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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contain ammonia N. The amount present may be small, yet in a protein of high molecular weight it may be equivalent to one or more groups of amide N per molecule, thus making less definite the estimation of the latter factor. A procedure for determining this ammonia N, which depends on the fact that the protein can be precipitated from methanolic hydrochloric acid by ether, while the ammonium salts remain in solution, is described in the present paper. It has been applied to native samples of several different proteins and, allowing for the ammonia N thus determined, it has been shown, for instance, that the corrected value for the amide N of native β-lactoglobulin is in very close agreement with that recorded for the heat-coagulated product, and that all the samples of crystalline ox insulin examined, except that prepared by countercurrent distribution, have low corrected values for amide N, suggesting loss in manufacture.

EXPERIMENTAL

Materials

Ox insulin. (1) Two samples of crystalline Zn insulin were obtained from Boots Pure Drug Co. Ltd., Nottingham (batch no. 9633B and 9011G). They had been prepared under identical conditions and recrystallized six times from a citrate buffer. Batch 9633B contained (moisture-free) 15·76% N (micro-Kjeldahl) and 15·83% N (micro-Dumas). (2) A sample for which we thank Dr L. C. Craig; it was the '*A* peak' material obtained by countercurrent extraction of a commercial product (Harfenist & Craig, 1952). (3) A sample for which we thank Dr R. R. Porter; it had been purified by chromatography (Porter, 1953).

β-Lactoglobulin. Samples *A* and *B* were prepared by the procedure of Palmer (1934), $(NH_4)_2SO_4$ and Na_2SO_4 fractionation respectively being used. The twice-recrystallised products had been dialysed against distilled water for 3 days.

β_1-Lactoglobulin. We have to thank Dr T. L. McMeekin for a sample of crystalline material prepared by the method of Polis, Schmukler, Custer & McMeekin (1950).

Lysozyme. Prepared from egg-white (Armour, no. 20793).

Methods

Total nitrogen. This was estimated by the procedure of Chibnall, Rees & Williams (1943).

Ammonia nitrogen. This was estimated by the ninhydrin method of Moore & Stein (1954), with cyanide (Troll & Cannan, 1953; Yemm & Cocking, 1955) in place of hydrindantin. The colour intensity with NH_3 was affected by the presence of salts, and to overcome this a sodium acetate buffer (5M) was used. The colour yield was then reproducible and 80% of that obtained from leucine. Small quantities of the reagent can readily be made up as required.

The procedure was as follows. Sodium acetate buffer, pH 5·5 (5M), was stored at 37°. To a sample containing approx. 1·5–5 μg. of ammonia N in a volume of 2 ml. was added 1 ml. of a solution made from buffer (23 ml.), 0·01M-KCN (2 ml.) and methyl Cellosolve (2-methoxyethanol) (75 ml.) followed by 10% (w/v) ninhydrin in methyl Cellosolve (0·2 ml.). After shaking, the colour was developed, diluted and estimated in the usual way against a standard curve. In some of the studies on β-lactoglobulin we used the hypochlorite procedure of Van Slyke & Hiller (1933), as modified by Russell (1944). It is satisfactory, but the sensitivity of the colour developed is considerably less than with ninhydrin.

Amide nitrogen. The protein was hydrolysed by boiling with 2N-HCl (3 mg./ml.) for 3 hr. The subsequent treatment depended on the amount of material concerned. If this was in excess of 0·1 g., the hydrolysate was brought to pH 4 (bromocresol green) with 4·8N-NaOH, run into a micro-Kjeldahl apparatus and made alkaline by the addition of 10 ml. of 0·3M-Na_2HPO_4–NaOH, pH 11, buffer solution. The NH_3 was then distilled over into 0·0143N-HCl. If the quantity of protein was of the order of 5–10 mg. suitable samples of the hydrolysate were taken and the Conway technique was employed, the NH_3 being estimated with ninhydrin. The value found (Table 1) is a measure of (amide N + ammonia N), and to obtain the first the second must be subtracted from the total.

Estimation of ammonia nitrogen in proteins. The protein (10 mg.) was dissolved in 1 ml. of methanolic 0·033N-HCl and at once precipitated by adding ether (2 ml.). It was removed by centrifuging and the operation was twice repeated. The collected liquors were then evaporated to dryness *in vacuo* to remove solvents, and ammonia N was determined with ninhydrin after distillation in the Conway

Table 1. *Amide nitrogen of various proteins*

Protein	Sample, literature reference or source	Amide N (found) % total N	Ammonia N (% total N)	Amide N (corrected) % total N	Amide N (corrected) Groups/mole
β-Lactoglobulin	*A*	7·06	0·26	6·80	28·04
	B	6·88	0·05	6·83	28·16
	Rees (1946)	6·77	—	6·77	27·93
	Cannan *et al.* (1942)	6·79	—	6·79	28·0
	Mean	—	—	6·80	28·03
β_1-Lactoglobulin	Dr T. L. McMeekin	7·32	0·60	6·72	27·73
Insulin	Boots: (9633B, 9011G)	9·08	0·16	8·92	5·81
	Dr L. C. Craig (fraction *A*)	9·38	0·14	9·24	6·0
	Dr R. R. Porter	9·19	0·85	8·34	5·42
Lysozyme	Armour 20793	9·19	0·05	9·14	17·9

apparatus. To ascertain whether the treatment completely removed all the NH_4^+ ions a sample of insulin containing 1·825 mg. of N was passed through it, the ammonia N content of the three supernatant liquors being separately determined. The amounts found were 2·34, 0·46 and 0·13 μg. of ammonia N, equivalent to 0·128, 0·025 and 0·007 % total protein N respectively, showing that the treatment was suitable for the purpose. The residual solubility of the protein in the methanolic HCl–ether mixture was of the order of 1 %. As a check on the Conway procedure a sample of 180 mg. of Boots insulin was given the standard treatment and the collected liquors were reduced to a small volume *in vacuo*. Appropriate portions were then taken for the estimation of ammonia N by the ninhydrin–Conway procedure and by distillation under micro-Kjeldahl conditions. The results were in excellent agreement.

RESULTS AND DISCUSSION

β-Lactoglobulin. The data given in Table 1 present some interesting contrasts. The two samples of native crystalline material, which had such very different contents of ammonia N, gave corrected values for amide N which were not only in excellent agreement with each other, but also with those obtained in two earlier studies in which heat-coagulated and washed products had been used. Cannan, Palmer & Kibrick (1942) hydrolysed the protein with N-HCl at 100°, and Rees (1946) employed 10N-HCl at 37° (Gordon, Martin & Synge, 1941); in each case the amide N was determined at successive intervals of time and extrapolated to zero. If the protein N is taken as 15·6 % (Chibnall *et al.* 1943) and the mean value for the amide N as 6·50 % of the total N, then 28 groups of amide N/mole of protein leads to a molecular weight of 36 960, which is in good agreement with recent deductions based on physical measurements. One group of amide N is thus equivalent to 0·243 % of the total N, and it will be observed that the ammonia N of sample *A* is in excess of this value.

β_1-Lactoglobulin. The molecular weight of this protein has not yet been determined and we have assumed it to be the same as that of β-lactoglobulin itself; the data given in Table 1 call for no comment.

Insulin. According to Sanger, Thompson & Tuppy (1952) the minimum mol.wt. of ox insulin is 5732 and the amide N is 9·24 % of the total N. The two commercial samples used in the present work contained 9·08 % amide N without allowance being made for any ammonia N present, and it was, in part, upon this uncorrected value that our original estimate of the minimum mol.wt. of insulin (12 000 or 6000) was based (Chibnall, 1946). Harfenist & Craig (1952) have since shown by countercurrent distribution that commercial insulin can be separated into two fractions, an '*A* peak' containing about six groups and a '*B* peak' with only about five groups of amide N respectively (Harfenist, 1953).

Dr Craig (private communication) informed us that our sample of Boots insulin (batch no. 9633B) contained about 85–90 % of the '*A* peak' and 5–8 % of the '*B* peak' material. The '*A* peak' material provided by Dr Craig (origin not stated) was the only sample of insulin we have handled during the present research which gave a (corrected) theoretical value for amide N. Harfenist & Craig suggested that their '*B* peak' material, of lower amide content, may have been an artifact of hydrolysis during manufacture of the insulin: we are in agreement with this view and shall present further data in a later paper. The preparation of purified insulin by the chromatographic method of Porter (1953) has clearly not differentiated between the *A* and *B* peak material of Harfenist & Craig. In spite of the fact that metallic salts were used in the fractionation, the final product contained a high proportion of ammonia N. Indeed, the uncorrected value for amide N given in Table 1 illustrates the danger of omitting an estimation of ammonia N when the amide N of a protein is in question.

Lysozyme (mol.wt. 14 700). The amide N given in Table 1 is in agreement with that of Lewis, Snell, Hirschmann & Fraenkel-Conrat (1950).

SUMMARY

1. Attention is drawn to the need to ascertain the free ammonia content of native protein preparations as a factor in the determination of the amide nitrogen.

2. A sample of ox insulin prepared by countercurrent distribution had a theoretical amide nitrogen of six groups/mole (5732 g.); all other samples examined had low values, which suggested loss during manufacture from the pancreas.

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

3. THE ESTERIFICATION OF PROTEINS

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The carboxyl groups of a protein can be esterified with diazomethane, but more conveniently with methanolic hydrochloric acid. In the present research, however, neither reagent has proved to be entirely satisfactory, for in our hands the first has not given full esterification and the second would do this only at the expense of the amide N, which we were particularly anxious to keep intact. Fraenkel-Conrat & Olcott (1945) were the first to show that the concentration of mineral acid (0·02 to 0·1N) required to catalyse the reaction with methanol was very much less than previous workers had employed for the purpose. They claimed that 97% of the carboxyl groups of polyglutamic acid from *Bacillus subtilis* could be esterified by treatment with methanolic 0·05N-HCl at 22–24° for 24 hr. and that several proteins, including insulin, were fully esterified under similar conditions in the presence of 0·1N-HCl. They mentioned that there was no loss of amide N under such treatment, though data in support of the assertion were not presented. Mommaerts & Neurath (1950) repeated the experiments with insulin, and confirmed that full esterification was apparently achieved with methanolic 0·1N-HCl at 25° in 24 hr. They claimed that the ammonia liberated under these conditions amounted to not more than 2% of the amide N.

Our own observations do not support the contention that 0·1N-HCl can be used as a catalyst in the esterification of proteins in this way without loss of amide groups. With insulin the ammonia produced in 24 hr. at 25° represents about 6·6% of the amide N, a value much greater than the above-mentioned workers seem to have suspected. Lowering the concentration of hydrochloric acid spares the amide N but unfortunately full esterification is not then achieved. We have thus been obliged to prepare the ester with methanolic 0·1N-HCl, knowing that when this is used subsequently to determine *inter alia* the amide distribution of the protein, we had already set a limit to the accuracy with which this could be done. Another interesting side-reaction of protein esterification has been traced to *N–O* acyl migration in serine and particularly threonine residues.

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EXPERIMENTAL

Materials

Protein samples, and methods for determining N, ammonia N and amide N were described in the preceding paper (Chibnall, Mangan & Rees, 1958*b*).

Methoxyl. The Zeisel procedure of Pregl (1937) was used. Estimations on 20–30 mg. samples agreed to within 2%.

Methanol. This was boiled under reflux for some hours with Mg turnings and then distilled.

Methanolic hydrochloric acid. An approx. 2N-solution was prepared with dry HCl gas and stored at −15°. As the acidity falls on keeping it is necessary to titrate a sample immediately before use.

Methods

Estimation of the extent of esterification. The extent of esterification of the total free carboxyl groups was followed by determination of methoxyl. As β-lactoglobulin contains methionine, which yields methyl iodide on treatment with HI, the (apparent) methoxyl content of the protein itself was subtracted from that given by the ester. In studies dealing with the relative rates of esterification of glutamyl and asparagyl residues (Tables 1 and 4) the number of the respective residues esterified was computed from an analysis of the reduced protein hydrolysate (see Chibnall, Haselbach, Mangan & Rees, 1958*a*). Amide hydrolysis during esterification was followed by applying to the ester, while still in solution, the procedure for the