

## PURIFICATION OF THE FIVE MAIN CALF THYMUS HISTONE FRACTIONS BY GEL EXCLUSION CHROMATOGRAPHY

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### 1. Introduction

Ion exchangers (CMC-cellulose and Amberlite IRC-50) and molecular sieves (Sephadex and Biogel) have been widely used for the chromatography of histones (for references see [1]). Both methods have been successfully employed for the purification of histone fractions and mixtures obtained by the method of Johns [2]. However, at present chromatographic methods alone can not resolve whole histone into its five main fractions [1]. Recently exclusion chromatography on Biogel P-60 has yielded  $F_1$  and  $F_{2a1}$  in high purity, leaving  $F_3$ ,  $F_{2a2}$  and  $F_{2b}$  unresolved [3]. Chromatography on Biogel P-10 gives  $F_1$  and, after appropriate pooling and rechromatography, other pure histones [4].

Histones aggregate in solution [5–8]. At low pH and low ionic strength aggregations are minimal, however, under these conditions the histones exist as extended chains and behave anomalously in gel exclusion chromatography exhibiting molecular weights which are by a factor of 2–6 higher than their actual molecular weights [7]. In addition, hydrophobic interactions between peptide chains, and prolyl determined conformational features may affect the behaviour of histones in gel pores [7] and may be responsible for the unsatisfactory separation of  $F_3$ ,  $F_{2a2}$  and  $F_{2b}$ . Variation of salt, pH and protein concentration affect the conformation of histones in specific regions [10] and all histones undergo similar conformational changes on the addition of salt [6]. These conformational changes are continuous [9] and the extent of chain interaction differs for the various histones [11, 12].

Using variation of salt and pH to achieve selected aggregation we have resolved whole histone from calf thymus into its five main components in a two step exclusion chromatographic procedure at pH 1.7 and 5.4.

### 2. Materials and methods

The histones were extracted from calf thymus chromatin [13] with 0.25 N  $H_2SO_4$ , dialyzed against distilled water at 4°C and freeze-dried. For chromatography at pH 1.7 the histones were dissolved in freshly prepared 8 M urea, pH 4–5, 1% mercaptoethanol to give a protein concentration of 10 mg/ml. The solution was stored overnight at 4°C and then applied to the column. All chromatography was done at room temperature. For chromatography at pH 5.4 [15] the dialyzed and freeze-dried  $F_{2a2}$ – $F_3$  mixture from the first chromatogram was dissolved in 2 M NaCl, stored overnight at 4°C and then applied to the column. Analytical runs were done on 150 X 1.5 cm columns (Biogel and Sephadex) at a pressure head of 40 cm and a flow rate of 6–10 ml/cm<sup>2</sup>/hr. Preparative runs on Biogel were done on 14.5 X 77 cm columns at a pressure head of 30 cm and a flow rate of 4 ml/cm<sup>2</sup>/hr without any loss in resolution. Preparative runs on Sephadex were done on 5 X 100 cm columns at a pressure head of 17 cm and a flow rate of 3 ml/cm<sup>2</sup>/hr. Under these conditions the separation of  $F_{2a2}$  and  $F_3$  is less satisfactory and rechromatography becomes necessary. The protein concentration was determined from the absorbance at 230 nm. Pooled fractions were dialyzed against distilled water

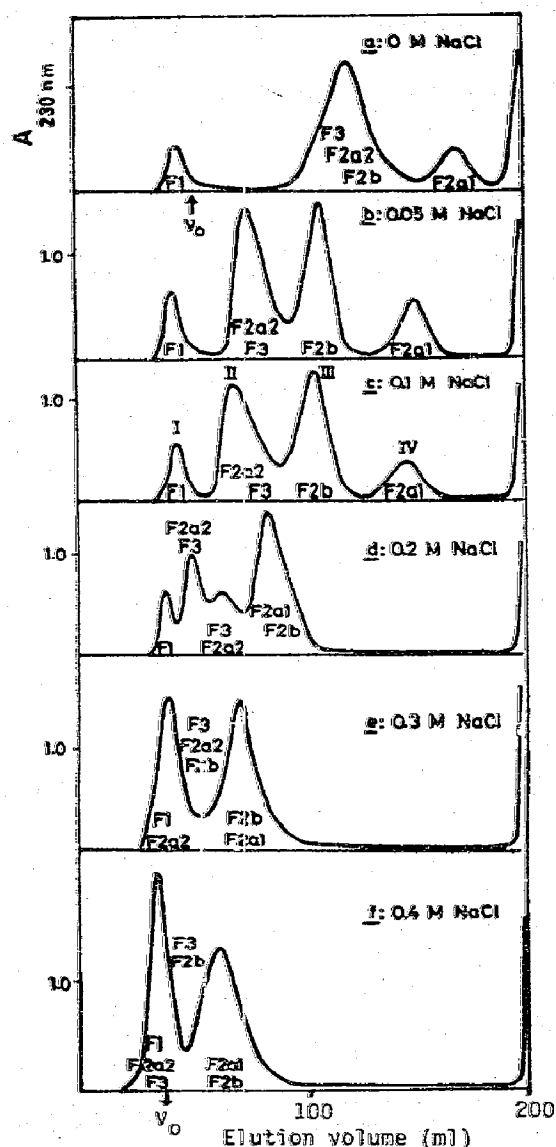


Fig. 1. Chromatography of total calf thymus histone on Biogel P-60 in 0.02 N HCl–0.02%  $\text{NaN}_3$ , pH 1.7 and various concentrations of NaCl. Samples were dissolved in 8 M urea–1% mercaptoethanol and stored overnight at 4°C before application. Column dimensions: 150 × 1.5 cm; sample weight: 12–15 mg; sample volume: 2 ml; fraction volume: 1.2 ml; flow rate: 6 ml/cm<sup>2</sup>/hr; pressure head: 40 cm.  $V_0$ , 0 M NaCl (Blue Dextran 2000): 68 ml;  $V_0$ , 0.4 M NaCl (Blue Dextran 2000): 62 ml. Polyacrylamide electrophoretograms [14] for fraction I, II, III and IV (fig. 1c) are shown in fig. 3. The strong UV-absorption towards the end of the inner volume is due to the elution of urea and mercaptoethanol.

at 4°C, freeze-dried and then analyzed by gel electrophoresis [14]. Further experimental details are given in the legends of the figures.

### 3. Results and discussion

Exclusion chromatography of calf thymus histones on Biogel P-60 in 0.02 N HCl in the absence of NaCl results in 3 distinct fractions (fig. 1a). The fractions eluting first and last were electrophoretically identified as  $F_1$  and  $F_{2a1}$  respectively. These findings are in agreement with those of Hamana and Iwai [3]. Electrophoretic analysis of the front-centre and back part of the middle peak indicated that the histones  $F_3$ ,  $F_{2a2}$  and  $F_{2b}$  are eluted in that order but remain unresolved. The elution order of calf thymus histones on Biogel P-60 in 0.02 N HCl thus is:  $F_1$ ,  $F_3$ ,  $F_{2a2}$ ,  $F_{2b}$ ,  $F_{2a1}$  and seems to follow their molecular weights:  $F_1 = 21\,000$ , sedimentation equilibrium analysis [16, 17],  $F_3 = 15\,324$ , sequence [18, 19],  $F_{2a2} = 14\,005$  sequence [20, 21],  $F_{2b} = 13\,774$ , sequence [22] and  $F_{2a1} = 11\,300$ , sequence [23]. In view of the fractionation range of Biogel P-60 and the column dimensions, it is obvious, however, that the small differences in molecular weight of the histone monomers can not be responsible for the elution sequence. Phillips and Clarke [7] using the same chromatographic system found the elution order to be:  $F_1$ ,  $F_{2a2}$ ,  $F_{2b}$ ,  $F_3$ ,  $F_{2a1}$ , but the histones were applied and run singly.

The presence of 0–0.4 M NaCl in the 0.02 N HCl eluant markedly changes the elution order, the elution volume and the resolution of the individual fractions (figs. 1b–f). At 0.05–0.1 M NaCl the middle peak partially resolves giving a total of four fractions (figs. 1b and 1c). On polyacrylamide electrophoresis fraction I consists of homogeneous  $F_1$ , fraction II of a mixture of  $F_{2a2}$  and  $F_3$ , fraction III and IV of homogeneous  $F_{2b}$  and  $F_{2a1}$  respectively (fig. 3). Analysis of the front, centre and back part of fraction II (fig. 1c) indicated that  $F_{2a2}$  is eluted prior to  $F_3$ . Thus, the elution order of histones on Biogel P-60 in 0.02 N HCl–0.1 M NaCl is:  $F_1$ ,  $F_{2a2}$ ,  $F_3$ ,  $F_{2b}$ ,  $F_{2a1}$ . At higher NaCl concentration all histones but  $F_1$ , which is eluted in the outer volume, shift towards lower elution volume and the resolution of fractions deteriorates (figs. 1d–f). A NaCl concentration of 0.1 M was

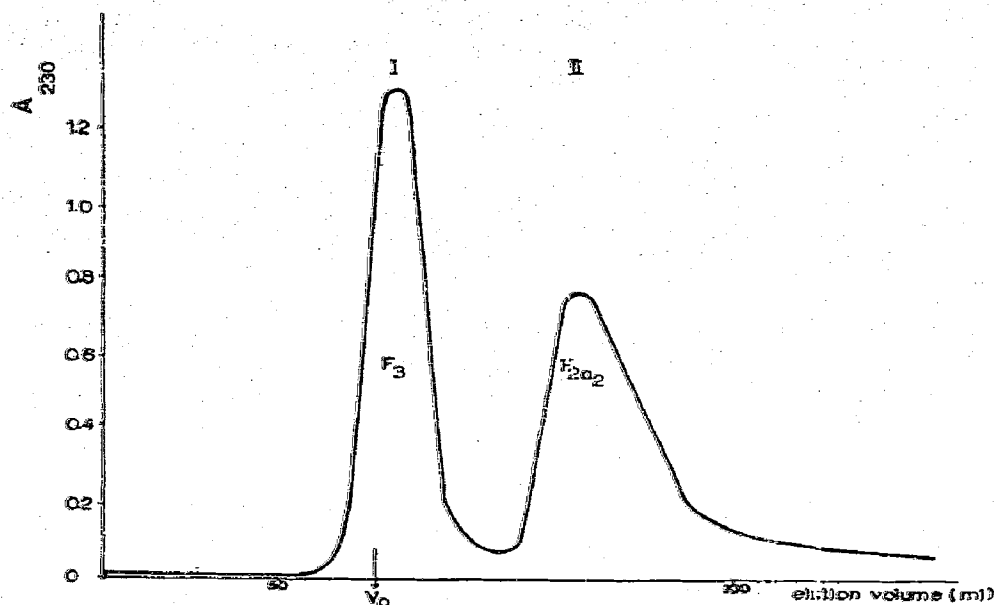


Fig. 2. Chromatography of calf thymus  $F_{2a2}$ - $F_3$  mixture on Sephadex G-100 at pH 5.4. The freeze-dried proteins from peak II (figs. 1b and 1c) were dissolved in 2 M NaCl and stored overnight at 4°C before application. Column dimensions: 150 x 1.5 cm; eluant: 0.05 M Sodium acetate-0.05 M  $\text{NaHSO}_3$ , pH 5.4 buffer; sample weight: 10 mg; sample volume: 1 ml; fraction size: 1.2 ml; flow rate: 10 ml/cm<sup>2</sup>/hr; pressure head: 40 cm. Polyacrylamide electrophoresis [14] see fig. 3.

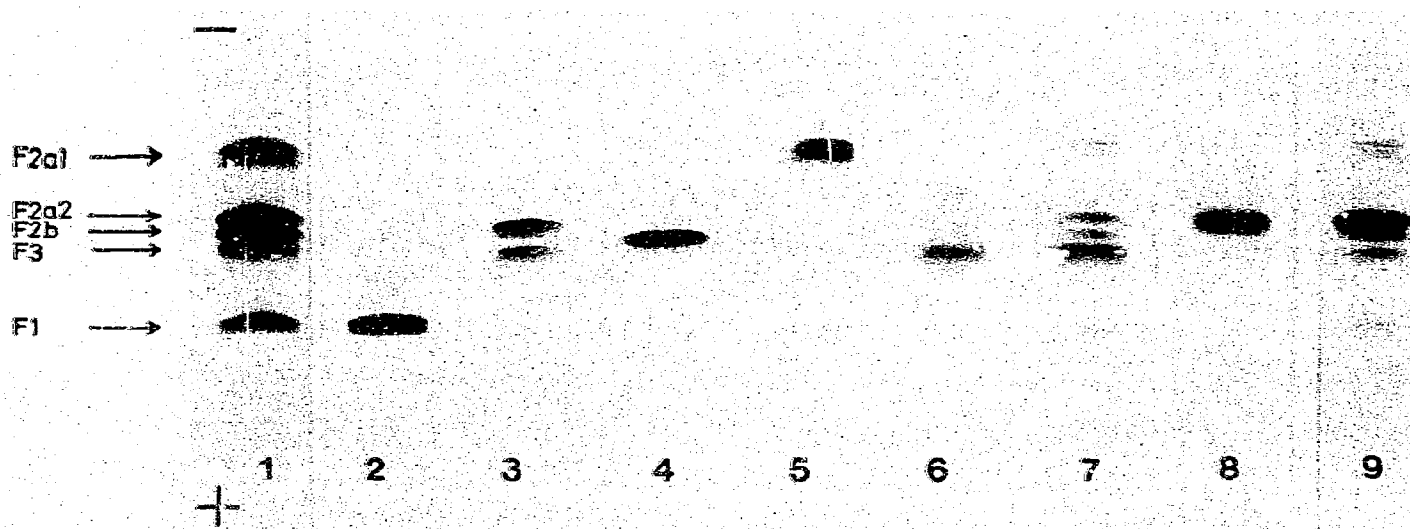


Fig. 3. Polyacrylamide electrophoretic pattern of calf thymus histone fractions prepared by column chromatography on Biogel P-60 (fig. 1c) and Sephadex G-100 (fig. 2). Gel identification: 1) total calf thymus histone; 2) Fraction I (fig. 1c):  $F_1$ ; 3) Fraction II (fig. 1c):  $F_{2a2}$ - $F_3$  mixture; 4) Fraction III (fig. 1c):  $F_{2b}$ ; 5) Fraction IV (fig. 1c):  $F_{2a1}$ ; 6) Fraction I (fig. 2):  $F_3$ ; 7) Fraction I (fig. 2) + total calf thymus histone; 8) Fraction II (fig. 2):  $F_{2a2}$ ; 9) Fraction II (fig. 2) + total calf thymus histone. 20  $\mu\text{g}$  samples were applied in 8 M urea-1% mercaptoethanol. All gels were run for 2.5 hr.

found to be optimal giving the 3 homogeneous fractions  $F_1$ ,  $F_{2b}$  and  $F_{2a1}$  and a mixture of  $F_{2a2}$  and  $F_3$  (figs. 1c and 3). The unresolved  $F_{2a2}$ - $F_3$  fraction was then subjected to gel exclusion chromatography on Sephadex G-100 using 0.05 M sodium acetate-0.05 M  $\text{NaHSO}_3$  pH 5.4 buffer as the eluant [15] to result in the separation of histone  $F_3$  and  $F_{2a2}$  (fig. 2). In a typical preparative separation 2 g crude whole histone gave electrophoretically homogeneous histone fractions with the following yields:  $F_1$  = 0.35 g;  $F_{2b}$  = 0.40 g;  $F_{2a1}$  = 0.45 g;  $F_{2a2}$ - $F_3$  mixture = 0.50 g. The latter was resolved on Sephadex G-100 to give  $F_{2a2}$  = 0.20 g and  $F_3$  = 0.22 g. The recovery of protein from the Biogel step was 90% and from the Sephadex step 80%.

Osmotic pressure measurements and sedimentation studies have shown that with the exception of  $F_1$  all histones are aggregated in the presence of salt even at pH 2 and below [5, 11]. The shift of the histone fractions towards lower elution volumes with increasing salt concentration at pH 1.7 (fig. 1a-f) is in agreement with these findings. The fact that histones differ in their tendency to aggregate at a given salt concentration [9, 11, 12] is also indicated here (fig. 1a-f). The change of the elution order of  $F_3$  and  $F_{2a2}$  when whole histone is applied (fig. 1a) as compared to the elution order of individually chromatographed histones [7] suggests that the two histones interact with each other. Interchain interactions between  $F_{2a2}$  and other histone fractions have previously been suggested [8]. The fact that  $F_{2a2}$  and  $F_3$  in the HCl-NaCl system (figs. 1b and 1c) are being eluted as one peak may be the result of similar conformations of the two histones under these conditions or due to interchain interactions.

This chromatographic procedure yields all five calf thymus histone fractions in high purity in only two steps. Because whole histone is used as the starting material the method is suitable for quantitative studies on the analytical scale and can easily be adapted to the preparative scale. The method can be employed at various stages of the solvent extraction procedure of Johns [2] and has been successfully used to isolate chicken and sea urchin  $F_{2a2}$  and  $F_{2a1}$  in high purity from the corresponding  $F_{2a}$  (Johns) fractions. Chicken  $F_1$ ,  $F_{2c}$ ,  $F_{2a2}$  and  $F_{2a1}$  have been obtained directly by chromatography of whole histone on Biogel. Sea urchin, shark and chicken  $F_{2b}$  (Johns) have been ef-

fectively freed from contaminating histones. Gel exclusion chromatographic procedures based on selective aggregation are being employed in this laboratory to purify histones from various sources for comparative structure investigation.

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