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Studies on Histones

7. PREPARATIVE METHODS FOR HISTONE FRACTIONS FROM CALF THYMUS*

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Previous work from this Laboratory (Johns, Phillips, Simson & Butler, 1960) has shown that calf-thymus histones can be separated into three main groups of basic proteins by column chromatography on CM-cellulose. These three groups were characterized by their total and N-terminal amino acid analyses and were designated: lysine-rich histones, f1; slightly lysine-rich histones, f2; and arginine-rich histones, f3. A method was also given for the extraction of fraction f3 directly from the tissue by using a mixture of ethanol and hydrochloric acid.

Somewhat similar fractionations were achieved by starch electrophoresis by Cruft, Hindley, Mauritzen & Stedman (1957), and by chromatography on Amberlite IRC-50 by Rasmussen, Murray & Luck (1962). Many other authors have described lysine-rich and arginine-rich histones (for review see Phillips, 1962).

In a further paper (Johns & Butler, 1962) the preparation of two subgroups of the slightly lysine-rich histones, f2 (a) and f2 (b), was described, making use of a combination of ethanol-hydrochloric acid extraction methods and column chromatography on CM-cellulose. A method for the extraction of the lysine-rich histones, f1, with 5% perchloric acid was also given.

These extraction methods have now been studied further and two preparative methods have been developed each of which enables the four groups of histones, f1, f2 (a), f2 (b) and f3, to be isolated from calf thymus, in large quantities, and does not involve the preparation and subsequent fractionation of whole histone.

A method for the further fractionation of f1 is also described, a preliminary account of which has been given (Johns, 1963).

EXPERIMENTAL AND RESULTS

All operations were carried out at 4° except for the washing and drying of all precipitates. Summaries of the extraction methods employed are given in Schemes 1 and 2.

Method 1. Minced calf thymus (100 g.) was homogenized with 700 ml. of 0.9% sodium chloride at top speed for 2 min. in the MSE Ato-Mix. The homogenate was centrifuged at 1100g for 30 min. The supernatant with some fatty debris on the surface was discarded, and the sediment was washed five times more in a similar manner but with homogenizing for 30 sec. and centrifuging for 15 min. The sediment obtained from the last washing was homogenized with 400 ml. of 5% (v/v) perchloric acid (0.74N) in the blender for 2 min. at full speed and the suspension was centrifuged for 30 min. at 1100g. The sediment was extracted twice more in the same way with 200 ml. of 5% perchloric acid. The combined supernatants from these extractions were clarified by filtering through a grade-4 sintered-glass funnel. Trichloroacetic acid was then added to give a final concentration of 18% (w/v) (1.1M). The lysine-rich histones, f1, that were precipitated were recovered by centrifugation, washed once in acidified acetone (200 ml. of acetone plus 0.1 ml. of conc. hydrochloric acid) and then three times in acetone, and dried under vacuum. The vacuum was released occasionally during the drying and any lumps were broken up with a glass rod. By drying in this way a fine white powder was obtained which dissolved easily in water to give a clear solution. The yield of lysine-rich histones was 440 mg., which is approx. 20% of all the histones that can be extracted from calf thymus.

The sediment remaining after the extraction of fraction f1 with 5% perchloric acid was then mixed with 200 ml. of ethanol, stirred for 2 min. to break up all lumps, and

* Part 6: Butler & Cohn (1963).

allowed to stand overnight (18 hr.) at 4°. The suspension was centrifuged at 1100*g* and the sediment extracted twice more by stirring for 5 min. with 200 ml. of 80% (v/v) ethanol. The combined supernatants, which contained fractions f2 (a) and f3, were slightly cloudy and were clarified by centrifuging at 2500*g* for 30 min. Fractions f2 (a) and f3 were both precipitated from the clear ethanol extract by adding 5 vol. of acetone, followed by 3 ml. of conc. hydrochloric acid. The precipitate was recovered by centrifugation, washed once in acetone, and redissolved, without drying, in 60 ml. of water. Ethanol (320 ml.) was then added followed by 20 ml. of 5*N*-hydrochloric acid to make the solution 80% with respect to ethanol and 0.25*N* with respect to hydrochloric acid. The solution was then dialysed once for 18 hr. against 600 ml. of ethanol, followed by a second dialysis for 4 hr. against 600 ml. of ethanol. The precipitate, f3, which formed was washed in ethanol and then three times in acetone, and dried under vacuum. The yield of fraction f3 was 520 mg.

The supernatant contained fraction f2 (a) which was recovered by adding 3 vol. of acetone. The precipitate was washed twice in acetone and dried under vacuum. The yield of fraction f2 (a) was 880 mg.

The sediment remaining after the extraction of fractions f2 (a) and f3 with ethanol contained fraction f2 (b). This was extracted by homogenizing for 30 sec. in 200 ml. of 0.25*N*-hydrochloric acid at half-speed in the MSE Ato-Mix. The suspension was centrifuged at 1100*g* and the sediment extracted three times more in the same way. The combined supernatants were clarified by filtering through a grade-4 sintered-glass funnel and fraction f2 (b) was precipitated from the clear solution by adding 5 vol. of acetone. The precipitate was recovered by centrifugation, washed twice in acetone and dried under vacuum. The yield of fraction f2 (b) was 605 mg.

The total and *N*-terminal amino acid analyses of the four groups of histones prepared by this method are given in Table 1.

Method 2. Minced calf thymus (100 g.) was washed six times with 0.9% sodium chloride in the same way as described for method 1. The sediment obtained from the last washing was blended for 30 sec. with 700 ml. of 90% (v/v) ethanol at half-speed in the MSE Ato-Mix. The homogenate obtained was centrifuged at 1100*g* for 15 min. and the supernatant discarded. The wet residue was transferred to a 1 l. polythene bottle containing 400 ml. of ethanol-1.25*N*-hydrochloric acid (4:1, v/v) and about 20 porcelain balls (0.5 in. diam.). The bottle was then rotated on rollers at about 20 rev./min. for 18 hr. The contents, excluding the balls, were then centrifuged for 15 min. at 1100*g* and the sediment was extracted twice more in the same way, but by using 200 ml. of solution and extracting for 3 hr. The combined supernatants, which contained fractions f2 (a) and f3, were clarified by filtering through a grade-4 sintered-glass funnel. The clear supernatant (approx. 750 ml.) was then dialysed against 1200 ml. of ethanol for 18 hr. followed by two more dialyses of 3 hr. each against the same volume of fresh ethanol. The precipitate that formed, f3, was centrifuged down, washed once in ethanol and three times in acetone, and dried under vacuum. The yield of fraction f3 was 530 mg.

The supernatant remaining after the precipitation of fraction f3 contained fraction f2 (a), which was recovered by adding 3 vol. of acetone. The precipitate was washed twice in acetone and dried under vacuum. The yield of fraction f2 (a) was 830 mg.

The sediment remaining after the extraction of fractions f2 (a) and f3 with ethanol-hydrochloric acid contained fractions f2 (b) and f1, and was extracted with 300 ml. of 0.25*N*-hydrochloric acid in the ball mill for 18 hr. This was

Scheme 1. Summary of method 1 (see the text) for the extraction of histone groups from calf thymus.

followed by two more extractions of 2 hr. each with 150 ml. of 0.25N-hydrochloric acid. The combined extracts were clarified by filtering through a grade-4 sintered-glass funnel and then raised to room temperature, and 3 vol. of acetone was added with rapid stirring. The precipitate that formed, f1, was recovered by centrifugation in covered pots to prevent evaporation of acetone and washed once in 200 ml. of acetone-0.25N hydrochloric acid (3:1, v/v). The precipitate was then washed three times in acetone and dried under vacuum. The yield of fraction f1 was 400 mg.

The supernatant remaining after the precipitation of fraction f1 contained fraction f2 (b), which was recovered

by adding 2 vol. of acetone. The precipitate was washed twice in acetone and dried under vacuum. The yield of fraction f2 (b) was 570 mg.

The total and *N*-terminal amino acid analyses of the four groups prepared by this method are given in Table 1.

Fractionation of fraction f1. Fraction f1 (100 mg.), prepared by method 1, was dissolved in 20 ml. of 0.01M buffer, pH 9, containing 7 mM-boric acid and 3 mM-sodium hydroxide, and applied to a column (15 cm. long $\times$ 3 cm. diam.) of CM-cellulose (5 g.) previously equilibrated with the buffer solution. Elution was carried out with a linear gradient between the same buffer and the buffer containing

Table 1. *Composition of histone fractions prepared from calf thymus*

The amino acids are expressed as moles/100 moles of all amino acids found, and no corrections have been made for hydrolytic losses. Amide was not determined. The yields of *N*-terminal amino acids are given as μ moles/g. of protein.

Fraction...	Method 1				Method 2			
	f1	f2 (a)	f2 (b)	f3	f1	f2 (a)	f2 (b)	f3
Amino acids								
Aspartic acid	3.4	5.8	5.5	5.0	7.5	5.9	15.9	5.2
Glutamic acid	5.6	8.3	9.3	11.2		8.8		11.7
Glycine	6.4	12.5	7.4	6.5	7.8	12.1	7.4	6.2
Alanine	23.6	10.0	10.6	12.6	23.3	10.1	11.0	13.2
Valine	9.8	7.7	7.2	6.4	5.7	7.5	6.2	4.7
Leucine + isoleucine		14.1	11.2	13.9	5.5	14.3	10.8	14.2
Serine	5.4	3.9	7.4	4.7	6.1	3.1	8.3	5.1
Threonine	5.1	5.1	5.7	6.4	5.6	5.8	5.6	6.9
Phenylalanine	1.0	2.0	1.9	2.6	0.8	1.9	1.6	2.2
Tyrosine	0.2	2.7	3.1	2.3	0.6	2.6	3.1	2.5
Proline	9.7	3.0	5.0	4.8	8.1	2.8	4.7	5.1
Histidine	0.3	2.5	2.1	2.4	0.4	2.5	1.7	2.0
Lysine	27.5	11.2	15.8	9.8	25.9	11.8	15.8	10.2
Arginine	2.0	11.2	7.8	11.1	2.8	10.8	7.9	10.8
<i>N</i> -Terminal amino acids								
Proline	1.7	9.0	45.3	10.3	5.0	1.0	39.3	0.0
Alanine	1.1	2.8	7.7	46.7	2.7	5.2	8.9	34.6
Glycine	5.9	1.9	1.2	0.6	0.9	1.2	2.8	0.4
Others*	3.6	4.9	4.1	3.0	2.4	2.1	4.2	0.7
Main <i>N</i> -terminal group	Acetyl†	Acetyl†	Proline	Alanine	Acetyl†	Acetyl†	Proline	Alanine

* Includes lysine, serine, threonine, aspartic acid and glutamic acid.

† Phillips (1963).

Scheme 2. Summary of method 2 (see the text) for the extractions of histone groups from calf thymus.

sodium chloride (1 M). Fractions (5 ml.) were collected at a flow rate of about 0.5 ml./min., and the progress of the elution was followed by measuring the extinction of the fractions at 235 m μ . The sodium chloride in the fractions was determined approximately by measuring their refractive indices. The result of the fractionation is shown in Fig. 1. The proteins were recovered by precipitation with trichloroacetic acid at a final concentration of 18% (1.1 M). Peaks 1 and 2 accounted for about 15% of the applied protein, and peak 3, which is apparently still complex,

Fig. 1. Effluent diagram of fraction f1 (100 mg.) chromatographed on a CM-cellulose column (15 cm. $\times$ 3 cm.). The histone was applied in 20 ml. of 0.01 M buffer, pH 9, containing 7 mM-boric acid and 3 mM-sodium hydroxide, and eluted with a linear gradient between the buffer and the buffer containing sodium chloride (1 M). The average fraction size was 5 ml. The continuous line indicates the extinction at 235 m μ and the discontinuous line indicates the molarity of sodium chloride.

accounted for about 85%. The total amino acid analyses of these fractions are given in Table 2.

A method which gives a similar separation, and enables larger quantities of fraction f1 to be fractionated, is as follows. Fraction f1 (650 mg.) was dissolved in 200 ml. of 0.01 N-hydrochloric acid containing 10 g. of guanidinium chloride. Acetone (670 ml.) was then added to the clear solution and the precipitate that formed was recovered by centrifugation, washed in 40 ml. of 0.01 N-hydrochloric acid plus 134 ml. of acetone and then three times in acetone, and dried under vacuum. The yield of precipitate, designated A, was 480 mg. Then 1 vol. of acetone was added to the supernatant, and the precipitate that formed was recovered, washed three times in acetone, and dried under vacuum. The yield of precipitate, designated B, was 130 mg. The total and N-terminal amino acid analyses of precipitates A and B are given in Table 2. The total amino acid analysis of the lysine-rich 'Fraction A' of Crampton, Stein & Moore (1957), recalculated in moles/100 moles, is also given for comparison.

Amino acid analyses. Total amino acid analyses were carried out as described by Phillips & Johns (1959) by a modification of the methods of Levy (1954) and Fraenkel-Conrat, Harris & Levy (1955). No corrections have been made for hydrolytic losses. Amide was not determined. N-Terminal amino acid analyses were carried out as described by Phillips (1958) by a modification of the fluorodinitrobenzene method of Sanger (1945).

Starch-gel electrophoresis. Fractions f1, f2 (a), f2 (b) and f3 were examined by starch-gel electrophoresis at pH 2.3, and the patterns obtained were similar to those described previously (Johns, Phillips, Simson & Butler, 1961; Johns & Butler, 1962), except that the arginine-rich histones, f3, are in the unaggregated form (Johns, 1962).

Table 2. *Composition of lysine-rich histones from calf thymus*

The amino acids are expressed as moles/100 moles of all amino acids found, and no corrections have been made for hydrolytic losses. Amide was not determined. The yields of N-terminal amino acids are given as μ moles/g. of protein.

Amino acids	Column chromatography of fraction f1 at pH 9			Acetone precipitation of fraction f1		Crampton, Stein & Moore (1957). Fraction A
	Peak 1	Peak 2	Peak 3	Ppt. A	Ppt. B	
Aspartic acid	11.4	30.9	6.2	6.0	20.0	2.0
Glutamic acid	20.1					3.2
Glycine	5.6	6.5	7.2	7.0	7.4	6.6
Alanine	10.1	8.3	24.3	25.9	15.4	26.0
Valine	2.4	2.5	5.4	5.4	4.5	4.8
Leucine + isoleucine	5.6	4.2	6.0	5.3	5.6	5.0
Serine	5.6	7.6	5.6	5.7	6.9	6.4
Threonine	2.9	2.3	5.6	6.1	4.0	5.7
Phenylalanine	3.2	3.5	0.9	0.6	1.5	0.5
Tyrosine	2.4	3.0	0.9	0.8	2.0	0.5
Proline	5.8	7.2	9.2	9.3	7.2	9.0
Histidine	1.3	0.9	—	—	0.9	—
Lysine	19.4	19.0	26.8	26.4	20.9	28.3
Arginine	4.2	4.2	1.8	1.5	3.8	1.7
N-Terminal amino acids						
Proline	—	—	—	—	3.4	—
Alanine	—	—	—	0.25	2.5	—
Glycine	—	—	—	0.05	23.2	—
Others*	—	—	—	1.7	6.8	—
Main N-terminal group	—	—	—	Acetyl†	Glycine	—

* Includes lysine, serine, threonine, aspartic acid and glutamic acid.

† Phillips (1963).

DISCUSSION

The methods described enable the four main groups of histones, f1, f2 (a), f2 (b) and f3, to be isolated in high yields from calf thymus during one preparation, by successive extraction of the washed tissue. The yield of all histones extracted together from calf thymus by using 0.25N-hydrochloric acid is approx. 2.4 g./100 g. of tissue (Phillips & Johns, 1959). The combined yields of the groups obtained by the methods described above amount to about 2.3–2.4 g./100 g. of calf thymus. The analyses of all the groups agree well with those reported previously (Johns *et al.* 1960; Johns & Butler, 1962).

The low yield of *N*-terminal amino acids in fractions f1 and f2 (a) is probably due to the presence of *N*-terminal acetyl groups. Acetyl groups have been found in large amounts in fractions f1 and f2 (a) and in smaller amounts in fractions f2 (b) and f3 (Phillips, 1963).

Though these separations are useful in producing different groups of histones it is important to remember that the groups are all heterogeneous. Fractions f1 and f2 (b) both contain at least three proteins, and fractions f2 (a) and f3 probably three or four, since the end-group analyses and starch-gel-electrophoresis patterns at pH 2.3 are all complex. Methods for fractionating the various groups are being investigated, but so far a homogeneous histone fraction has not been isolated. The analyses of individual histones from the various groups may, of course, have very different analyses from their parent group, and therefore, though names like 'lysine-rich', 'slightly lysine-rich' and 'arginine-rich' histones were useful during the preliminary fractionations, it is proposed in future to refer to the groups only by the designations f1, f2 (a), f2 (b) and f3.

The chromatography of fraction f1 at pH 9 has given three fractions, two of which (peaks 1 and 2 in Fig. 1) have a very high content of acidic amino acids. However, these proteins still contain 24–25 % of basic amino acids and may still be basic proteins if many of the acidic amino acids are present as the amides.

Another characteristic of these two proteins is their low threonine content, about half that found in all other histone fractions.

Peak 3 (Fig. 1) has a low content of acidic amino acids, does not contain histidine and is very similar to 'Fraction A' of Crampton *et al.* (1957) (see Table 2). This is the major part of fraction f1 and is more easily prepared by the acetone precipitation method (precipitate A). The percentage yield by this method is not, however, as high, and from the analyses and yields it would appear that precipitate B, containing the proteins high in acidic amino

acids, is slightly contaminated with the other fraction.

The *N*-terminal group analysis of the precipitated fraction B does, however, indicate that the glycine *N*-terminal groups found in the unfractionated group f1 belong to the proteins with the high content of acidic amino acids.

SUMMARY

1. Two preparative methods have been described, each of which enables the four main groups of histones, f1, f2 (a), f2 (b) and f3, to be isolated from calf thymus. The combined yields of the four groups amounted to 2.3–2.4 g./100 g. of calf thymus, which is equivalent to the total histone that can be extracted with 0.25N-hydrochloric acid.

2. The lysine-rich histones, f1, have been fractionated further by column chromatography on carboxymethylcellulose at pH 9. Three fractions were obtained, two of which contained 24 % of basic amino acids and over 30 % of acidic amino acids (or their amides).

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