A Study of the Proteinase Content and the Chromatography of Thymus Histones

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Previous work from this Laboratory has shown that calf-thymus histones have mainly proline and alanine *N*-terminal groups, together with several minor ones in variable amounts (Phillips, 1958). We wish to describe experiments which show that the histone preparations all contain a proteolytic enzyme that is able to produce alanine, lysine and glycine end groups. It seems likely that proline and alanine are the only original end groups of the histones isolated by the methods used here.

Some partial separations of the mixture of histones into its component proteins on carboxymethylcellulose and alumina columns have been reported (Davison, 1957; Phillips, 1957), but an improved method of chromatography on carboxymethylcellulose has now given two fractions—one having mainly proline, the other mainly alanine *N*-terminal groups.

MATERIALS AND METHODS

The crystalline bovine ribonuclease, bovine plasma albumin, bovine chymotrypsin and hen-egg lysozyme were purchased from Armour and Co. Ltd., and the protamine (salmine) sulphate from Burroughs Wellcome and Co. Ltd.

Preparation of histones. The mixed histones were prepared from previously isolated nucleoprotein from fresh frozen calf-thymus glands by the method of Davison, James, Shooter & Butler (1954). In a few preparations (avoiding dialysis) 0.25 *N*-H₂SO₄ was used for the extraction instead of the usual 0.25 *N*-HCl. Subsequently the H₂SO₄ was removed as barium sulphate which was centrifuged down in a model L Spinco ultracentrifuge at 24 000 *g* (av.) for 1.5 hr. A sample of the supernatant was taken for end-group determination (sample H32 X, Table 1) and the rest freeze-dried. The yield of histone was about 1.2 g./50 g. of thymus gland, the same as that obtained in the method using HCl. In some preparations the NaCl solution used for washing the nucleoprotein was acidified to pH 3.5 with HCl, so that the pH of the washings fell from about 6 to about 4. This was done in order to prevent the enzyme action which (as shown in the results) occurs at neutral and alkaline pH. An example of such a preparation is H30 X (see Table 1). The use of diisopropyl phosphorofluoridate, also to inhibit enzyme action, is described in the Results. In some instances the histones extracted from deoxy-nucleoprotein with 0.25 *N*-HCl were dialysed against 2 *mM*-HCl and the solution was then applied to the chromatographic columns (see below).

Chromatography on carboxymethylcellulose. Carboxymethylcellulose (CMC) was prepared from Whatman cellulose powder by the method of Peterson & Sober (1956) and was finally obtained in the free acid form by washing with 0.01 *N*-HCl and then with water. Columns were prepared by allowing a slurry in 2 *mM*-HCl to settle in the tube under gravity, with about 5 g. of CMC for 50–100 mg. of histones. The histones (about 4 mg./ml. in 2 *mM*-HCl) were run in under gravity. The CMC used could adsorb 140 mg. of histone/g. from a solution in 2 *mM*-HCl (pH 3.8) but the columns were run at about 10% of this loading. The chromatogram was developed by gradient elution with HCl up to 0.05 *N*, fractions of 5–10 ml. being collected automatically at about 1 ml./min. In most of the work the HCl concentration was changed in steps, previously determined from the gradient-elution experiments. The progress of the elution was followed by measuring the extinction of the fractions at 278 *mμ* and in some cases by micro-Kjeldahl nitrogen determination. The product of the extinction and the volume gave the 'extinction units' applied to the column, and similar measurements on the bulked fractions corresponding to the peaks and the interspaces gave the recovery of 'extinction units'. The histones were recovered from the fractions by dialysis against water and freeze-drying, but frequently the acid solutions were simply neutralized and the histones converted into the dinitrophenyl derivatives without prior isolation.

Reaction with 1-fluoro-2:4-dinitrobenzene. The *N*-terminal groups were measured as before (Phillips, 1958). Guanidine hydrochloride was not generally used in the medium since further experiments have shown that it probably has no effect on the type or yield of end groups. In some instances end groups have been determined in the presence of NaCl derived from the neutralization of the HCl used for extraction or fractionation. With histone sample H21 coupled in (a) guanidine bicarbonate, and (b) the same but saturated with NaCl, the end groups were identical both in type and quantity.

To minimize proteolytic action during the coupling with 1-fluoro-2:4-dinitrobenzene (FDNB) in alkaline media, diisopropyl phosphorofluoridate (DFP) as a 0.1 *M*-solution in propan-2-ol is now added to the reaction medium to give a final concentration of *mM*-DFP.

The method has been further checked by the determination of the *N*-terminal groups of several other proteins. Thus crystalline bovine ribonuclease gave 1 mole of lysine/12 000 weight of protein (cf. 1/13 700, Anfinsen, Redfield, Choate, Page & Carroll, 1954). Crystalline bovine plasma albumin gave 1 mole of aspartic acid/72 000 weight of protein (cf. 1.08/69 000: Desnuelle, Rovey & Fabre, 1951). Crystalline hen-egg lysozyme gave 1 mole of lysine/15 700

weight of protein (cf. approx. 1/15 000; Schroeder, 1952). Both horse and human haemoglobins, prepared by the method of Boor & Hektoen (1930) but not crystallized, gave only about 2 moles of valine/66 000 weight of globin, together with extra spots due to stable dinitrophenylated peptides. On more vigorous hydrolysis (16 hr. at 110° in 6*N*-HCl) about 4 moles of valine was found (cf. 5/63 400 of horse globin; Rovey, Desnuelle & Bonjour, 1950, and 4/66 000 for several human haemoglobins; Masri & Singer, 1955). Salmine sulphate after purification through Dowex 1 OH⁻ resin gave 1 mole of *N*-terminal groups/2300 protein, of which 74% were proline end groups. This demonstrated that proline was readily detectable under our conditions.

Total amino acid analyses have been determined with FDNB by the method of Levy (1954) and Fraenkel-Conrat, Harris & Levy (1955), with the following modifications. The histone was hydrolysed for at least 24 hr. in 6*N*-HCl at 110°. The dry hydrolysate from about 2 mg. of histone was dissolved in 1 ml. of borate buffer at pH 9.3 in a stoppered tube and adjusted to pH 9 (testing on indicator papers). After adding 0.01 ml. of FDNB, the tube was shaken at 40° for 4 hr. with frequent adjustment of the pH with NaOH as necessary. The coupled alkaline solution was not extracted with ether since it was found that this removed a small amount of bisdinitrophenyl-lysine. The diluted reaction mixture was then acidified and extracted with ether three times. The ether extracts were then treated as in the *N*-terminal-group measurement (Phillips, 1958). The acid-soluble material was evaporated to dryness *in vacuo* and the yellow derivatives in it removed by several extractions with small amounts of a solvent consisting of acetone-0.1*N*-HCl (95:5, v/v). These extracts were then chromatographed on strips of no. 7 Whatman paper previously buffered with 0.05*M*-phthalate buffer (pH 6), butan-2-ol saturated with the phthalate buffer being used as the solvent. Another sample of the acid-soluble material was run on no. 1 Whatman paper and developed with conc. ninhydrin (Lewis, 1952) to check that all the amino acids had reacted with FDNB. As a control for the method, a hydrolysate of crystalline ribonuclease was used, and the molar ratios of the amino acids were found to be in fair agreement (with the exception of tyrosine, cystine and methionine) with the published analyses of the chromatographic fraction A of ribonuclease (Hirs, Stein & Moore, 1954). Cystine and methionine are absent from, or present only in small amounts in, histones (Crampton, Moore & Stein, 1955). The recovery of amino acids varied between 70 and 80% of the amount taken (based on N), but in all cases only the molar ratios have been quoted in the form: moles of amino acid/100 moles of all amino acids (see Table 4). Levy (1954) found that the molar ratios in his analyses were much more constant than the recoveries. Only tyrosine showed variability of molar ratio.

Proteolytic-enzyme tests. The action of chymotrypsin and histones on *N*-acetyl-L-tyrosine ethyl ester was tested by adding a dilute solution of chymotrypsin or histone or a weighed amount of solid histones to a stock solution of the ester (about 2 mg./ml. in borate buffer, generally pH 8.3, which was 0.12*M*, 0.02*N*, and 0.04*M* in boric acid, NaOH and NaCl respectively). The tubes were kept at 20° and 20 μ l. samples were removed immediately on mixing and at intervals and applied to the origin positions on no. 1 Whatman paper, the spots being dried immediately by a

jet of air. The chromatograms were run in butan-1-ol-acetic acid-water (5:1:4, by vol., upper phase used) and the spots were detected by the Pauly reaction, which showed that *N*-acetyltyrosine ran at R_F 0.83 and the ester at R_F 0.91, the latter giving a redder spot.

Benzoyl-L-arginine ethyl ester and the amide were prepared by the method of Bergmann, Fruton & Pollok (1939). The action of trypsin and histones on these substances was tested as described above. The stock solutions of the substances was tested as described above. The stock solutions of the substrates were 15 mg./ml. in borate buffer (pH 8), and after mixing with the protein the tubes were kept at 40°. The chromatograms were run on no. 7 Whatman paper pretreated with 0.05*M*-phthalate buffer (pH 6), and were developed with the upper layer from butan-2-ol which had been shaken with excess of the buffer. The spots were detected by the Sakaguchi reaction, and had the following R_F values: arginine, 0.02-0.06; benzoylarginine, 0.39-0.45; benzoylarginine-amide, 0.58-0.62; benzoylarginine ethyl ester, 0.83. In all cases control samples of the substrates were incubated without enzyme or histone.

Amide determination. The histones were hydrolysed for 3 hr. in 2*N*-HCl. The solution was brought to pH 8.5, a little antifoaming agent added, and the solution then treated like a digested sample in the micro-Kjeldahl nitrogen determination. Asparagine gave 94% of the theoretical amide nitrogen under these conditions.

RESULTS

Causes of variation in the N-terminal groups of histones

Acid conditions. Since the histones are extracted from nucleoprotein by 0.25*N*-HCl, the effect of the prolonged action of this acid on the end groups was examined. Histone (sample H23), prepared in the usual way, was dissolved in 0.25*N*-HCl at a concentration of 25 mg./ml., and duplicate samples of 1 ml. were removed at the start and after 2, 8, 14 and 28 days at room temperature. After coupling with FDNB the *N*-terminal groups were determined on the solid dinitrophenyl derivatives. It was found that all end groups increased linearly with time and the total was doubled in 23 days. Of the new end groups, glycine and proline were the most important, followed by serine, alanine and lysine. Another experiment was done to discover the end groups of histones which had not been in contact with acid at any stage of their preparation. For this purpose, deoxynucleoprotein was prepared from calf thymus by the usual washing with 0.14*M*-NaCl. Some of the material was then dispersed in an equal volume of 20% NaCl, bicarbonate and FDNB were added and the mixture shaken gently overnight. The *N*-terminal groups of the yellow, insoluble material obtained after washing were determined and are given in Table 1 (sample NP), as well as the values from histone samples V1 and V3 prepared at about the same time but by the usual method involving acid extraction.

Alkaline conditions. During their preparation the histones are kept for a considerable time near neutrality (in the salt-washing, dialysis, and freeze-drying) and also at pH 8–9 during the coupling with FDNB. To study the effect of these conditions, histone sample H23 was dissolved at 25 mg./ml. in borate buffer (pH 7.9; 0.11M, 0.01N and 0.045M in boric acid, NaOH and NaCl respectively), a little chloroform added to prevent bacterial action, and the solution kept at room temperature. Samples were taken at the start and after 7 days. A strong effect was noted. The yield of insoluble dinitrophenylated product fell from 48 mg. in the initial samples to 37 mg. in the 7-day samples, and the total *N*-terminal groups increased threefold on a molar basis. Of the new end groups, alanine, lysine and glycine accounted for 80% of the increase (with histone sample V1, 70%). Degradation also occurred at pH 6 and 9, but was much slower than at pH 7 and 8. For these trials, phosphate buffers were used at pH 6 and 7 and were prepared by mixing 0.067M-solutions of Na_2HPO_4 and KH_2PO_4 in the volume proportions 1:9 for pH 5.9, and 6:4 for pH 7.0 buffers. The borate buffer used at pH 8 is described above and the pH 9 buffer was 0.17M, 0.07N and 0.015M in boric acid, NaOH and NaCl respectively. The pH of the buffers was not changed by more than 0.1 unit when the histones were dissolved in them. The rate of liberation of end groups decreased with time and the effects suggested that a proteolytic enzyme was active in the histones. This view was strengthened by finding that DFP at a final concentration of 2.6 mM prevented the increases at pH 8 except for a 50% rise in the lysine value in 7 days (compared with a 1200% rise when DFP was not present). Histone H23 was also tested with synthetic substrates, all at pH 8. In borate buffer at 40°, it had no action on the trypsin substrate benzoyl-L-arginine-amide in 5 days, but benzoyl-L-arginine ethyl ester was slowly digested and the chymotrypsin substrate, *N*-acetyl-L-tyrosine ethyl ester, was readily digested. One of the properties of chymotrypsin is the clotting of milk. Under conditions (Northrop, Kunitz & Herriott, 1948) in

which chymotrypsin (15 $\mu\text{g.}$) clotted the milk in about 10 min., 5 mg. of histone caused no clotting even in 16 hr. The histone also did not prevent added chymotrypsin from clotting the milk readily.

Acetyltyrosine ester was also used to test other histones, and activity was found in all our preparations, in variable amounts as judged on the chromatograms from the size and intensity of the spots of acetyltyrosine revealed by the Pauly reaction. Activity was also present in rat-thymus histones and in an old sample of calf-spleen histones. Mouse blood was also found to split the ester readily. The activity of histones towards *N*-acetyl-L-tyrosine ethyl ester was just apparent at pH 6.2 and increased up to pH 9.8, the highest pH tested. At the higher pH, the spontaneous hydrolysis of *N*-acetyl-L-tyrosine ethyl ester became rapid. No activity towards L-leucyl-L-tyrosine, L-leucylglycine or L-prolylglycine was observed at pH 9 in 24 hr. with a histone preparation which readily split *N*-acetyl-L-tyrosine ethyl ester. In borate buffer (pH 8.3) there was a partial inhibition of activity towards *N*-acetyl-L-tyrosine ethyl ester in the presence of 0.067M-3-phenylpropionate, and mm-DFP achieved complete inhibition. However, treatment of histone V1 with mm-DFP at pH 8.2 for 3 hr. and subsequent dialysis and freeze-drying did not produce a completely inactive material, a slight fission of *N*-acetyl-L-tyrosine ethyl ester occurring in 21 hr. Histone V2, extracted from nucleoprotein which had been washed with 0.14M-NaCl containing 130 μM -DFP and subsequently dialysed against water containing 30 μM -DFP, gave end groups with the highest proportion of proline and alanine obtained up to that time (see Table 1). A repeat on a larger scale was less successful (V3, Table 1). Advantage is now taken of the inhibitory effect of DFP at higher concentrations to protect the histones from some of the enzyme degradation during the alkaline coupling with FDNB. For this purpose DFP is added to give a final concentration of mm.

When trypsin or chymotrypsin was allowed to digest histone sample V1 at pH 9 for 24 hr. at room temperature, the extra *N*-terminal groups

Table 1. *N*-Terminal groups of unfractionated histones

The proportions of *N*-terminal groups are expressed as percentages of the total end groups found.

Histone preparation (for origin, see text) ...	V1	V3	NP	V2	H30X	H32X
<i>N</i> -Terminal amino acid						
Proline	50	46	52	54	54	60
Alanine	36	37	37	38	39	36
Lysine	5	8	5	3	3	3
Others*	9	9	6	5	4	1
$10^{-3} \times \text{wt. of histone/mole}$ of end groups	32	31	32	36	31	41

* Glycine, valine, leucine (or isoleucine), serine, threonine aspartic and glutamic acids.

Table 2. *N*-Terminal groups of various histone fractions

The proportions of *N*-terminal groups are expressed as percentages of the total end groups found.

Histone fraction (for origin, see text) ...	H21/2	H21/3	V2/F2	V3/F2	V1/B	V2/F3	V3/F3	V1/A
<i>N</i> -Terminal amino acid								
Proline	28	54	81	76	78	13	8	14
Alanine	21	30	13	10	5	83	76	74
Lysine	31	6	3	9	10	2	4	4
Others*	20	10	3	5	7	2	12	8
10 ⁻³ × wt. of histone/1 mole of end groups	34	33	40	47	38	34	28	32

* As in Table 1.

Fig. 1. Effluent diagrams of calf-thymus histones (100 mg.) chromatographed on carboxymethylcellulose columns (14 cm. × 2 cm.). All the solutions were applied to the columns in 2 mN-HCl and eluted with HCl of the concentrations shown. (a) Run with unfractionated histones; (b) run with fraction 2 (F2) from (a), on a fresh column; (c) run with fraction 3 (F3) from (a), on a fresh column.

released, in decreasing order of importance, were: (with trypsin) alanine, lysine and glycine, together accounting for 60% of the total increase; (with chymotrypsin) alanine, lysine, glutamic acid and glycine, together accounting for 75% of the total increase. These compare closely with the 70–80% increase due to alanine, lysine, and glycine seen in the histones H23 and V1 mentioned above.

Approximately 1 part of chymotrypsin in 10 000 parts of histone would account for the effects observed.

Chromatography of histones on carboxymethylcellulose columns

The end-group results given by fractions of calf-thymus histones prepared by chromatography on CMC columns under the conditions employed by Davison (1957), using gradient or stepwise elution with acetate buffers of pH 4.2, showed them still to be complex mixtures. An example is given in Table 2, where sample H21/2 is the first histone peak eluted and H21/3 the main peak. In further work, HCl was used as the eluent, since it seemed from the results above that aggregated histone might still be present at pH 4.2. Gradient elution of the column with 2 mN-rising to 0.05N-HCl showed three peaks (F1, F2 and F3), and these were more conveniently separated on later columns by stepwise elution with 2 mN, 0.01N- and 0.02N-HCl. Fig. 1 (a) shows the elution diagram with histone sample H25, and similar patterns were obtained with five other preparations. After F1 no further material could be removed from the columns by stepwise elution until 0.01N-HCl was used.

The end groups of the main fractions, F2 and F3, from CMC columns have been examined and examples from histone preparations V2 and V3 are given in Table 2. Fraction 1 has never been large enough for end-group measurements to be made. In several runs the peaks and interspaces have been analysed for nitrogen content and recovery of 'extinction units'. The results are given in Table 3, together with the values for the two fractions resulting from the NaCl-aq. NH₃ fractionation of the histones by the method of Davison & Shooter (1956).

Table 3 also shows that the recovery of histones from the columns was at least 86%. The elution of further traces of histones from the columns by 0.1N or more concentrated HCl or by 0.1N-NaOH has occasionally been seen, but the concentrated acid and alkali also produce ultraviolet-absorbing

materials from the cellulose itself. It seems certain, however, that small amounts of histones are left adsorbed after peak F3 has been eluted with 0.02N-HCl since the cellulose column, after washing with water, extruding from the tube and drying, gave a positive Sakaguchi reaction throughout its length.

In order to demonstrate the effectiveness of the separation on the CMC column, the two main fractions, F2 and F3, from a run with histone H25, were diluted so that the HCl concentrations were 2 mN, and they were then run separately on fresh columns and eluted with 0.01N- and 0.02N-HCl. With F2 [Fig. 1 (b)], 85% of the material ($E_{1\text{cm}}^{278\text{m}\mu}$ basis) appeared as before on elution with 0.01N-HCl, and only about 3% appeared when 0.02N-HCl was subsequently run through. With F3 [Fig. 1 (c)], none of the material was eluted with 0.01N-HCl and 80% with 0.02N-HCl. The end groups of the fractions were unchanged after the second chromatography.

*Amino acid composition of histones
and histone fractions*

Table 4 gives the average amino acid composition of the unfractionated calf-thymus histones as determined in duplicate from samples E1, V1, V2 and V3. For comparison the values for calf-thymus histone determined by Crampton *et al.* (1955), also shown in the table, have been recalculated on the same basis but excluding the 1.1 moles of methionine found by those authors, since this amino acid was not definitely observed on any of our chromatograms. The compositions are also given for two examples of the carboxymethyl-cellulose column fractions F2 and F3, and for the two fractions obtained from an NaCl-aq. NH_3 treatment of histones by the method of Davison & Shooter (1956), using histones E1 and V1 (the latter in the presence of mm-DFP), E1/A and V1/A were the precipitated histones and E1/B and V1/B remained in the supernatant.

Table 3. *Relative proportions of various histone fractions*

The ranges of values given are percentages of those given by the unfractionated materials.

Source of fractions	Fraction	Relative proportions found			Histone preparations
		By N determination	By extinction measurement*	By wt.	
	Interspaces	3-8	2-11	—	—
Carboxymethylcellulose column chromatography [see Fig. 1 (a)]	F1	1-3	3-15	—	H23, H25 E1 and V3
	F2	51-67	53-62	—	
	F3	20-31	21-29	—	
	Totals	86-95	86-99	—	
NaCl- NH_4OH precipitation	A (precipitate)	44†	47-55	40-43	H1, H16, H18, E1 and V1
	B (supernatant)	56†	45-53	57-60	

* The product $E_{1\text{cm}}^{278\text{m}\mu} \times \text{vol. (ml.)}$ for each fraction.

† Sample H16 only.

Table 4. *Amino acid composition of histones and histone fractions*

The values are moles of amino acid/100 moles of all amino acids found. Valine, leucine and isoleucine were eluted as one spot, when not sufficiently well separated. Similarly with aspartic acid and glutamic acid.

Histone preparation or fraction ...	*	†	V1/A	E1/A	V4/F3	V3/F3	V1/B	E1/B	V4/F2	V3/F2
Amino acid										
Aspartic acid	4.8	4.4]	15.7	14.8	17.9	16.4	14.2	13.8	12.7	13.2
Glutamic acid	8.2	9.2]								
Glycine	8.5	8.6	9.7	10.1	6.2	5.8	7.2	8.6	9.2	9.5
Alanine	13.6	14.6	11.3	11.4	12.7	15.4	17.4	17.8	14.0	14.2
Valine	6.2	6.1	7.9]	21.4	17.1	15.2	16.1	13.6	17.5	17.9
Leucine/isoleucine	12.2	11.6	12.5]							
Phenylalanine	2.1	1.0	2.3	2.5	2.7	2.1	0.6	0.5	1.3	1.4
Tyrosine	2.5	1.5	2.1	0	1.7	1.7	1.4	0	2.0	2.4
Serine	5.9	5.0	3.7	4.0	4.3	5.1	5.8	6.5	4.6	6.0
Threonine	5.75	5.5	6.6	6.3	5.7	6.1	4.9	4.9	5.1	5.5
Proline	5.1	4.8	2.6	3.4	5.2	5.6	5.3	6.6	5.0	4.3
Arginine	8.4	9.5	12.6	15.5	11.8	10.0	7.4	8.7	9.0	7.9
Lysine	14.9	16.6	11.6	8.3	12.5	15.4	18.9	17.7	17.4	17.1
Histidine	2.0	1.3	1.6	2.2	2.3	1.1	0.8	1.4	2.0	1.0
Amide N (as % of total N)	4.17	4.0†	—	—	—	4.5	—	—	—	3.7

* Crampton, Moore & Stein (1955) recalculated with omission of methionine. See text.

† Average or results from histone preparations E1, V1, V2 and V3.

‡ Histone preparation V3 only.

The amide-nitrogen content of histone preparation V3 and the two column fractions obtained from it are also given in Table 4.

DISCUSSION

The calf-thymus histones are, as regards the *N*-terminal groups, so stable in 0.25*N*-hydrochloric acid that their customary preparation with this acid at 5° would not entail the formation of any extra end groups. Moreover, the end groups are found in such quantities that they must pre-exist in the thymus histones unless some especially acid-labile bond is present, such as is postulated by Schramm, Braunitzer, Anderer, Schneider & Uhlig, (1958) in tobacco-mosaic-virus protein. However, the finding that the same end groups in about the same quantities are present in histones which have at no time been more acid than pH 6 (Table 1, sample NP) disposes of this possibility.

The findings that histone preparations are degraded in alkaline solution, that this effect is almost abolished by *mm* diisopropyl phosphorofluoridate, and that *N*-acetyl-L-tyrosine ethyl ester is split by them, point strongly to the presence of a proteolytic enzyme in the preparations, with some of the properties of chymotrypsin. The chief new end groups released during this degradation are alanine, lysine and, to a less extent, glycine. Such degradations could occur in the preparation of the histones, during the washing with aqueous sodium chloride, the dialyses and the coupling with FDNB, and it is noteworthy that the lysine end groups of fractions (see Table 2) have frequently been found to be much higher than in the parent histones (compare V1 in Table 1 with its fractions given in Table 2), and the variability of this end group was noted in previous work (Phillips, 1958). A small variation in alanine end groups would scarcely be noticed as it is already a major end group.

The literature contains many accounts of proteinases occurring in animal tissues of all kinds, and there is some resemblance between the properties of the enzyme described here and the proteinase II from lung tissue reported by Dannenberg & Smith (1955). As with our material, their enzyme hydrolysed acetyltyrosine ester and was partially inhibited by DFP.

The use of DFP during the preparation of the histones (Table 1, sample V2) or the use of the more convenient slight acidification of the salt washes (Table 1, sample H30X) and avoidance of dialysis by the use of sulphuric acid extraction followed by barium sulphate precipitation (Table 1, sample H32X) has led to increasing proportions of proline and alanine end groups (see Table 1). The other *N*-terminal groups have become so small that

it is suggested that the native calf-thymus histones probably have only two *N*-terminal groups, proline and alanine, the others arising by degradation. Concomitant with the simplification of the end groups is an increase in the weight of histone per mole of end groups to 30 000–40 000.

The experiments on the fractionation of the histones on carboxymethylcellulose columns give two chief products, and each has largely a single end group. Thus in V2/F2 and V3/F2 (Table 2), about 80% of the end groups are proline and in V2/F3 and V3/F3 about 80% are alanine. Since each fraction gives virtually a single peak when run again on a fresh column [see Fig. 1 (b), (c)], it seems that the presence of 13% of proline ends in V2/F3 is not due to incomplete separation, but due to a second proline-terminated histone with chromatographic properties like V2/F3. A similar argument applies to the 13% of alanine ends in fraction V2/F2. However, until one can avoid any kind of degradation during manipulation, the existence of these minor fractions will be doubtful.

The end groups of the two column fractions are in close agreement with those of the supernatant and precipitated histones obtained by sodium chloride-ammonia fractionation (Davison & Shooter, 1956) in the presence of *mm*-DFP (Table 2, samples V1/B and V1/A).

The overall amino acid composition of unfractionated histone samples used here is in broad agreement with the accurate values of Crampton *et al.* (1955), as shown in the first two columns in Table 4. We find that there is also agreement in the composition of the main peak from the CMC columns (Table 4, samples V3/F2 and V4/F2) and that of the histone from the supernatant of the sodium chloride-ammonia fractionation (V1/B and E1/B, Table 4). Both sources give histone with a similar excess of lysine over arginine residues in them. We thus have fractions, obtained by two different methods, which have the same end groups, a similar total amino acid composition, and which are produced in similar amounts from the parent histones, as shown by the proportions given in Table 3. The agreement is not perfect but it is suggested that these materials are largely identical.

When we turn to the other fractions in Table 4, however, discrepancies appear. The yield of sodium chloride-ammonia-precipitated histone A is undoubtedly greater than that of column fraction F3, as shown in Table 3. The end groups, however, agree almost as well as the other case cited above, the F3 fractions and the A fractions both having about 80% of alanine end groups (see Table 2). The total amino acid compositions, however, differ considerably, the sodium chloride-ammonia-precipitated histone having more glycine, valine,

leucine (isoleucine) and arginine, but less alanine, proline and lysine. The reason for these differences is uncertain. Some protein is left adsorbed on the CMC columns and we postulate that it has mainly alanine end groups and a composition rich in arginine.

SUMMARY

1. Calf-thymus histones prepared by acid extraction of sodium chloride-washed deoxynucleo-protein contained a proteinase (partly inhibited by mM diisopropyl phosphorofluoridate), which gave rise to extra *N*-terminal alanine, lysine and glycine residues in the histones, especially at pH 7-8.

2. Histones prepared under conditions minimizing this proteolysis had proline and alanine *N*-terminal groups accounting for up to 96 % of all end groups and amounting to 1 mole/30 000-40 000 weight of histone.

3. These two end groups pre-existed in the deoxynucleohistone and were not produced by the acid extraction.

4. On carboxymethylcellulose columns run with dilute hydrochloric acid as eluent, the histones gave two main peaks, accounting for at least 86 % of the histones. The first peak had mainly proline, the second mainly alanine *N*-terminal groups.

5. The two column fractions have been compared in yield, end groups and amino acid composition with the two fractions resulting from the sodium chloride-ammonia fractionation of the histones.

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