3. The procedure has been used to separate and identify the β-amino alcohols present in the hydrolysates of esterified and reduced proteins.

4. The use of silver oxide to oxidize β-amino alcohols to the corresponding α-amino acid is described.

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

5. ESTIMATION OF ASPARAGINE AND GLUTAMINE RESIDUES

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(*Received* 19 *June* 1957)

In the introduction to the first paper of this series (Chibnall & Rees, 1958) an outline was given of the modifications that an esterified protein could undergo on reduction with lithium borohydride, and how the various changes produced would become manifest after hydrolysis. In the present paper we deal with one particular aspect of this phenomenon, which offered the possibility of estimating the total number of asparagine and of glutamine residues in the protein molecule concerned. Subject to certain qualifications to be discussed later, asparaginyl and glutaminyl residues which occur in the peptide chains will remain intact until hydrolysis, when they will be converted into aspartic and glutamic acids respectively, whereas those occupying *C*-terminal positions (I) will undergo reduction at the esterified α-carboxyl group to give, eventually, γ-hydroxy-β-aminobutyric acid (γ-β-HAB) and δ-hydroxy-γ-aminovaleric acid (δ-γ-HAV) respectively (II). Aspartyl and glutamyl residues in the peptide chains (III) will undergo reduction at esterified ω-carboxyl groups to give γ-hydroxy-α-aminobutyric acid (γ-α-HAB) and δ-hydroxy-α-aminovaleric acid (δ-α-HAV) respectively (IV), whereas if they occupy *C*-terminal positions (V) they will give the corresponding diols (VI). Products (II) and (VI), having hydroxyl and amino groups attached to adjacent carbon atoms, are susceptible to periodate oxidation, which makes it possible to devise a procedure for determining the various residue species in the protein concerned.

Aspartic and glutamic acids (estimated with ninhydrin), as well as δ-γ-HAV and γ-β-HAB (estimated as periodate-formaldehyde), are separated on a column of Dowex-50 by elution with hydrochloric acid. There is a partial overlap between δ-α-HAV and γ-β-HAB so that the former cannot be estimated by ninhydrin. δ-α-HAV and γ-α-HAB have been computed by difference. The two diols (VI) are estimated after separation on a column of silica gel (Rees, 1958).

Before presenting evidence that such a scheme is of practical value, however, it is necessary to mention five points which may adversely affect the issue. In the first place it has already been shown that full esterification of a protein cannot be achieved without a small concomitant loss of about 6% of the amide N (Chibnall, Mangan & Rees, 1958*b*), which sets a limit to the accuracy with

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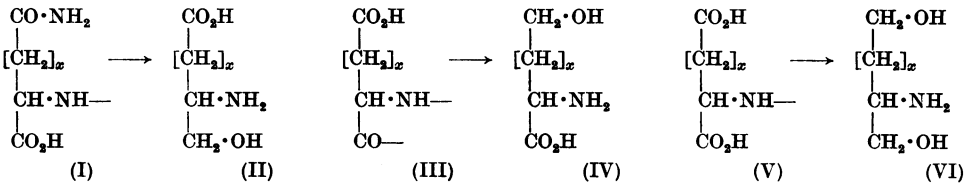


which the amide distribution can be determined with proteins of high molecular weight, as we shall see later for β-lactoglobulin. Secondly, reductive cleavage of peptide bonds (Crawhall & Elliott, 1955) may occur, so that residues in peptide chains will undergo modification as though they were present in the *C*-terminal position. Aspartyl and glutamyl residues (III) therefore will be found in the reduced ester hydrolysate as diols (VI), and the corresponding amide residues as the periodate-labile hydroxyamino acids (II). As will be shown later, these changes can be quantitatively determined and allowed for. Thirdly, Crawhall & Elliott (1955) have suggested the possibility that lithium borohydride might reduce the acid amide group. Evidence presented later shows that this may occur, but to a negligible extent. Fourthly, the possible occurrence of β-aspartyl and γ-glutamyl peptide linkages in the chains must be considered. Their presence would lead to the occurrence of more of the respective hydroxyamino acid (II) in the hydrolysate of the reduced ester than in that of the reduced protein, and confusion with *C*-terminal asparagine or glutamine residues would be possible. Lastly, there is the possible occurrence of a terminal amide residue, other than that of asparagine or glutamine. This would be indicated by an unbalance in the reduced ester between the number of amide groups on the one hand and the total number of aspartic acid, glutamic acid and hydroxyamino acid (II) residues on the other. The evaluation would thus be susceptible to the overall errors of analysis and would become progressively less valid with increase in molecular weight of the protein concerned.

EXPERIMENTAL

Materials

L-*Pyrrolidonecarboxylic acid.* This was prepared from L-glutamic acid by the method of Lichtenstein (1942). The product gave no colour with ninhydrin.

δ-*Hydroxy-γ-aminovaleric acid.* We have to thank Professor Linderstrøm-Lang for a sample prepared by Sørensen (1905).

γ-*Hydroxy-β-aminobutyric acid.* This was prepared from α-aminobutyrolactone by the method of Livak, Britton, Vander Weele & Murray (1945).

α-γ-*Diaminobutyric acid.* We have to thank Dr L. C. Craig for a sample of this compound.

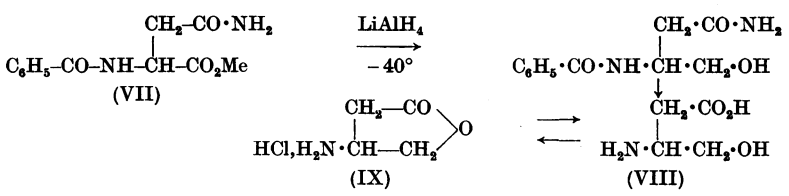
Proteins. The samples have been described elsewhere (Chibnall, Mangan & Rees, 1958*a*).

Synthesis of γ-hydroxy-β-aminobutyric acid (γ-β-HAB)

The following synthesis was carried out in 1951 before the technique for handling $LiBH_4$ had been fully explored. *N*-Benzoylasparagine methyl ester (VII) was reduced with $LiAlH_4$ under mild conditions. The amide group was largely unaffected, and after hydrolysis the required hydroxyamino acid (VIII) was isolated and purified as β-aminobutyrolactone hydrochloride (IX).

N-*Benzoylasparagine methyl ester.* *N*-Benzoylasparagine (10 g., Pauly & Weir, 1910), somewhat contaminated with the free acid owing to hydrolysis of the amide group during benzoylation, was dried *in vacuo* and suspended in methanolic 0·1 N-HCl. The mixture was shaken at 25° for 24 hr., the product going into solution as the esterification proceeded. The solvent was removed by evaporation *in vacuo*, and the residue, after standing for some hours in a vacuum desiccator over NaOH, was ground to a fine powder and extracted exhaustively with warm ligroin. The final residue was taken up in warm acetone, from which it crystallized on cooling. Yield of *N*-benzoylasparagine methyl ester, 6·7 g. (63%). The ligroin liquors were collected, reduced in volume and cooled, when *N*-benzoyl-aspartic acid dimethyl ester separated in needles (2·83 g.).

β-*Aminobutyrolactone hydrochloride.* *N*-Benzoylasparagine methyl ester (1·1 g.) was placed in a reaction vessel fitted with a stirrer and tetrahydrofuran (10 ml.) added. The mixture was then cooled to −40° and 1·6 M-$LiAlH_4$ (14 ml.) added slowly with stirring. After 30 min. the cooling bath was removed and the reaction temperature allowed to rise slowly to room temperature during 30 min. The mixture was then cooled again to −40°, ether saturated with water was added and then a slight excess of ethanolic HCl. The solvents were removed by evaporation, and the residue was hydrolysed with 5 N-HCl (25 ml.) for 4 hr. After removing excess of mineral acid the residue was dissolved in water, and neutralized with $Ba(OH)_2$ to precipitate $Al(OH)_3$, which was centrifuged off and washed. The clarified liquors were collected, made just alkaline with $Ba(OH)_2$ and evaporated *in vacuo* to remove ammonia. Barium was then removed as $BaSO_4$, the solution was reduced to a small volume, transferred to a column (3·5 cm. × 25 cm.) of Dowex-50, and eluted with N-HCl. Fractions (5 ml.) were collected, and from each a portion (0·05 ml.) was removed for flame test (Li) or periodate analysis. Lithium was eluted between fractions 60 and 80, and periodate-reducing material between fractions 97 and 130. The latter fractions were combined and reduced to a syrup *in vacuo*. The γ-lactone hydrochloride crystallized from a small volume of hot ethanol; yield, 263 mg. (45%), m.p. 185–186° (Found: C, 35·23; H, 5·78; N, 10·19. $C_4H_7O_2N$,HCl requires C, 34·91; H, 5·86; N, 10·18%).



The γ-lactone hydrochloride is very stable, and gives no formaldehyde under the usual conditions of periodate oxidation (Rees, 1958). Treatment with a phosphate buffer (pH 11) at 100° for 5 min. opens the ring and a 75% recovery of formaldehyde is obtained. Boiling under reflux with 5N-HCl produces an equilibrium mixture containing about 50% of the free acid; a similar mixture results when either the lactone or free acid is eluted from a column of Dowex-50 with N-HCl. Paper chromatography with butanol–acetic acid–water (4:1:5, by vol.) does not open the ring, and on spraying with ninhydrin a yellow spot is obtained, $R_F = 0{\cdot}22$.

γ-Hydroxy-β-aminobutyric acid. The method used was that of Fischer & Blumenthal (1907) for opening the ring of α-aminobutyrolactone hydrobromide. The lactone hydrochloride (97 mg.) was dissolved in water and shaken with freshly precipitated and washed Ag_2CO_3. The AgCl and excess of carbonate were centrifuged off and the supernatant and washings treated with H_2S to remove Ag. More conveniently, the combined HCl can be removed on a column of Amberlite 1R-4B. The lactone solution was evaporated on an open water bath, taken up in water and the operation repeated several times. On reducing to a syrup the free acid crystallized in large prisms; yield, 55 mg. (55%). It was recrystallized from water; m.p. 232–233·5° (decomp.); periodate–formaldehyde 97% of theoretical (Found: C, 40·31; H, 7·47; N, 11·56. $C_4H_9O_3N$ requires C, 40·33; H, 7·62; N, 11·76%). On paper chromatography the acid ran as one spot and gave a normal ninhydrin colour; $R_F = 0{\cdot}31$ in butanol–acetic acid–water (4:1:5, by vol.). Jollès & Fromageot (1951) claim to have prepared the acid by reduction of *N*-benzyloxycarbonyl-DL-asparagyl-β-ethylglycinate α-benzyl ester with $LiAlH_4$, followed by hydrolysis. The crude mixture was separated by paper chromatography. The few milligrams of material obtained were shown to be oxidized by periodate and to give a discrete spot on paper chromatography but were not otherwise characterized.

Synthesis of δ-hydroxy-γ-aminovaleric acid (δ-γ-HAV)

Jollès & Fromageot (1952) claim to have prepared this hydroxyamino acid by reduction of the α-benzyl ester of ethyl *N*-benzyloxycarbonylglutamyl-γ-glycinate with $LiAlH_4$, but the few milligrams of material obtained were not satisfactorily characterized. In the present case the methyl ester of pyrrolidonecarboxylic acid (X) was reduced with $LiAlH_4$ to pyrrolidonyl alcohol (XI), the ring of which opened on hydrolysis to give δ-hydroxy-γ-aminovaleric acid (XII).

L-Pyrrolidonecarboxylic acid (2·2 g.) in 85% ethanol, containing 1·5 ml. of N-HCl, was treated at 0° with diazomethane (1·1 g.) in ether. On removing the solvent *in vacuo* the ester was obtained as a colourless oil which was not further purified. It was reduced with $LiAlH_4$ under the conditions described above, and, after hydrolysis, was separated on a (3·5 cm. × 25 cm.) column of Dowex-50 (fractions 78–120), N-HCl being used for elution. On evaporation δ-γ-HAV hydrochloride was obtained as an oil which crystallized in needles on standing for several months. After treatment on a column of Amberlite IR-4B the free acid was evaporated *in vacuo* and an equal volume of alcohol added. The precipitated material dissolved on warming, and on cooling to 0° it crystallized in clumps of needles, m.p. 160–161·5°; periodate–formaldehyde, 98·5–100% theory; (Found: C, 45·3; H, 8·32; N, 10·45. $C_5H_{11}O_3N$ requires C, 45·1; H, 8·33; N, 10·52%). On paper chromatography it gave a normal ninhydrin colour; $R_F = 0{\cdot}21$ in butanol–acetic acid–water (4:1:5, by vol.).

Methods of estimation

Amide nitrogen. This was estimated as described by Chibnall *et al.* 1958*b*.

Hydrolysis of proteins. This was done as described by Chibnall & Rees (1958).

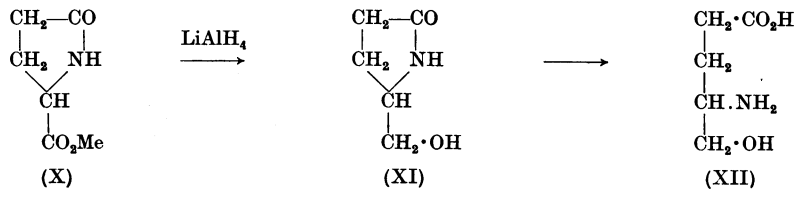
Ornithine and α-γ-diaminobutyric acid. These were estimated by the gradient-elution procedure of Moore & Stein (1954). They emerge together, following ammonia and in front of lysine. As ornithine alone gives a colour by the specific method of Chinard (1952) the two bases can be separately estimated by treating portions of the relevant fractions by this method as well as by ninhydrin. Amino alcohols emerge in this region; if present they can be detected by periodate oxidation, but the quantitative estimation as formaldehyde is unreliable because of the presence of thiodiglycol and BRIJ 35 (polyoxyethylene lauryl alcohol) in the elution liquors.

L-*Glutamic acid.* This was estimated by the decarboxylase method of Gale (1945). The bulk supply of *Clostridium welchii*, S.R. 12, was prepared by the procedure of Krebs (1950). After hydrolysis by the usual procedure the recovery of the acid was 96±1%; a correction factor of 4% has accordingly been applied. In agreement with both these workers we find that the enzyme preparation is without action on L-aspartic acid (cf. Meister, Sober & Tice, 1951).

L-*Aspartic acid.* The shortened procedure of Krebs (1950) was used so as to avoid possible interference from cysteic acid. In agreement with Krebs (1953) it was found that special care must be taken to ensure quantitative liberation of CO_2 by adjusting the reaction mixture to below pH 5. The recovery was 95±1% and the correction factor 5%.

Procedures

Unless otherwise stated the protein esters were prepared with methanolic 0·1N-HCl at 25° for 24 hr. and were reduced with 0·3M-$LiBH_4$ (eightfold molar excess) in tetrahydrofuran under reflux for 6 hr. (Chibnall & Rees, 1958).



(X) (XI) (XII)

Column analysis for aspartic acid, glutamic acid, γ-hydroxy-β-aminobutyric acid and δ-hydroxy-γ-aminovaleric acid. To avoid buffered systems which would interfere with the oxidation of the hydroxyamino acids by periodate, Dowex-50 was used in the hydrogen form and the amino acids were eluted with HCl (Stein & Moore, 1949*a*). The column (50 cm. × 1 cm.) was water-jacketed to maintain 18–19° and packed in 4 cm. zones. It was first washed with N-NaOH until the eluate was alkaline, and a further 50 ml. run through. Water was then applied until the eluate was neutral. The column was regenerated with N-HCl, which was run through until the flame test for Na^+ ion was negative. The sample to be chromatographed (2·2–2·5 mg. of N) in N-HCl was then added and washed into the column with 4 × 1 ml. of N-HCl. The usual elution procedure with N-HCl was then applied and 140 fractions (1 ml.) were collected. From each of these sample (0·2 ml.) was withdrawn, neutralized with N-NaOH (0·2 ml.) and treated with ninhydrin (Chibnall *et al.* 1958*a*). If the temperature control had been adequate a good separation was obtained (Fig. 1). From fraction 141 onwards the fraction size was increased to 2 ml. and the tubes, in racks of 25, were dried overnight in a vacuum desiccator (0·5 mm.) over P_2O_5 and NaOH. The residual material was dissolved in phosphate buffer (pH 11, 0·1 ml.) and heated at 100° for 5 min. to open the γ-lactone ring, a treatment that does not affect the estimation of δ-γ-HAV. The periodate–formaldehyde procedure of Rees (1958) was then applied.

Recovery of hydroxyamino acids. After hydrolysis for 24 hr. with HCl–formic acid and treatment under the usual conditions used for proteins the recovery of δ-γ-HAV, estimated by periodate oxidation, was 85%. Attention has already been drawn to the equilibrium established between γ-β-HAB and its lactone on acid hydrolysis, during column fractionation and on periodate analysis of the column effluents. To allow for these various incidental interferences, and also for destruction during hydrolysis, it was necessary to work under standard conditions. Accordingly, insulin with 2% of γ-β-HAB and β-lactoglobulin with 1% of δ-γ-HAV were passed through the hydrolysis and column treatment. The recovery of the former acid was 63·8% and of the latter 72%; corresponding correction factors have been applied in all the analyses quoted below. These low recoveries are due not to extensive destruction on hydrolysis or to incidental losses but to ring formation under the conditions used (cf. Chibnall, Rees & Richards, 1958). The recovery of aspartic and glutamic acids after hydrolysis and treatment on the column was 100±2% and no correction factors have been applied.

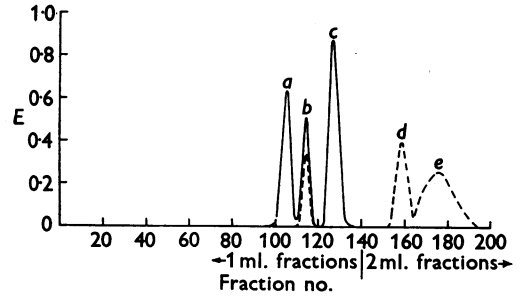


Fig. 1. Chromatographic separation of aspartic acid (*a*), threonine and serine (*b*), glutamic acid (*c*), δ-hydroxy-γ-aminovaleric acid (*d*) and γ-hydroxy-β-aminobutyric acid (*e*) on a 50 cm. × 1 cm. column of Dowex-50 × 8 eluted with N-HCl (——, estimated by ninhydrin; - - - -, estimated by formaldehyde given on periodate oxidation).

Comments. The procedure gives a sharp separation of the four products required. If the temperature of the column is allowed to rise above 20° the serine–threonine peak tends to move forward and the separation from aspartic acid may be impaired. If portions of fractions 105–115 are developed with ninhydrin to give the serine–threonine peak and a similar series of portions with periodate to give a serine peak, a reasonably accurate value for threonine can be obtained by difference. When the elution is carried through to the fourth day the amino alcohols emerge, but because of the time lag the order of emergence and the recoveries have not been investigated in detail. δ-α-HAV and γ-α-HAB emerge just behind δ-γ-HAV and γ-β-HAB respectively, but they must be developed with ninhydrin and reasonable resolution of δ-α-HAV from γ-β-HAB has not yet been achieved. This might be possible on a longer column, or at a higher pH at which the expected differences in the p*K* values of the carboxyl groups would be effective.

RESULTS AND DISCUSSION

Loss of amide nitrogen on reduction. A direct reduction of the amide group in residues of glutamine and asparagine would lead to the presence of ornithine and α-γ-diaminobutyric acid respectively in the hydrolysate of reduced protein or protein ester. When search was made for these two basic amino acids the amounts found were so small that it was necessary to charge the Moore & Stein (1954) column with four times the normal load of hydrolysate. A reduced insulin ester gave 0·1 mole of ornithine/mole of protein (5732 g.) and a reduced β-lactoglobulin ester 0·23 mole/mole of protein (37 000 g.). In neither case was α-γ-diaminobutyric acid detected.

Reductive cleavage of the amide bond would also lead to a loss of amide N. According to our experience, however, the observed loss is no more than that which should accompany the direct reduction mentioned above. For insulin, the protein (dispersed as described by Chibnall & Rees, 1958) showed a fall of 0·04 and the ester 0·1 mole/mole of protein (5732 g.) respectively. The ester and reduced ester of β-lactoglobulin gave the same value within experimental limits, as one would expect if the actual loss were no greater than 0·23 out of 26·2 moles/mole of protein (37 000 g.). It can be assumed therefore that, within the limits claimed for the present procedure, the loss of amide N on reduction with $LiBH_4$, about 1·0–1·5% of the total amide N, is negligible. The recent work of Davis (1956) led to a similar conclusion.

Analysis of β-lactoglobulin

Protein dicarboxylic acids. The values for glutamic acid obtained by the decarboxylase procedure, which is optically specific, and by column analysis, which is not specific, are in agreement with each other (Table 1) and in slight excess (2 residues/mole) over those obtained with a starch column (Stein & Moore, 1949*b*) and by isotope dilution (Foster, 1945). The aspartic acid values are in agreement throughout. It is clear from these results, for which an accuracy closer than 1 residue/mole cannot be claimed, that the protein hydrolysate does not contain any appreciable proportion of racemized glutamic acid. This finding is of interest in connexion with the work of Bailey, Chibnall, Rees & Williams (1943), who claimed to have isolated, in terms of residues/mole, 49 of L- and 5 of D-glutamic acid as the hydrochloride, and only 28 of L-aspartic acid as the Cu salt. It would appear that when aspartic acid is present in fairly high concentration, as it would be in this particular hydrolysate, it can be salted out as the hydrochloride along with that of L-glutamic acid, and the assumed presence of D-glutamic acid must be ascribed at least in part to this occurrence, for aspartic acid hydrochloride has a lower positive rotation than glutamic acid hydrochloride. We have, indeed, recently tested by paper chromatography some of the (assumed) partially racemized glutamic acid–hydrochloride fractions isolated by Bailey and his colleagues in the above-mentioned work, and they contain aspartic acid, but in amount too small to be detectable by methods available in 1943.

Reduced protein. Reductive cleavage of peptide bonds has occurred to a small extent and the hydroxyamino acids thereby produced could have come from corresponding residues of either the free dicarboxylic acids or the acid amides. The evidence given later shows that the amide residues alone are concerned.

Reduced protein ester. Reductive cleavage of peptide bonds to the same small extent was again encountered. In this case, however, the γ-β-HAB and the δ-γ-HAV must have had their origins in asparaginyl and glutaminyl residues respectively, as aspartyl and glutamyl residues would have given rise to the corresponding diols, the presence of which was not observed (Chibnall & Rees, 1958). The hydroxyamino acids have been accordingly included in the summation of the aspartic and glutamic acid residues given in Table 1, which are collectively in good agreement with the amide N of the ester concerned. The amide N lost during esterification (two groups) may have come exclusively, as with insulin, from asparagine residues, but we have preferred, arbitrarily, to assume that the loss is distributed equally between the two amide species (Table 3).

Analysis of lysozyme

The aspartic and glutamic acid analysis of the protein (Table 2) is in agreement with that of Thompson (1955). When the protein is esterified with methanolic 0·1 N-HCl at 25° for 24 hr. there is a fall in the amide N (Chibnall *et al.* 1958*a*) from 17·9 to 17·0 groups/mole. The data for the reduced protein (Table 2) leave no doubt that the distribution of residues set out in Table 3 is correct. Smith, Kimmel & Brown (1954) deduced from the electrophoretic mobility studies of Alderton, Ward & Fevold (1945) that the free carboxyl groups in this protein were predominantly aspartyl groups.

Table 1. *Analysis of β-lactoglobulin*

Protein samples A and B, see Chibnall *et al.* (1958*a*). Treatment: H, hydrolysed; R, reduced and hydrolysed. Methods of analysis: E, enzyme; C, column of Dowex-50. Results are expressed as residues/mole (37 000 g.).

			1			4				
Material	Treatment	Method of analysis	Aspartic acid	2 γ-β-HAB	3 1+2	Glutamic acid	5 δ-γ-HAV	6 4+5	7 3+6	Amide N
Protein A	H	E	30·1	—	—	49·9	—	—	—	—
Protein A	H	C	31·7	—	—	50·5	—	—	—	—
Protein B	H	E	31·1	—	—	49·5	—	—	—	—
		Mean	31·0	—	31·0	50·0	—	50·0	81·0	28·0
Protein A	R	E	29·8	—	—	—	—	—	—	—
Protein B	R	E	30·4	—	—	49·6	—	—	—	—
Protein B	R	C	30·4	0·8	—	49·0	1·7	—	—	—
		Mean	30·2	0·8	31·0	49·3	1·7	51·0	82·0	—
Ester A	R	E	8·6	—	—	15·8	—	—	—	—
Ester A	R	C	8·2	—	—	15·0	—	—	—	—
Ester B	R	E	8·4	—	—	15·5	—	—	—	—
Ester B	R	C	8·0	0·9	—	15·8	1·5	—	—	—
		Mean	8·3	0·9	9·2	15·5	1·5	17·0	26·2	26·0

Table 2. *Analysis of lysozyme, β_1-lactoglobulin and ox insulin*

Treatment: H, hydrolysed; R, reduced and hydrolysed. Results are expressed as residues/mole (lysozyme, 14 700 g.; β_1-lactoglobulin, 37 000 g.; insulin, 5732 g.).

Material	Treatment	1 Aspartic acid	2 γ-β-HAB	3 1+2	4 Glutamic acid	5 δ-γ-HAV	6 4+5	7 3+6	8 Amide N
β_1-Lactoglobulin	H	30·6	—	30·6	49·8	—	49·8	80·4	27·7
β_1-Lactoglobulin	R	30·0	0·8	30·8	49·0	1·4	50·4	81·2	—
β_1-Lactoglobulin ester	R	8·2	0·9	9·1	15·6	1·33	16·9	26·0	25·9
Lysozyme	H	20·1	—	20·1	4·95	—	4·95	25·05	17·9
Lysozyme	R	18·4	0·67	19·07	3·87	1·0	4·87	23·94	—
Lysozyme ester	R	11·3	0·86	12·16	3·64	1·03	4·67	16·83	17·0
Insulin	H	2·94	—	2·94	6·90	—	6·90	9·84	5·82
Insulin	R	2·75	0·08	2·83	6·72	0·17	6·89	9·72	5·78
Insulin ester	R	1·6	0·89	2·49	3·0	0·18	3·18	5·67	5·45

Table 3. *Number of asparagine and glutamine residues in different proteins*

Results are expressed as residues/mole of protein, allowance being made for amide loss during esterification.

Residue	β-Lactoglobulin (mol.wt. 37 000)	β_1-Lactoglobulin (mol.wt. 37 000)	Lysozyme (mol.wt. 14 700)	Ox insulin (mol.wt. 5732)
Asparaginyl	10	10	13	3
Glutaminyl	18	18	5	3
Aspartyl	21	21	7	—
Glutamyl	32	32	—	4

Ohno (1954) claimed to have shown by hydrazinolysis that the protein contains 1 aspartyl, 12 asparaginyl and 4 glutaminyl residues/mole.

Analysis of β_1-lactoglobulin

The mol.wt. of this protein has not been determined, but the amide N would allow 28 groups/mole of 37 000 g., the same as β-lactoglobulin. The data given in Tables 2 and 3 fail to reveal any difference between the two proteins.

Analysis of insulin

This protein is of particular interest in the present studies. In the first place, its amino acid structure is known, so that deductions based on the procedure under review can be verified. Secondly, the sample of insulin used in these studies, which was a commercial preparation that had been specially purified for research purposes, has been shown to be deficient in amide N (5·82 instead of the expected 6 groups/mole, Chibnall *et al.* 1958*a*). The data collected in Table 2 show that reductive cleavage of peptide bonds has occurred, and as the amount of δ-γ-HAV produced from the ester was the same as that from the protein itself, it may be inferred, as discussed above, that glutaminyl and not glutamyl residues were concerned. The computed number of glutamine residues in the reduced ester (Table 2, column 6) is, indeed, in slight excess of the number (3/mole) required to satisfy the known structure and reflects, according to our experience, the difficulty of fully esterifying the glutamyl residues in this particular protein. The γ-β-HAB given by the reduced protein may have been derived from asparaginyl residues in the peptide chains, for insulin does not contain any aspartyl residues. It is more than possible, however, as mentioned below, that residues of the latter type might have been produced during the manufacture or esterification of the sample of insulin concerned.

The balance (Table 2) between the amide N of the ester and the products representing asparagine and glutamine residues is reasonable, especially if allowance be made as suggested above for the possibility that one of the glutamyl residues is esterified with less readiness than the others. If allowance is made for known losses of amide N, the data given in Table 3 correspond to those derived in a similar way for the other proteins and are in agreement with the known structure of insulin.

There remains the problem of deciding the origin of the lost amide N. Insulin, when reduced and hydrolysed, gave no aspartidiol, but *O*-acetylinsulin methyl ester, prepared with diazomethane without loss of amide N, gave 0·05 group/mole, and this must have come from a *C*-terminal aspartic acid residue which, in its turn, must represent a *C*-terminal asparagine residue which had lost its amide N. By difference therefore the remaining loss of 0·13 group of amide N/mole of insulin (Table 4) must have come, in large part, from asparaginyl residues in the peptide chains. On esterification with methanolic HCl under the usual

Table 4. *Loss of amide nitrogen during the preparation of insulin from ox pancreas and on subsequent esterification with methanolic hydrochloric acid*

Results are expressed as groups/mole (5732 g.).

	Loss of amide-N		
	1	2	3
	Total	*C*-terminal	In peptide chains (1 – 2)
Insulin (see text)	0·18	0·05	0·13
Insulin ester (see text)	0·55	0·10	0·45
Loss on esterification with methanolic 0·1 N-HCl	0·37	0·05	0·32

conditions there was a further loss of amide N and this was derived, as before, from both types of asparagine residues. These deductions suggest strongly that the specially prepared sample of insulin used in our research had lost 0·18 group of amide N/mole through hydrolysis during its preparation from the frozen pancreas, which involves, at various stages, an acid medium. It has already been shown that the hexa-amide (*A* peak) insulin of Harfenist & Craig (1952) contains the theoretical amount of amide N. From the discussions given above it would seem clear that their penta-amide (*B* peak) material is not a chemical entity, but an artifact of hydrolysis during preparative treatment in which all of the asparagine residues in the molecule have become modified to some extent.

SUMMARY

1. A method for estimating the total number of asparagine and of glutamine residues in proteins has been explored.

2. The method is based on modifications which aspartyl, glutamyl, *C*-terminal asparaginyl and *C*-terminal glutaminyl residues undergo when esterified and then reduced with lithium borohydride.

3. After hydrolysis the two latter residues are represented by γ-hydroxy-β-aminobutyric acid and δ-hydroxy-γ-aminovaleric acid respectively.

4. Asparaginyl and glutaminyl residues in the peptide chains do not become modified and after hydrolysis can be estimated as aspartic and glutamic acids respectively.

5. Interference due to reductive cleavage of peptide bonds occurs to a very small extent and can be allowed for.

6. Data are presented for the asparaginyl, glutaminyl, aspartyl and glutamyl residues in ox insulin, β- and β_1-lactoglobulin and lysozyme.

7. δ-Hydroxy-γ-aminovaleric acid and γ-hydroxy-β-aminobutyric acid have been synthesized and their properties are described.

One of us (A.C.C.) would like to acknowledge the generous financial assistance provided by Eli Lilly and Co., Indianapolis, U.S.A.

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