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Improved Fractionations of Arginine-Rich Histones from Calf Thymus

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It has been shown by a variety of methods that calf-thymus histones can be separated into fractions relatively rich in arginine (Grégoire & Limozin, 1954; Daly & Mirsky, 1955; Davison & Shooter, 1956) and in lysine (Stedman & Stedman, 1951; Davison & Butler, 1954; Crampton, Moore & Stein, 1955; Daly & Mirsky, 1955). Further chromatographic separation on Amberlite IRC-50 resin columns was also achieved by Luck, Rasmussen, Satake & Tsvetkov (1958) with a gradient of guanidinium chloride for elution. Davison (1957) first reported the use of carboxymethylcellulose columns with a salt gradient at pH 4.2 which gave two main peaks, in addition to an initial small fraction very rich in aspartic and glutamic acids. The first main peak was a lysine-rich histone and the second, larger, peak was heterogeneous, with a higher arginine content in the part eluting more slowly. Later it was possible to elute histone as two fractions from carboxymethylcellulose columns with dilute hydrochloric acid, the fractionation being followed by *N*-terminal amino acid analysis (Phillips & Johns, 1959). The two fractions had mainly proline and mainly alanine *N*-terminal groups respectively. These two systems of elution have now been combined to give an improved fractionation into three parts. A bulk fractionation process has also been worked out, which readily gives arginine-rich histones, with alanine as virtually the sole *N*-terminal group. This method is based on the extraction of the whole tissue with acid in the presence of a high concentration of ethanol. It was thought that under these condi-

tions the action of tissue proteinases, such as that shown to be present in calf thymus (Phillips & Johns, 1959), would be prevented. It was realized that proteins other than histones would be dissolved from the tissue in this procedure, but judging by the analytical results on the product it is thought that these have been eliminated. This paper is mainly confined to the study of the arginine-rich histones obtained by the chromatographic and the extraction procedures.

EXPERIMENTAL AND RESULTS

Improved chromatographic separation on carboxymethylcellulose. The histone was prepared from calf-thymus glands by the methods previously described, with slightly acidified sodium chloride washes (Phillips & Johns, 1959). The histone (100 mg.) was dissolved in 20 ml. of buffer, pH 4.2, containing 0.1 M-acetic acid and 0.03 M-sodium hydroxide, and applied to carboxymethylcellulose in the form of a column, 14 cm. long and 2 cm. in diameter, which had been prepared by allowing a slurry of 5 g. of carboxymethylcellulose in the same buffer to settle in the chromatographic tube under gravity. Fractions of 3–5 ml. were collected automatically 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 278 m μ . Elution was carried out in four steps as follows: (1) a buffer, pH 4.2, containing 0.1 M-acetic acid and 0.03 M-sodium hydroxide; (2) a buffer, pH 4.2, containing 0.17 M-acetic acid, 0.051 M-sodium hydroxide

and 0.42M-sodium chloride; (3) 0.01N-HCl and (4) 0.02N-HCl.

The result of a fractionation with whole histone preparation H 42 is shown in Fig. 1. The peak fX contained only about 0.5% of the total nitrogen taken and had an amino acid composition very like that of the initial contaminant peak reported by Davison (1957). Step (2) then removed the very lysine-rich histones as two small peaks f1A and f1B. Histones f2 and f3 were then eluted in steps (3) and (4). Table 1 gives some of the total amino acid and *N*-terminal group analyses of fractions f1A, f2 and f3 as determined by methods previously described (Phillips, 1958; Phillips & Johns, 1959). The lysine-rich histone f1B had a lower lysine to arginine ratio than histone f1A but was otherwise very similar in composition. The yield of the arginine-rich histone, f3, was about 20% of the whole histone taken for chromatography.

When, instead of applying whole histone to the carboxymethylcellulose column, either the 0.01N-HCl or the 0.02N-HCl fractions were used [prepared

Fig. 1. Effluent diagram of calf-thymus histones (510 mg.) chromatographed on a carboxymethylcellulose column (20 cm. $\times$ 2.4 cm.). The histone was applied to the column in 20 ml. of 0.1M-sodium acetate buffer, pH 4.2, and eluted with: (1) the same buffer; (2) sodium acetate-sodium chloride buffer, pH 4.2 [for (1) and (2) see text]; (3) 0.01N-HCl and (4) 0.02N-HCl. The average fraction size was 4 ml.

Table 1. *Composition of histones from chromatography and acid-ethanol extraction*

The amino acids are expressed as moles/100 moles of all amino acids found and no correction has been made for the hydrolytic losses of amino acids. Amide was not determined. The analytical values for the starting material are the averages of results from histone preparations HV5 and H 42, used for preparing the column fractions f1A, f2 and f3. The 70 and 80% ethanol preparations are the same as preparations 70(1) and 80(3) respectively in Table 2. The proportions of *N*-terminal groups are molar percentages of all such groups found.

Histone fraction (for origin, see text)	Starting material	Column fractions			70% Ethanol preparation	80% Ethanol preparation	70% Ethanol preparation. Column fraction f3
		f1A (average of 3)	f2 (average of 3)	f3 (average of 2)			
Amino acid							
Aspartic acid } A	14.2	8.0	14.3	16.9	5.7	4.2	4.3
Glutamic acid }					10.5	12.0	12.0
Glycine	8.5	7.2	10.3	6.6	7.5	6.1	5.8
Alanine	13.8	23.6	11.1	13.0	13.1	13.7	13.9
Valine	6.4	4.8	7.5	5.2	5.6	5.7	5.3
Leucine + isoleucine	10.6	5.4	12.7	13.8	13.7	13.6	13.3
Phenylalanine	1.9	0.7	1.5	2.9	2.2	2.4	3.0
Tyrosine	1.7	0.5	2.7	0.5	1.8	1.3	0.8
Serine	5.2	6.1	5.2	4.4	4.0	4.2	4.6
Threonine	5.8	5.6	5.4	6.2	6.1	6.9	6.6
Proline	5.1	8.9	3.6	4.9	5.0	4.7	4.9
Histidine	2.0	0.4	2.7	2.2	2.2	2.2	1.8
Lysine } B	15.6	25.9	13.1	10.3	9.9	9.0	9.0
Arginine }	9.2	2.9	9.8	13.2	12.6	14.0	14.7
Lysine:arginine	1.70	9.0	1.34	0.78	0.78	0.64	0.61
Basic:acidic; B/A	1.89	3.6	1.79	1.52	1.52	1.54	1.56
<i>N</i> -terminal groups							
Alanine	34*	—	10	69	85	96	90
Proline	51*	—	70	14	6	3	4
Others	15*	—	20	17	9	1	6
Wt. (g./mole of <i>N</i> -terminal groups)	27 000	—	28 500	17 800	22 300	18 000	17 600

* Sample HV5 only.

from a column run with acid only, see Phillips & Johns (1959)], then the acetate-chloride step (2) removed a small peak of lysine-rich material in both cases. The main fraction was then eluted by step (3) or step (4) respectively.

Preparation of arginine-rich histones by extraction with acid-ethanol. Calf thymus (100 g.) was homogenized with 700 ml. of ethanol-water (70:30, v/v) for 2 min. in a Waring Blendor (the concentration of ethanol used in this step was always the same as that used in the subsequent extraction). The homogenate was centrifuged at 1000 g for 20 min. and the supernatant was discarded. The sediment was washed once more by this method and then extracted in a ball mill with 200 ml. of ethanol-N-HCl-water (70:25:5, by vol.). This extraction was carried out at 4° for 20 hr., the suspension was centrifuged and the sediment again extracted with 100 ml. of the solvent for 4 hr. followed by a final extraction with 100 ml. for 2 hr. The combined supernatants were dialysed three times for 4 hr. at 4° against 600 ml. of ethanol. The precipitate which formed accounted for 33% of the nitrogen extracted, while 58% remained in the alcoholic

supernatant and only 9% diffused out of the bag. After centrifuging, the precipitate was washed with ethanol, then with acetone and dried under vacuum. The vacuum was released every few minutes and any lumps of material were broken up with a glass rod. By drying in this manner, a fine white powder was obtained which was readily soluble in water. The average yield was 0.58 g. or 24% of the usual yield of all histones from calf thymus.

The protein remaining in the supernatant in the dialysis bag had the composition of a histone, containing 24 moles of basic amino acids/100 moles of all amino acids, with a lysine/arginine ratio of 1:1.

The effect of varying the concentration of ethanol used for the extraction from 10 to 98% (v/v) has been studied (in all cases the solvents were 0.25N with respect to HCl). The results of these experiments and the preparation described above are shown in Table 2, in which the yield, the *N*-terminal groups, the lysine:arginine ratio and the molar proportion of all basic amino acids are also given. In one preparation with ethanol-1.25N-HCl (80:20, v/v), the final precipitate was redissolved in fresh

Table 2. *Yield and properties of histones prepared by acid-ethanol extractions*

N-Terminal groups are expressed as molar percentages of all such groups found. Those other than alanine and proline were glycine, valine, leucine (and isoleucine), serine, threonine, aspartic acid and glutamic acid. The basic acids referred to are arginine, histidine and lysine.

Ethanol concentration (% by vol.). (Solutions all 0.25N with respect to HCl)	Yield of histone (mg./10 g. of calf thymus)	<i>N</i> -Terminal groups			Basic acids (% of moles of all amino acids)	Moles of lysine Moles of arginine
		Proline	Alanine	Others		
98	0	—	—	—	—	—
90	0	—	—	—	—	—
80(1)	48	5	86	9	25.5	0.76
80(2)	42	7	86	7	—	—
70(1)	66	6	85	9	24.7	0.78
70(2)	50	8	83	9	—	—
60	86	9	78	13	26.7	1.1
50	115	13	76	11	22.9	1.7
40	121	37	44	19	23.1	1.6
30	118	36	46	18	24.8	1.9
20	149	43	44	13	24.6	1.8
10	120	39	50	11	24.5	1.9
0 (i.e. 0.25N-HCl)	240	41	32	27	26.3	1.9
80(3) Redissolved and redialysed	33	3	96	1	25.2	0.64

Table 3. *Composition of histones obtained by successive acid-ethanol extractions*

N-Terminal groups are expressed as molar percentages of all the *N*-terminal groups found. The molecular weight is the g. wt. of histone/mole of *N*-terminal groups. The values for total lysine and arginine are molar percentages of all the amino acids found in these histones.

Ethanol concentration (% by vol.). (Solutions all 0.25N with respect to HCl)	Yield (mg./100 g. of calf thymus)	<i>N</i> -Terminal groups		Molecular weight	Total amino acids	
		Proline	Alanine		Lysine	Arginine
80	480	5	86	17 800	9	14
Subsequent extraction with 50	60	18	67	22 000	12	10.5
Subsequent extraction with 10	320	40	31	46 000	20	5

Table 4. *Further properties of arginine-rich histones*

The molar ratios lysine:arginine are calculated from the analyses of complete acid hydrolysates of the fractions. The *C*-terminal amino acid values are expressed as moles/18 000 weight of histone (the weight containing 1 mole of *N*-terminal groups).

Histone fraction (for origin, see text and Table 2)		70(1)		80(3)	
Elution step		Amount eluted (%, by wt.)	Moles of lysine Moles of arginine	Amount eluted (%, by wt.)	Moles of lysine Moles of arginine
Carboxymethylcellulose column chromatography					
(1)		Trace	—	0	—
(2)		10	1.02	0	—
(3)		42	0.93	16	0.87
(4)		48	0.61	84	0.64
<i>C</i> -terminal groups		Moles/18 000 g. of histone		Moles/18 000 g. of histone	
By hydrazinolysis { Alanine		0.2		0.6	
{ Others		0.1		0.1	
By carboxypeptidase { Alanine		0.4	22 hr. digestion	0.3	28 hr. digestion
{ Others		0.1		0.05	

solvent and dialysed against ethanol. The yield fell but the protein had 96 % of alanine *N*-terminal groups [cf. 80(1) and 80(2) with 80(3) in Table 2].

In another experiment the extraction of one sediment with successively lower concentrations of ethanol (all 0.25*N* with respect to HCl) extracted histones of quite different composition, the final one, with 10 % ethanol, being a lysine-rich histone containing 20 moles of lysine/100 moles of all amino acids. The results of this procedure are shown in Table 3.

Other properties of the arginine-rich histones. The materials which have been most studied were prepared by 70 and 80 % ethanol extractions [samples 70(1) and 80(3) of Table 2]. These have been examined as follows:

(a) Starch-gel electrophoresis. The method of Smithies (1955) was used, but the gel was prepared in 0.01*N*-HCl to avoid aggregation of the histone and to suppress alkaline-proteinase activity. The sample was dissolved in 0.01*N*-HCl and applied to the gel by the filter-paper technique. Both samples of arginine-rich histone gave two major and two minor bands staining with naphthalene black. Whole calf-thymus histone under these conditions gave ten bands, three of them very faint. Both the filter-paper technique and the staining method are as described by Smithies (1955).

(b) Alkaline-proteinase activity. The method of Phillips & Johns (1959) was used. The *N*-acetyl-L-tyrosine ethyl ester was digested in pH 7.7 buffer, since at this pH the autolysis of the substrate in 16 hr. at 40° was negligible. Sample 70(1) showed activity, but none was detected in sample 80(3).

(c) Chromatography on carboxymethylcellulose. The system with four eluents described above was used. The results are given in Table 4.

(d) *C*-terminal amino acids. The action of carboxypeptidase (Armour and Co. Ltd.) was studied by the method of Fraenkel-Conrat, Harris & Levy (1955). Both the enzyme and the histone were separately treated for 1.5 hr. at 20° and pH 7.7 with MM (final concentration) diisopropyl phosphorofluoridate, to inactivate proteinases. The solutions were then mixed and carboxypeptidase action was allowed to proceed at 40°. The *N*-terminal alanine residue remained unchanged during the digestion. The hydrazinolysis method used was mainly that of Niu & Fraenkel-Conrat (1955), but 95 % hydrazine was used and the reaction time kept at 10 hr. at 100° throughout. The results of the *C*-terminal amino acid experiments are also given in Table 4.

DISCUSSION

The combination of the acetate-sodium chloride and acid elutions from carboxymethylcellulose columns clearly separates calf-thymus histones into (a) very lysine-rich histone with a lysine:arginine molar ratio of 6–9:1, (b) slightly lysine-rich histone with a lysine:arginine ratio of about 1.3:1 and having mainly proline *N*-terminal groups, and (c) arginine-rich histone, with a lysine:arginine ratio of about 0.8:1 and bearing mainly alanine *N*-terminal groups. The analysis of the lysine-rich histone (see f1A in Table 1) approximates in composition to the lysine-rich histone of other authors (Davison & Butler, 1954, and Crampton *et al.* 1955). The analysis of group (b), the slightly lysine-rich histone, agrees fairly well with that of the histone II reported by Luck *et al.* (1958). The groups (b) and (c) are richer in arginine than the corresponding fractions reported by Phillips & Johns (1959), owing to the removal here of lysine-rich histone from both by the

sodium acetate-sodium chloride elution step. The amino acid analyses of group (c) are also similar to those reported by Mauritzen & Stedman (1959) for the β -histones of three fowl tissues. The arginine-rich group of histones, which chiefly concerns us here, can however be more conveniently prepared by the acid-ethanol extraction method described above. The acid-ethanol extraction of thymus previously washed with aqueous ethanol undoubtedly dissolved some non-histone material from the tissue, particularly with the lower ethanol concentrations. However, the subsequent dialysis (against ethanol) precipitated only basic proteins, as is shown by the presence of 23-27 moles of basic amino acids in every 100 moles of all acids in these preparations (see Table 2). Whole calf-thymus histone and the carboxymethylcellulose chromatographic fractions obtained from it have 25-29 moles of histidine, lysine and arginine per 100 moles of all amino acids in them (see Table 1).

The arginine-rich histones obtained by the two different methods give very similar amino acid analyses and weights per mole of *N*-terminal residues (see Table 1), and both are obtained in similar yields from calf-thymus tissue. However, the extraction method is better than the column method for this group since the *N*-terminal residue of the extraction product is almost entirely one amino acid, alanine, and the lysine:arginine ratio is lower. This result may be due to the absence of degradation by tissue proteinases in the acid-ethanol preparation. The column method demonstrates some heterogeneity in the materials obtained by the extraction method. This is shown by the presence of a little material eluted by 0.01N-HCl [elution step (3)] having a higher lysine:arginine ratio than the main fraction f3 (see Tables 1 and 4). The heterogeneity of the arginine-rich material is also suggested by the results of *C*-terminal amino acid determinations and the multiple bands observed in starch-gel electrophoresis. Since the *N*-terminal group is unaltered during the carboxypeptidase digestion, we are sure that the alanine released is *C*-terminal. The low yield of *C*-terminal alanine may be due to the presence of other *C*-terminal groups which cannot be split off by carboxypeptidase A or which give very low recoveries in the hydrazine method.

Owing to the small scale of the experiments done so far with starch-gel electrophoresis, the detailed properties of the two major and two minor bands (which are seen even in the best preparation of arginine-rich histone) have not yet been examined. The histones in these bands must presumably all have *N*-terminal alanine residues and they are unlikely, at the acid pH of the electrophoresis, to be artifacts due to aggregation.

SUMMARY

1. Calf-thymus histones, chromatographed on carboxymethylcellulose, gave two very lysine-rich fractions with sodium acetate-sodium chloride elution, a slightly lysine-rich histone with 0.01N-hydrochloric acid elution and finally an arginine-rich histone (f3), eluted with 0.02N-hydrochloric acid.

2. Material with very similar amino acid analysis and chromatographic properties to fraction f3 above could be readily prepared in bulk by extraction of the tissue with ethanol-hydrochloric acid and subsequent dialysis of the extract against ethanol.

3. The arginine-rich histone, which was precipitated, had 1 mole of *N*-terminal groups/18 000 weight, which in reprecipitated material was almost entirely alanine. The chief *C*-terminal group detected by carboxypeptidase and on hydrazinolysis was also alanine, but the yield was only about one-half that of the *N*-terminal group.

4. When lower concentrations of ethanol were used in the extraction the *N*-terminal groups of the final precipitates became more complex. Extraction of washed tissue with successively lower concentrations of ethanol showed that the arginine-rich histones were extracted at higher, and the lysine-rich histones at lower ethanol concentrations. In all cases the solvent used was 0.25N with respect to hydrochloric acid.

5. Starch-gel electrophoresis, column chromatography and perhaps the *C*-terminal group analyses indicated that the arginine-rich material with virtually a single *N*-terminal group still consisted of several components.

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Polarographic Measurement of Oxygen Uptake by Pea-Root Mitochondria

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Preliminary work on the oxidation of succinate and α -oxoglutarate by pea-root mitochondria showed that the standard manometric method of measuring oxygen uptake was not sufficiently sensitive. A polarographic method was thus developed. It was observed that, whereas the oxidation of α -oxoglutarate as determined manometrically was increased by the addition of diphosphopyridine nucleotide, that measured polarographically was not. This coenzyme also stimulated α -oxoglutarate oxidation (measured polarographically) if the mitochondria were first shaken in a water bath. Similar results with other substrates and cofactors are reported in this paper.

EXPERIMENTAL

Growth of peas and extraction of mitochondria. Peas (*Pisum sativum* L. var. Meteor) were rinsed in ethanol, treated with 10% (w/v) calcium hypochlorite for 30 min. and germinated under sterile conditions in a 5:2 (v/v) vermiculite-water mixture at 26° for 47 hr. Seedlings with roots between 22 and 30 mm. long were selected from those harvested, and a terminal 20 mm. section was excised and placed immediately in ice-water mixture. About 120-140 roots were used in each experiment. Excess of moisture was blotted off the roots, which were weighed and homogenized in a cold glass homogenizer of the Potter-Elvehjem type in 1 vol. of a cold solution containing 0.6M-sucrose and 0.05M-phosphate buffer, pH 6.8. The homogenization was carried out for the minimum length of time needed to ensure disruption of the roots (usually about 10-12 sec.) and in a loose homogenizer (with a clearance of 0.3 mm. round the pestle) which was surrounded by ice.

Unbroken cells, cell walls and nuclei were removed from the homogenate by centrifuging at 1000 *g* for 10 min. at 0°. The supernatant was centrifuged at 15 000 *g* for 15 min.

and the precipitate was taken as mitochondria. It was washed by resuspension in 0.5 vol. of the homogenizing medium and centrifuging at 15 000 *g* for 15 min. The mitochondria were finally resuspended in 0.5 vol. of the medium.

In many experiments part of the mitochondrial suspension was shaken for $\frac{1}{2}$ hr. in a test tube in a Warburg bath at 25°, at a speed of 142 oscillations/min. and through a distance of about $\frac{1}{4}$ in.

Measurement of oxygen concentration. Oxygen concentration in the incubation medium was measured polarographically. The circuit of a simple polarograph suitable for such measurements is shown in Fig. 1. The polarizing circuit is shown on the right side. The voltage divider (R_1) enables an e.m.f., derived from the battery (B_1) and measured on the voltmeter (V), to be applied across the electrodes of the cell. The current which passes through the cell is measured on the galvanometer (G). Some current

Fig. 1. Circuit of polarograph. B_1 , B_2 , 1.5 v dry batteries; C_1 , C_2 , 50 μ F, 50 v condensers; G, Scalamp galvanometer 7893/S (W. G. Pye and Co. Ltd., Cambridge); R_1 , 125 Ω helical potentiometer; R_2 , R_3 , 5 M Ω linear track potentiometer; R_4 , 1 M Ω log track potentiometer; V, 1.5 v d.c. voltmeter.