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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 0·1 N) 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 0·05 N-HCl at 22–24° for 24 hr. and that several proteins, including insulin, were fully esterified under similar conditions in the presence of 0·1 N-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 0·1 N-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 0·1 N-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 0·1 N-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. 2 N-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

estimation of ammonia N in proteins described in the previous paper (Chibnall *et al.* 1958*b*).

Esterification with methanolic hydrochloric acid. The protein (0·25 g.) was placed in a flask (50 ml.) and dried overnight *in vacuo* at room temperature over P_2O_5 and NaOH. Methanol (20 ml.) was added, followed by methanolic HCl drop by drop until the protein dissolved. Sufficient excess of both methanolic HCl and methanol was then added to bring the final volume to 25 ml. and the acid concentration to that required. The protein was sometimes partly precipitated, especially with the stronger acid catalyst, but always as a fine emulsion, and mechanical stirring during the incubation period was unnecessary. The flask was then immersed in a water bath at 25° and shaken slowly for the prescribed period. About 60 ml. of ether was next added and after standing for a short time the precipitated ester hydrochloride was removed by centrifuging, washed twice with ether (50 ml.), dried *in vacuo* over P_2O_5 and NaOH and then allowed to equilibrate in the air at room temperature.

Esterification with diazomethane. To suppress ionization of the free carboxyl groups it is necessary to employ an acid medium and 85% (v/v) ethanolic 0·03 N-HCl is a suitable one for insulin and lactoglobulin as both proteins are soluble in it. The reagent rapidly neutralizes the HCl, and as the protein becomes progressively more basic during esterification the pH of the medium will rise, favouring both O and N methylation, unless controlled by the addition of more acid. In the early stages of the work an ethereal solution of diazomethane was added in small portions at a time, alternately with drops of HCl, until the yellow colour of the chilled protein solution was permanent for at least 5 min. As an improvement the pH was controlled with a spot indicator (bromocresol purple). Under such conditions not more than three of the four ω-carboxyl groups of glutamic acid in insulin were esterified (Chibnall & Rees, 1952, 1953). More complete esterification can be effected in the following way. The reaction vessel is equipped with a glass electrode–calomel cell assembly, a stirrer and two burettes with long capillary tips, one for the addition of the cooled ethereal reagent and the other for 6 N-HCl. The vessel, containing a 1% solution of the protein in the above-mentioned solvent, was stood in a freezing mixture. Ethereal diazomethane (a twofold excess allowing for the HCl as well as the protein carboxyl groups) was then run in slowly with stirring, the (apparent) pH being controlled between 4 and 5 by the necessary addition of HCl. At the end of the reaction a slight excess of HCl was added and the ester hydrochloride precipitated with 8–10 volumes of ether, washed twice with ether and dried *in vacuo* over P_2O_5 and NaOH. According to our experience such products often contain a small amount of trimethylamine, which is not readily removed and can lead to high values for amide N.

RESULTS AND DISCUSSION

The data given in Table 1 show that full esterification of β-lactoglobulin with diazomethane had not been achieved under any of the conditions employed. Our results with insulin were similar, and an earlier claim (Chibnall & Rees, 1953) that full esterification was possible provided that the reaction mixtures was kept below pH 5 was based on a molecular weight of 6000 and not on the present accepted value of 5732. With methanolic 0·1 N-HCl the esterification is complete in 24 hr. at 25° (Table 2), but, as has been mentioned earlier, appreciable alcoholysis of the amide groups occurs. As these observations on the lability of the amide N are in partial conflict with those of Mommaerts & Neurath (1950) the full protocol for one experiment on insulin is given in Table 3. For the studies reported in other papers of the series esterification with methanolic 0·1 N-HCl has been found to be the only workable compromise for our purpose.

The data given in Table 4 illustrate the progress of the esterification of the various free carboxyl groups in insulin and β-lactoglobulin. The fact that the theoretical maximum is exceeded in all cases is due to amide hydrolysis. It is to be noted that the α-carboxyl of the *C*-terminal asparagine residue in insulin esterifies at a faster rate than the γ-carboxyl groups of the glutamyl residues, whereas in β-lactoglobulin the latter react rapidly and at a rate which exceeds that of the α-carboxyls of the aspartyl residues.

Acyl migration on esterification

A phenomenon accompanying the esterification of insulin and β-lactoglobulin in methanolic hydrochloric acid is an increase in free amino N (Van Slyke). In a previous communication (Chibnall & Rees, 1953) we had discussed the possibility that this might be due to an *N–O* acyl shift at serine and threonine residues to give the corresponding *O*-peptidyl ester and a free amino group, in keeping with the earlier findings of Desnuelle & Casal (1948) and of Elliott (1952), who had shown that such a change could take place under the influence of strong mineral acid. Under our conditions the increase in amino N is much less than Elliott observed, but it is reversible in alkaline solution (Tables 5 and 6) and the data presented in Table 7 show that serine and threonine residues are concerned in the reaction. In insulin, somewhat surprisingly, the threonine residue is the more labile. When the ester is made with diazomethane, the pH being kept below 5 to prevent *N*-methylation, there is no appreciable increase in amino N, suggesting that it is the catalytic action of the hydrochloric acid which promotes the acyl shift and not the suppression of free carboxyl groups as mentioned in our earlier communication. That such a shift is a sensitive one and prone to reversal, as though it involved a state of molecular strain, would follow from the observation that when a sample of insulin ester hydrochloride was analysed some months after preparation, the amino N had fallen from an initial 3·6 to 3·2 groups/mole.

Table 1. *Esterification of β-lactoglobulin with diazomethane*

Protein preparation *A*: no. 1, esterified without definite pH control; no. 2, reaction mixture was maintained below pH 7; no. 3, reaction mixture was maintained between pH 4 and 5. For comment on the number of residues esterified see text.

	Glutamyl residues		Aspartyl residues	
No.	Residues esterified/mole of protein (37 000 g.) (max. 32)	Esterification (%)	Residues esterified/mole of protein (37 000 g.) (max. 21)	Esterification (%)
1	27·5	86·0	17·4	82·7
2	32·4	101·2	16·2	77·2
3	30·6	95·6	19·5	92·8

Table 2. *Extent of esterification attained with various strengths of methanolic hydrochloric acid*

Insulin: Boots, 9011 G; amide N and total carboxyl groups are 6 and 6 groups/mole of protein (5732 g.) respectively. β-Lactoglobulin; sample *A*; amide N and total carboxyl groups are taken as 28 and 56 groups/mole of protein (37 000 g.) respectively (Chibnall *et al.* 1958*a*). Results are expressed as groups/mole of protein.

	Insulin			β-Lactoglobulin		
		Carboxyl groups esterified			Carboxyl groups esterified	
Material and normality of methanolic HCl used for esterification	Amide N (found)	Max. possible	Found (methoxyl)	Amide N (found)	Max. possible	Found (methoxyl)
Protein	5·82	6·18	—	28	56·0	—
Ester, 0·025 N	5·65	6·35	5·43	—	—	—
Ester, 0·033 N	5·60	6·40	5·8	27·1	56·9	44·8
Ester, 0·05 N	5·57	6·43	—	26·5	57·5	55·5
Ester, 0·1 N	5·43	6·57	6·4	26·0	58·0	59·1

Table 3. *Hydrolysis of the amide group of insulin during esterification with methanolic hydrochloric acid*

		Total ammonia N			Amide N	
Material	Total N in sample (mg.)	mg.	% protein N	Decrease in amide N (% protein N)	% protein N	Groups/mole of protein (5732 g.)
Insulin	36·5	0·0586	0·16	—	8·92	5·80
Ester (0·025 N-HCl)	36·08	0·1015	0·28	0·12	8·80	5·72
Ester (0·033 N-HCl)	36·08	0·1170	0·32	0·16	8·76	5·69
Ester (0·05 N-HCl)	36·24	0·1595	0·44	0·28	8·64	5·61
Ester (0·1 N-HCl)	36·17	0·2781	0·77	0·61	8·31	5·40

Table 4. *Rate of esterification of glutamyl, aspartyl and C-terminal asparagine residues*

Insulin, Boots (9011 G); β-lactoglobulin, sample *A*; esterified for the period stated in methanolic 0·1 N-HCl at 25°. For comment on the number of residues esterified, see text.

	Insulin				β-Lactoglobulin			
	Glutamyl residues		*C*-terminal asparagine residues		Glutamyl residues		Aspartyl residues	
Time of esterification (hr.)	Esterified residues/ mole of protein (5732 g.)	Esterification (%)	Esterified residues/ mole of protein (5732 g.)	Esterification (%)	Esterified residues/ mole of protein (37 000 g.)	Esterification (%)	Esterified residues/ mole of protein (37 000 g.)	Esterification (%)
0·5	1·9	47·5	0·65	65·0	22·1	69·1	7·3	34·7
1·0	—	—	—	—	27·7	86·6	8·1	38·6
2·2	2·6	65·0	0·9	90·0	—	—	—	—
4·0	3·1	77·5	1·05	105·0	33·0	103·2	15·4	73·3
24·0	4·1	102·5	1·1	110·0	34·8	108·7	21·5	102·5

Table 5. *Increase in amino nitrogen on esterification of β-lactoglobulin and its reversal on treating the ester with dilute alkali*

Procedures before Van Slyke analysis: 1, sample *A*, esterified in methanolic 0·1 N-HCl; 2, sample *B*, esterified as above for 24 hr.; 45·2 mg. of ester was dissolved in 15 ml. of 0·01 N-sodium borate buffer (pH 9·2) at 20·3° to give pH 8·9; 5 ml. portions were taken for analysis.

Material	Procedure	Time of esterification (hr.)	Time of treatment in alkali (hr.)	Amino N: % protein N	Amino N: Groups/mole of protein (37 000 g.)	Amino N: Increase or decrease
Protein *A*	1	0	—	7·10	29·3	—
		0·5	—	7·43	30·6	+1·3
		4·0	—	7·85	32·4	+3·1
		24·0	—	7·99	32·9	+3·6
Ester of protein *B*	2	—	0	8·03	33·1	—
		—	1·0	7·84	32·3	−0·8
		—	4·0	7·29	30·0	−3·1
Protein *B*	—	—	—	6·98	28·8	—

Table 6. *Increase in amino nitrogen on esterification of insulin and its reversal on treating the ester in dilute alkali*

Procedure before Van Slyke analysis: 1, Insulin (Boots, 9011 G) or its methyl ester hydrochloride was dissolved in water; 2, insulin was dissolved in dil. HCl; 3, methyl ester hydrochloride (40 mg.) was suspended in 0·2 ml. of 0·3 N-NaOH and kept at 0° for the required time; the product was then dissolved in a minimum excess of dil. HCl; 4, methyl ester hydrochloride (110 mg.) was dissolved in 10 ml. of aq. methanolic (85 %) 0·3 N-NaOH and kept at 0° for 1 hr. The product was then precipitated with excess of acetone, dried with ether and dissolved in a minimum excess of dil. HCl.

Material	Procedure	Time of esterification (hr.)	Time of treatment in alkali (min.)	Amino N: Temperature	Amino N: Reaction time (min.)	Amino N: Groups/mole of protein (5732 g.)	Amino N: Increase or decrease
Insulin	1	—	—	20·3°	15	2·87	—
Insulin hydrochloride	2	—	—	20·3	15	2·96	+0·09
Ester hydrochloride	1	0·5	—	20·3	15	3·10	+0·23
Ester hydrochloride	1	2·0	—	20·3	15	3·19	+0·32
Ester hydrochloride	1	4·0	—	20·3	15	3·25	+0·38
Ester hydrochloride	1	24·0	—	20·3	15	3·60	+0·73
Ester hydrochloride	1	24·0	—	22·5	11	3·65	—
Ester hydrochloride	3	24·0	5·0	22·5	11	3·30	−0·35
Ester hydrochloride	3	24·0	45·0	22·5	11	3·20	−0·45
Ester hydrochloride	4	24·0	60·0	21	11	2·90	−0·75

Table 7. *Serine and threonine analyses illustrating acyl migration on esterification of insulin in methanolic* 0·1 N-*hydrochloric acid*

The products were deaminated (Peters & Van Slyke, 1932), and then analysed on a column of Dowex-50 with ninhydrin (Moore & Stein, 1954). 1, Insulin; 2, insulin methyl ester (24 hr. at 25°); 3, insulin methyl ester treated with alkali as described in Table 6, procedure 3. In calculating the results a loss of 3 % total N on deamination has been assumed. Serine and threonine values have been corrected for losses on hydrolysis (Rees, 1946). Results are expressed as residues/mole of protein (5732 g.).

Products	Yield of amino acid: Aspartic acid	Threonine	Serine	Glutamic acid
1	2·97	1·02	3·12	6·98
2	2·93	0·73	2·95	6·90
3	2·96	0·91	3·14	7·02

SUMMARY

1. Methods of esterifying proteins have been investigated in detail.

2. It has not been found possible to esterify a protein fully with diazomethane, even when the reaction is allowed to proceed below pH 5.

3. Full esterification can be achieved with methanolic 0·1 N-HCl in 24 hr., in confirmation of the results of earlier workers. The hydrolytic loss of amide nitrogen is, however, much greater than has hitherto been reported.

4. Data are presented to show the relative rates of esterification of aspartyl, asparaginyl and glutamyl residues in insulin and β-lactoglobulin.

5. There is an increase of amino nitrogen (Van Slyke) on esterification of proteins with methanolic 0·1 N-HCl. The effect is reversed in an alkaline

medium and has been shown to be due to an N–O acyl shift in serine and threonine residues.

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

4. SEPARATION AND QUANTITATIVE DETERMINATION OF β-AMINO ALCOHOLS

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The procedure for characterization of the *C*-terminal residues in proteins which was described in the first paper of this series (Chibnall & Rees, 1958) called for a method for the separation and estimation of the β-amino alcohols present in the hydrolysate of the esterified and reduced protein. At the time the investigation was started no such method was available, although subsequently the isolation and chromatographic separation of dinitrophenylamino alcohols has been described by a number of workers (Luck & Wilcox, 1953; Jutisz, Privat de Garilhe, Suquet & Fromageot, 1954; Jatzkewitz & Tam, 1954; Grassmann, Hörmann & Endres, 1954; Crawhall & Elliott, 1955).

A partition column of silica gel on gradient elution with ethanol–chloroform has been found to effect a good separation of such of the β-amino alcohols as were encountered when dealing with insulin, lysozyme and β-lactoglobulin. The identities of the products giving rise to the effluent peaks were checked by paper chromatography, and these products could be quantitatively estimated as formaldehyde after periodate oxidation. Because of the limitations to which the reductive procedure is prone (Chibnall & Rees, 1958), no attempt has yet been made to test the behaviour of such other β-amino alcohols as might occur, but there is no doubt that the method of separation is capable of further development. Early experiments on the use of Dowex-50 were not continued because of low recoveries; paper chromatography was also tried but had to be abandoned because of persistent streaking due to traces of inorganic salts and low recoveries.

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MATERIALS AND METHODS

β-Amino alcohols. Alaninol, aspartidiol and glutamidiol were prepared by reduction of the corresponding amino acid ester hydrochloride. They were purified by distillation *in vacuo*. The other β-amino alcohols used were obtained from L. Light and Co., Wraysbury. As most of the products are oils, aqueous solutions were prepared and standardized for total N and formaldehyde given on periodate oxidation. Suitable portions containing 1–3 μmoles of each β-amino alcohol were then taken for chromatography.

Buffer solution (pH 4·85). $Na_2HPO_4,12H_2O$ (A.R., 184·5 g.) and citric acid monohydrate (A.R., 51 g.) were dissolved in water and made up to 1 l. Chloroform (1–2 ml.) was added as preservative.

Solvent A. Lower phase of the mixture: buffer solution–ethanol–chloroform (1:1:1).

Solvent B. Upper phase of the mixture: buffer solution–ethanol–chloroform (1:2:1). The mixtures were shaken in separating funnels and left overnight at 27·5±1° to equilibrate; the solvent phases were filtered before use.

Column chromatography

Preparation of column. Silica gel (5 g., Tristram, 1946) was ground with 2·5 ml. of the upper (aqueous) phase from solvent *A* and a slurry was then made with 40 ml. of the