × 10 was regenerated with carbonate-free N-NaOH and then thoroughly washed with CO_2-free water. A sample containing approx. 30 mg. of the reduced protein hydrolysate in a volume of 1 ml. was transferred to the column, and the amino alcohol fraction eluted with CO_2-free water until 25 ml. had been collected.

β-Amino alcohols. These were estimated as periodate–formaldehyde by the procedure of Rees (1958).

δ-Hydroxy-γ-aminovaleric acid and γ-hydroxy-β-aminobutyric acid. δ-γ-HAV and γ-β-HAB were estimated by the procedure of Chibnall, Haselbach, Mangan & Rees (1958*a*).

RESULTS

Glycylglycyl-α-aminomyristic acid. The acid (100 mg.) was dissolved in tetrahydrofuran (7·6 ml.) containing 0·33 M-$LiBH_4$ and the solution boiled under reflux for 6 hr. The collected hydrolysis liquors were brought to 2·5 ml. and a sample of 1 ml. was passed through a column of Dowex-2. The amino alcohol fraction gave formaldehyde equivalent to 1·3 % of a glycyl residue as ethanolamine.

Insulin. The results given in Table 2 leave no doubt that what may be referred to, in light of Sanger's researches on insulin structure, as the adventitious amino alcohols ethanolamine, leucinol and tyrosinol, have in all three cases a common origin. Our earlier suggestion of an *N–O* acyl migration on esterification, followed by reductive cleavage of the resulting *O*-peptidyl ester, is clearly untenable, not only for the reasons mentioned briefly in the introduction, but also because it has been shown that this particular acyl shift is largely at the expense of the unique threonyl residue (Chibnall *et al.* 1958*c*), so that tyrosinol, from the sequence tyrosyl-threonyl, should have been produced to a greater extent than ethanolamine. Direct reductive cleavage of peptide bonds, as demonstrated by Crawhall & Elliott (1955), with lysozyme, must clearly be the major factor concerned. We have so far been unable to find conditions which will minimize this effect without at the same time impairing the reduction of the terminal ester group. Crawhall & Elliott found that the reductive cleavage with $LiBH_4$ at room temperature was negligible, even when the material concerned was soluble in the reaction mixture. This would be in agreement with the work of Bailey (1955) on simpler peptide systems. With insulin methyl ester, however, the reductive cleavage of peptide bonds runs more or less parallel with the

Table 2. *Amino alcohol analysis of insulin and various derivates after reduction with lithium borohydride*

Unless otherwise stated, all products were esterified with methanolic 0·1 N-HCl for 24 hr. and all reductions were made in boiling tetrahydrofuran for 6 hr. Materials: Expt. 1, insulin, finely ground crystals; Expt. 2, precipitated insulin (see text); Expt. 3, insulin ester reduced at 20° for 24 hr.; Expt. 4, insulin ester; Expt. 5, *O*-acetylinsulin esterified with diazomethane. Results are expressed as moles/mole of protein (5732 g.).

Expt. no.	Yield of amino alcohol					Total amino alcohol fraction from Dowex-2 (periodate–formaldehyde)	
	Leucinol*	Tyrosinol*	Alaninol*	Aspartidiol*	Ethanolamine†	Yield	Accounted for‡ (%)
1	—	—	0·017	—	0·013	0·04	103
2	0·048	0·023	0·156	—	0·161	0·61	85
3	0·03	0·011	0·655	0·026	0·135	0·95	97
4	0·1	0·028	1·111	0·08	0·19	1·7	92
5	0·111	0·033	1·077	0·04	0·16	1·50	97

* Corrected for 10 % loss on hydrolysis. † Gives 2 equiv. of formaldehyde.

‡ Based on the yields (uncorrected for loss on hydrolysis) of the amino alcohols concerned.

reduction of the *C*-terminal alanine ester group in spite of the complete insolubility of the ester in the cold reaction mixture (Table 2, no. 3).

The comparison between the reduction products of insulin and its methyl ester is set out in Table 3. It will be seen that the increase in alaninol represents in full measure the *C*-terminal alanine residue of the *B* chain. The increase in the γ-β-HAB is somewhat less than might have been expected for the *C*-terminal asparagine residue of the *A* chain, but this is due in part to the initial low amide N of the insulin ester (Chibnall *et al.* 1958*c*). The aspartidiol, which is a reduction product of the ester and not of the unesterified protein, represents a *C*-terminal aspartic acid residue and this, in its turn, must represent an asparagine residue which has lost its amide N. All the remaining products listed in Table 3 represent adventitious reductive cleavage of peptide linkages and the various differences between the two sets of data are surprisingly small. It is interesting to note that residues of glutamine, giving rise to δ-γ-HAV, are particularly labile, whereas those of glutamic acid, which with the protein ester would give rise to glutamidiol, appear to be quite stable.

Lysozyme. Three different reduction treatments were applied to the ester, and the most drastic of these to the protein. The reduction products given on hydrolysis are listed in Table 4. In agreement with Crawhall & Elliott (1955) there is evidence of random reductive cleavage of peptide bonds, and in addition to the amino alcohols quoted, tyrosinol, valinol and serinol were present in amounts too small for inclusion in the table. Residues of glutamine and asparagine were labile, as shown by the production of δ-γ-HAV and γ-β-HAB respectively, but the absence of aspartidiol indicates that the free aspartic acid residues (Chibnall *et al.* 1958*a*) were untouched.

Thompson (1952) and Harris (1952), using carboxypeptidase, found that leucine occupies the *C*-terminal position. Our findings are in partial agreement with this, because leucinol is the only reduction product which shows a substantial increase as between ester and protein. Yet under all three of our reduction treatments, the mildest of which (Table 4, no. 1) has given full reduction of the aspartyl residues (Chibnall *et al.* 1958*a*), the amount of leucinol produced does not exceed 0·5 mole/mole, allowing for the protein control. Ohno (1953), using hydrazinolysis, reported one *C*-terminal leucine residue/mole, but his experimental data are subject to a very large correction for losses.

β-Lactoglobulin. This protein has a larger molecular weight than lysozyme and one peptide bond represents only 0·243% of the total protein N. A small degree of peptide cleavage may thus assume inflated proportions when measured in terms of groups/mole. The data given in Table 5

Table 3. *C-Terminal residues in insulin*

Insulin was esterified with methanolic 0·1 N-HCl for 24 hr. Insulin and ester were reduced with $LiBH_4$ in boiling tetrahydrofuran for 6 hr. Results are expressed as moles/mole of protein (5732 g.).

	Yield of amino alcohol or hydroxyamino acid						
Material	Leucinol	Tyrosinol	Alaninol	Aspartidiol	Ethanolamine	γ-β-HAB	δ-γ-HAV
Insulin methyl ester	0·1	0·027	1·110	0·08	0·19	0·89	0·18
Insulin	0·048	0·023	0·156	—	0·161	0·08	0·16
Difference	0·052	0·004	0·955	0·08	0·029	0·81	0·02

Table 4. *Reduction products of lysozyme and its methyl ester*

Methyl ester was prepared with methanolic 0·1 N-HCl at 25° for 24 hr. Treatment before reduction: no. 1, material was washed successively with acetone and ether, and dried *in vacuo*; no. 2, material was washed successively with acetone, ether and tetrahydrofuran and reduced forthwith (see text). Results are expressed as moles/mole of protein (14 700 g.)

			Yield of amino alcohol or hydroxyamino acid					
Material	Treatment	Time of reduction (hr.)	Amino alcohol fraction (Dowex-2) periodate–formaldehyde yield	Leucinol	Alaninol	Ethanolamine	γ-β-HAB	δ-γ-HAV
Ester	No. 1	6	1·68	0·53	—	—	—	—
Ester	No. 2	2	2·88	0·57	0·25	0·71	—	—
Ester	No. 2	6	3·48	0·54	0·6	0·75	0·86	1·03
Protein	No. 2	6	2·58	0·1	0·6	0·63	0·67	1·0

Table 5. *Reduction products of β-lactoglobulin and its methyl ester*

Methyl ester was prepared with methanolic 0·1 N-HCl at 25° for 24 hr. Results are expressed as moles/mole of protein (37 000 g.).

Material	Amino alcohol fraction (Dowex-2) periodate–formaldehyde yield	Yield of amino alcohol or hydroxyamino acid					
		Leucinol	Tyrosinol	Alaninol	Ethanolamine	γ-β-HAB	δ-γ-HAV
Ester	9·9	1·49	0·63	5·50	0·82	0·9	1·5
Protein	4·0	0·34	0·3	1·60	0·52	0·8	1·7
Difference	5·9	1·15	0·33	3·9	0·3	0·1	−0·2

demonstrate that fairly widespread peptide cleavage has occurred and, as with lysozyme, the analysis for amino alcohols showed the presence of valinol, serinol and the two diols, but in amounts too small for inclusion in the table. Comparison of results for the ester and protein shows an increase of 1 mole of leucinol and 4 moles of alaninol/mole of protein, suggesting that the molecule consists of at least five peptide chains, one of which has a *C*-terminal leucine residue and the other four have alanine residues. This finding is in contrast with that of Porter (1948), who was able to recognize no more than three *N*-terminal leucine residues by the DNP procedure of Sanger (1945), and is referred to again later.

DISCUSSION

The observations recorded above show that facile and quantitative reduction of the esterified carboxyl groups of proteins can be obtained under relatively mild conditions with lithium borohydride. The results, however, leave no doubt that the issue is complicated by the reductive fission of peptide bonds, as suggested by Crawhall & Elliott (1955), and that the contrary assertions of Grassmann, Hörmann & Endres (1953, 1955) and of Grassmann, Strobel, Hannig & Deffner-Plockl (1956) were based on inadequate experimental evidence. It is true that as a side reaction in such a major operation the effect is small (Table 6), but as has already been emphasized for β-lactoglobulin, it can be sufficiently large to cast doubt on any evaluation of the end groups in a protein of molecular weight greater than that, say, of insulin. Moreover, the effect depends in part on the state of dispersion of the product at the reduction stage, and it might be argued that with insulin (Table 3), and perhaps also with lysozyme (Table 4), the two contrasted products have been more closely balanced in this respect than was apparently the case with β-lactoglobulin (Table 5). The data given in the tables are presented with the object of illustrating these and many other points discussed in the text, and too much significance should not be attached to the numerical value of the results deduced.

Table 6. *Reductive cleavage of peptide bonds by lithium borohydride*

Values for N are computed from the sum of the formaldehyde equivalent of δ-γ-HAV, γ-β-HAB and amino alcohol fraction (Dowex-2, less allowance for excess given by ethanolamine).

Product	N (% total N)	Peptide bonds cloven/mole
Polyglutamic acid from *B. subtilis*	0	0
Glycylglycyl-α-amino-myristic acid	0·65	0·013
Insulin	1·07	0·69
β-Lactoglobulin	1·51	6·0
Lysozyme	1·84	3·62

In agreement with Crawhall & Elliott we find that the reductive fission by lithium borohydride is selective. α-Glutamyl and α-aspartyl residues in proteins are remarkably resistant, as also are the γ-glutamyl residues in the bacterial polypeptide from *Bacillus subtilis* (Chibnall, Rees & Richards, 1958), suggesting that it is the free carboxyl group which confers protection. Glutamine and asparagine residues, on the contrary, are susceptible, giving rise to δ-γ-HAV and γ-β-HAB respectively. In all the three proteins examined the amount of each hydroxyamino acid produced was the same for the protein as for the ester, so that their origin cannot be ascribed to the presence of γ- or β-linkages respectively in the peptide chains. The observation needs emphasis because γ-linkages have been suggested by Fromageot & Jutisz (1953) on no more evidence than the detection of δ-γ-HAV in hydrolysates of proteins reduced with lithium aluminium hydride ($LiAlH_4$) (cf. Haurowitz & Horowitz, 1956).

It will thus be seen that in practice the procedure under review for determining *C*-terminal residues in proteins, like all others based on chemical principles which have been suggested in recent years, has been found wanting. If applied with due regard to its limitations it may well yield valuable information with polypeptides of molecular weight of the order 10 000 or less, but the main problem of determining by chemical means the *C*-terminal

residues in proteins, the need for which was emphasized in the introduction, is still unsolved and remains a challenge to the analyst.

SUMMARY

1. The carboxyl groups of *C*-terminal residues in proteins, after preliminary esterification, can be reduced with lithium borohydride, and on subsequent acid hydrolysis the modified residues are present in the hydrolysate as amino alcohols or hydroxyamino acids as the case may be.

2. The suitability of the procedure for determining the *C*-terminal residues in proteins has been explored, and in agreement with Crawhall & Elliott (1955), it was found that the issue was complicated by the simultaneous reductive cleavage of peptide bonds which occurs, under the conditions chosen, to the extent of 1–2% of the total peptide bonds.

3. Data are presented showing that with a protein of low molecular weight such as insulin (5732) the interference is not serious and a satisfactory determination of the *C*-terminal residues can be made. With a protein of much higher molecular weight such as β-lactoglobulin (37 000), however, the interference is serious.

4. The procedure is not recommended as a reliable one for proteins but it may be of use with peptides of low molecular weight.

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Studies on the Amide and *C*-Terminal Residues in Proteins

2. THE AMMONIA NITROGEN AND AMIDE NITROGEN OF VARIOUS NATIVE PROTEIN PREPARATIONS

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In all our previous work which called for a determination of amide N, the protein preparation used for the purpose had been coagulated by heat and washed free from inorganic salts, so that the ammonia N content was negligible and a direct determination of the amide N permissive. Such heat-coagulated products, however, do not readily esterify, and in the present investigation it has been necessary to use the native crystalline material. This may have been fractionated from natural sources by reagents other than ammonium salts, and the product thus obtained may have been dialysed for some days against distilled water; even so, we have been surprised to find that it can

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