Chromatography of Histones

By P. F. DAVISON*

Chester Beatty Research Institute, Fulham Road, London, S.W. 3

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Crampton, Moore & Stein (1955) made a partial separation of the histones of calf thymus and other tissues by chromatography on IRC-50 resin. With a progressively increasing concentration of Ba^{2+} or Ca^{2+} ions they were able to elute three proteins, richer in lysine than in arginine, which together comprised about 40 % of the histones applied to the columns. They found that the corresponding proteins from different calf tissues had very similar amino acid compositions. Cruft, Mauritzen & Stedman (1954) have identified three electrophoretic components in the histones of ox thymus and liver, and they found that the major or β -histones from these two tissues differ in electrophoretic mobility. This they regard as an indication of the tissue specificity of the histones. These β -histones readily aggregate, and Davison & Shooter (1956) have shown that the aggregating histones are those rich in arginine. Thus the evidence from electrophoresis does not conflict with the results of Crampton *et al.*, which seemed to demonstrate no cell specificity in the lysine-rich histones. However, the electrophoretic evidence cannot be accepted as conclusive since any small amount of non-histone contaminants loosely combined with these basic proteins might change the electrophoretic mobility. To test this question of tissue specificity by the analysis of different fractions it is necessary to find a chromatographic system capable of separating the arginine-rich as well as the lysine-rich histones. The experiments to be reported here have shown that the histones can be completely eluted from carboxymethylcellulose, although they are not well separated. A preliminary report on this chromatographic system has been published (Davison & Shooter, 1956).

EXPERIMENTAL

Adsorbent. Carboxymethylcellulose was prepared in the manner described by Peterson & Sober (1956). The dried white powder was dispersed in the appropriate buffer solutions with rapid stirring and the slurry was then poured into chromatographic columns. A thin layer of acid-washed Celite 545 (Johns-Manville Co. Ltd.) helped to prevent the adsorbent clogging the sintered-glass plate at the bottom of

the columns. The carboxymethylcellulose was allowed to settle under gravity to give a column 10–15 cm. high. Tubes with an internal diam. of 1 cm. were used for qualitative studies, with 5–10 mg. of protein. Fifty to 100 2 ml. fractions were sufficient to delineate the effluent diagrams. For preparative studies a 1.4 cm. diam. column was used, with a loading of up to 120 mg. of protein. Larger columns were used for some experiments but, unless the adsorbent was initially suspended in butanol, even packing was difficult to achieve. The linearity of the front was checked by passing a small volume of potassium chromate through the column.

The flow rates were reduced to 4–8 ml./cm.² cross-section, by fixing drawn-out capillary tips to the columns.

Proteins. The histones used in the exploratory studies were those from calf thymus, prepared as previously described (Davison, James, Shooter & Butler, 1954). In later experiments some histones extracted from mouse-cell nuclei by acid were chromatographed. The cell nuclei were isolated from various mouse tissues in dilute acid (Davison, 1957).

Gradient devices. The proteins were eluted from the adsorbent by buffered solutions of progressively increasing ionic strength. The proteins were applied to the columns in

Fig. 1. Gradient device to provide continuously changing eluting solution. The more concentrated, denser solution is placed in the left-hand reservoir.

* Present address: Biology Department, Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.

0.1 or 0.05M-buffers; when the solution had passed into the column more of this buffer was added to wash the protein into the adsorbent. The column reservoir was filled with buffer and a concentration gradient in the eluting solutions was then produced by adding at a controlled rate a solution of the same buffer containing 0.9M-NaCl. Mixing of the solutions in the reservoir was effected by a magnetic stirrer.

An alternative device used on the small columns consisted of an open-topped Perspex box with a diagonal diaphragm of stainless steel sealed in with wax (Fig. 1). For low rates of flow the two solutions merging in the three-way tap mixed satisfactorily by diffusion alone. The shape of the concentration gradient could be modified by bending the diaphragm.

Effluent analysis. The concentration of protein in the column effluents was measured by the absorption of the solutions at 2780 Å, or by the ninhydrin reagent (Moore & Stein, 1954).

Effect of pH. The chromatographic behaviour of the histones was studied over a pH range 3.0–8.1. Sodium phosphate, citrate and acetate buffers (0.05 or 0.1M) were used.

At pH 3–4.5 two main components could be distinguished in the effluent diagrams. These were preceded by a smaller faster-running fraction (Fig. 2a, b). At higher pH values the diagrams became more complex until at pH 8.1 at least five peaks could be detected. Since, however, it is possible that complexes, as well as homogeneous proteins, may be chromatographed as distinct peaks, and since it is known that the histones readily aggregate above pH 7, the delineation of five peaks on the diagram does not necessarily imply the presence of five distinguishable proteins.

Recovery of the proteins from the adsorbent. In preliminary experiments (Davison & Shooter, 1956) the proportion of the protein recovered from the columns was determined by the colour given with ninhydrin, by the protein applied to the column and that in the total column effluent. Recoveries calculated in this way for duplicate chromatograms were frequently found to differ widely. The cause of this behaviour was not determined, but from subsequent observations it is possible that varying amounts of degradation occurred in the solutions as they stood on the fraction collector. An increase of amino end-groups would increase the ninhydrin colour.

In subsequent experiments the protein recovery was calculated from the nitrogen content of the effluents. It was found that, contrary to our earlier report, the proportion of the protein eluted from the columns decreased as the pH was raised. It was also found that the recovery from the columns decreased as the temperature was raised, although the elution diagrams were not qualitatively changed between 0° and 50°. The effect of temperature and pH on nitrogen recovery is shown in Table 1.

Isolation of proteins from column effluents. The concentration of the proteins in the column effluents was often very low. Dialysis of the pooled fractions, followed by freeze-drying, was inefficient, and not more than 70% of the applied protein was ever recovered in this way. Precipitation of the proteins by phosphomolybdic acid or perfluorooctanoic acid was effective, but there remained the difficulty of removing the precipitants. The most efficient method of recovery was to adjust the solutions to pH 10 and to add 3 vol. of acetone. After standing for 24 hr. at 0° approx. 80% of the histones could be precipitated from solutions

as dilute as 0.002%. The precipitates were washed in 95% acetone to free them of salt. This procedure precluded the use of sodium citrate and phosphate buffers since these are insoluble in 75% acetone. Up to 95% recoveries of the chromatographed histones were obtained in this way.

Analysis. Nitrogen determinations were made by the micro-Kjeldahl method. Amino acid analyses were made by the fluorodinitrobenzene method of Levy (1954). The procedure was slightly modified in detail (Davison, 1957).

The method of Moore & Stein (private communication) was used to determine accurately the ratio of the basic

Fig. 2. Elution diagrams of calf-thymus histones chromatographed at pH 4.2. Protein concentration was measured by: (a) absorption at 2780 Å; (b) ninhydrin reaction. The broken lines in the diagrams indicate the change in the concentration in the effluent, as determined by the refractive index.

Table 1. Percentage of applied protein nitrogen recovered in column effluents

Each value reported is the result of a separate experiment.

At pH 8.1		At 4°	
Temp.	Recovery (%)	pH	Recovery (%)
4°	(i) 84	4.1	(i) 94
	(ii) 87	5.0	(i) 90
20	(i) 75	6.6	(i) 97
	(ii) 73		(ii) 92
50	(i) 54	8.1	(i) 84
			(ii) 87

amino acids in the histone fractions separated by chromatography. Lysine, histidine, ammonia and arginine were eluted in sequence from a 10 cm. column of Zeo-Karb 225 (8% cross-linked), with pH 5.26, 0.35M-citrate buffer at 50°. Fractions (2 ml.) were collected and the amino acid concentration was determined by the modified ninhydrin reagent of Moore & Stein (1954). The 2-methoxyethanol (methyl Cellosolve) in this reagent was purified before use by passing it through a layer of Zeo-Karb 225 and a column of activated alumina.

Protein hydrolysis. For the amino acid analysis the proteins were hydrolysed in sealed tubes with 6N-HCl at 110° for 24 hr.

RESULTS

Qualitative examination of histones. For qualitative examination of the histone preparations the carboxymethylcellulose columns were buffered at pH 6.5. Typical elution diagrams are shown in Figs. 3 and 4. These diagrams show more distinguishing features than those at lower pH.

For preparative chromatography the columns were buffered at pH 4.2 with sodium acetate and acetic acid. At this pH the large-molecular-weight histone aggregates begin to dissociate (Davison *et al.* 1954) and this is obviously a prerequisite if the component proteins are to be efficiently separated.

Degradation in the histone fractions. Crampton (1956) reported that occasionally, when a histone fraction separated by chromatography was re-chromatographed, it gave rise to a fast-running component in addition to a peak moving in the original position. Similar behaviour was observed with fractions from carboxymethylcellulose. The fast-running fraction gave a much higher ninhydrin colour/mg. of nitrogen than the parent material. It was also found that the fast material could be eluted from IRC-50 resin columns with Na⁺ ions, whereas the histones can only be eluted with bivalent ions. These observations suggested that the histones were being degraded either on the column or during their subsequent handling. Butler, Davison, James & Shooter (1954) have shown that histones are rather easily degraded at neutral pH. Whether the degradation is the result of proteolysis or the dissociation of complex proteins is not known. As a precaution against proteolysis the buffer solutions were saturated with *p*-chloromercuribenzoic acid and the chromatograms were run at 4°.

Carboxymethylcellulose buffered at pH 4.2 separated calf-thymus histones into three components (Fig. 2*a, b*). The first peak passed directly through the column without the application of the gradient; since in many cases nothing could be precipitated from this effluent fraction it is probable that much of it is comprised of degraded protein. In two preparations, however, a precipitate was obtained. Qualitative amino acid paper chromatography

Fig. 3. Elution diagram of calf-thymus histones chromatographed at pH 6.5. The small first peak, which was visible in Fig. 2*a* and *b*, is obscured by another peak formed from material migrating at pH 4.2 in the two main peaks. The broken line indicates the refractive index of the eluting solution.

Fig. 4. Elution diagram of mouse-kidney histone chromatographed at pH 6.5. (Compare with Fig. 3.) The delayed application of the eluting gradient has resolved the small first peak. The broken line indicates the refractive index of the eluting solution.

Table 2. *Composition of leading fraction from calf-thymus histone chromatographed at pH 4.2 on carboxymethylcellulose*

The analysis is expressed in molar ratios (no. of amino acid residues/100 residues).

Arginine	2.7
Alanine	8.8
Aspartic acid	19.7
Glutamic acid	32.7
Glycine	8.4
Histidine	1.4
Leucine	5.0
Isoleucine	
Lysine	8.5
Proline	0.0
Serine	3.4
Threonine	3.5
Valine	5.6

suggested this protein was rich in dicarboxylic acids, and this was confirmed by an analysis of one sample by the Levy (1954) method (see Table 2). This acid protein comprised less than 3% of the total histone.

It was noticed some years ago in these laboratories that a few thymus-histone preparations, on electrophoresis at pH 4.5, showed a very small component migrating behind the histone boundaries. It is possible that this was the acid protein.

The second fraction separated at pH 4.2 gave a high colour yield with ninhydrin, but had a low absorption at 2780 Å: it consisted solely of the lysine-rich histone (Davison & Butler, 1954; Crampton *et al.* 1955). It comprised 11–13% of the various histone preparations analysed.

The third and major peak comprised 80% of the total histone and contained more than one species of protein. Samples were recovered from different portions of the third peak and analysed for basic amino acids by the method of Moore & Stein (1956, private communication). The molar ratio of lysine:arginine diminished from approx. 1.5 to less than 1.0, from the leading edge to the trailing edge of the peak. Some results of analyses are summarized in Table 3.

From columns buffered between pH 5 and 6.5 a further component could be isolated. This emerged with the first application of the gradient, ahead of

the lysine-rich component. If the eluting gradient were applied to the column immediately after the protein, this intermediate peak could not be distinguished from the small unadsorbed component (cf. Figs. 3 and 4). This new fraction contained considerably more lysine than arginine. It was probably formed by aggregates of proteins migrating at lower pH in the two main peaks.

Above pH 6.5 more intermediate peaks appeared and the two main peaks decreased in size: this behaviour probably reflected further aggregate formation.

Histones extracted from a variety of calf and mouse tissues were examined qualitatively on the carboxymethylcellulose columns at pH 6.5 (see also Davison, 1957). One preparation from mouse kidneys and one from mouse livers gave elution diagrams essentially similar to those from calf-thymus histones (Fig. 4). In most diagrams, however, five peaks could be detected in proportions which varied from one preparation to another (Fig. 5). It is probable that this added complexity arose from non-histone proteins in the extracts. The presence of such contaminants in some preparations was confirmed by electrophoresis at pH 2.2: at this pH the different proteins do not appear to aggregate, and a small peak could be detected trailing the sharp histone boundary.

The two peaks which were eluted last from these pH 6.5 columns appeared in every diagram at the same position and were presumed to correspond to the two major components resolved at pH 4.2. The presence of two or more peaks preceding these was taken to indicate contaminants. Thus the columns at pH 6.5 were mainly of value in assessing the purity of the histone preparations. For an efficient fractionation the adsorbent was buffered at a lower pH, where the protein interactions were minimized. It was found possible to operate these columns at pH 3.1, but the proteins were then eluted at lower salt concentrations.

Chromatography on IRC-50. The histone-elution diagrams at pH 4.2 were very similar to those obtained from IRC-50 (Crampton *et al.* 1955). When each of the main components from the carboxymethylcellulose column was isolated and rechromatographed on IRC-50 with barium acetate the similarity was confirmed; the first, lysine-rich, histone emerging in the position of Crampton's histone A, and the second component in the position of histone B. The proportion of the latter fraction which was eluted from IRC-50 was not determined.

DISCUSSION

The chromatographic system described here has several advantages over that devised by Crampton *et al.* (1955), notably in the higher recoveries from

Table 3. *Composition of fractions from the major peak from calf-thymus histone*

The analysis is expressed as molar ratios relative to lysine.

	Original histone	Leading half	Final third	Final fifth
Lysine	1.0	1.0	1.0	1.0
Arginine	0.54	0.66	0.83	0.94
Histidine	0.20	0.18	0.21	0.25

Fig. 5. Elution diagram of histone extract from mouse Landschütz ascites-tumour cells chromatographed at pH 6.5. The broken line indicates the refractive index of the eluting solution.

the columns and the possibility of precipitating the histones from the effluent fractions. It does not, however, satisfactorily resolve the arginine-rich histones. It is not clear if the continuous change observed in the lysine:arginine ratio of the proteins in successive portions of the major fraction can be attributed to relatively few components emerging in overlapping bands, or to a very large number of proteins. If the histones function as gene inhibitors, as Stedman & Stedman (1950) have suggested, the latter possibility would appear more likely. However, the little evidence available suggests that at least 20% of the major histone fraction is homogeneous, i.e. the histone B of Crampton. The problem of the complexity of this major fraction is at present being pursued by a study of the distribution of the *N*-terminal amino acids through the chromatographic peak.

The detection of the acid protein in the calf-thymus histones exemplifies one of the fundamental difficulties: that of purifying the basic proteins. Since this contaminant was found in a preparation from such a convenient tissue as thymus, it is not surprising that higher proportions of contaminants should be found in extracts from other tissues. Many purification procedures used in the past have resulted in unintentional fractionation of the histones (Davison & Butler, 1956). The carboxymethylcellulose columns, operated at pH 4 or lower, could probably provide a simple and effective means of freeing the histones of non-basic contaminants.

Although 90–95% of the protein nitrogen was recovered from these chromatograms, it is possible that small amounts of histone were lost. These would presumably be the histones richest in arginine, since they require the higher concentrations of salt to be eluted. Such a loss could not be demonstrated by analysis.

None of the fractions listed in Table 3 has a lysine:arginine ratio less than 1.0, and of several other fractions separated by chromatography only one, from the tail of the major peak, had this ratio slightly lower (0.95). The much lower value found for the histone fraction precipitated by ammonia (Davison & Shooter, 1956) has not been found in the fractions separated by chromatography. Whether the earlier analyses were in error, or whether the two fractionation methods are resolving different species of proteins, has yet to be determined.

SUMMARY

1. A description is given of a chromatographic system capable of separating the histones of calf thymus and other tissues into the lysine-rich histone and the remainder, with a recovery higher than 90%.

2. Analysis has shown that the lysine:arginine ratio decreases in the histone eluted with higher concentrations of salt, but it could not be decided whether the histones consisted of a few limited species or were more complex.

3. Examination of the protein in the acid extracts of nuclear materials from calf thymus and other tissues has demonstrated the presence of non-basic protein contaminants.

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