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Note

Chromatography of histones on hydroxyapatite columns

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It has been shown that hydroxyapatite is a valuable column support for the fractionation of non-histone proteins[1,2]. Bernardi *et al.*[3] found that basic proteins can also be chromatographed on hydroxyapatite by competition for their binding sites (the phosphate groups of hydroxyapatite) with cations.

This paper describes the chromatography of bovine histones on hydroxyapatite with gradients of sodium phosphate or sodium chloride, both in 5 M urea. The arginine-rich histones H3 and H4 are, together with histone H2A, eluted first, followed by the slightly lysine-rich histone H2B and then by the lysine-rich histone H1. From the chromatographic behaviour of poly(L-lysine) and poly(L-arginine) it is concluded that on the hydroxyapatite column the histones are mainly separated according to their lysine content, and that the guanidino group of arginine contributes only slightly to the binding of histones to hydroxyapatite.

EXPERIMENTAL

Materials

Poly(L-lysine) (M_r = 16,500) and poly(L-arginine) (M_r = 15,000) were purchased from Miles-Yeda (Rehovoth, Israel).

Preparation of histones

Chromatin was isolated from bovine lymphocytes as described previously[2]. Histones were extracted from chromatin according to Panyim *et al.*[4].

Chromatography on hydroxyapatite columns

Hydroxyapatite was prepared according to the method of Tiselius *et al.*[5]. The urea used throughout this study was prepared as a 10 M stock solution and deionized on a column of Ionenaustauscher V mixed-bed resin (Merck, Darmstadt, G.F.R.). The prepared buffers were stored in the cold and used within 2 days of preparation.

Columns containing 20 ml of hydroxyapatite were loaded with about 20 mg of protein in equilibration buffer (see legends of figures), the protein was washed in with the same buffer and the column was eluted with linear gradients as indicated in the figures. All operations were carried out at 4°. Elution molarities were checked by measuring the conductivity and compared with standard graphs for the appropriate buffers.

The protein concentration was determined from the absorbance at 230 nm using the relationship 1 absorbance unit $\equiv$ 0.30 mg/ml, which was established for histones by nitrogen determination by the Kjeldahl method[6].

Polyacrylamide gel electrophoresis

Electrophoresis was performed on gels of 15% acrylamide, 0.9 N acetic acid and 2.5 M urea as described by Panyim and Chalkley[7].

Amino acid analyses

Total amino acid compositions were determined by using a modified Model 120c Beckman amino acid analyser. Samples containing 200–300 μg of protein were hydrolysed in 6 N hydrochloric acid at 110° for 22 h in sealed tubes under a nitrogen atmosphere.
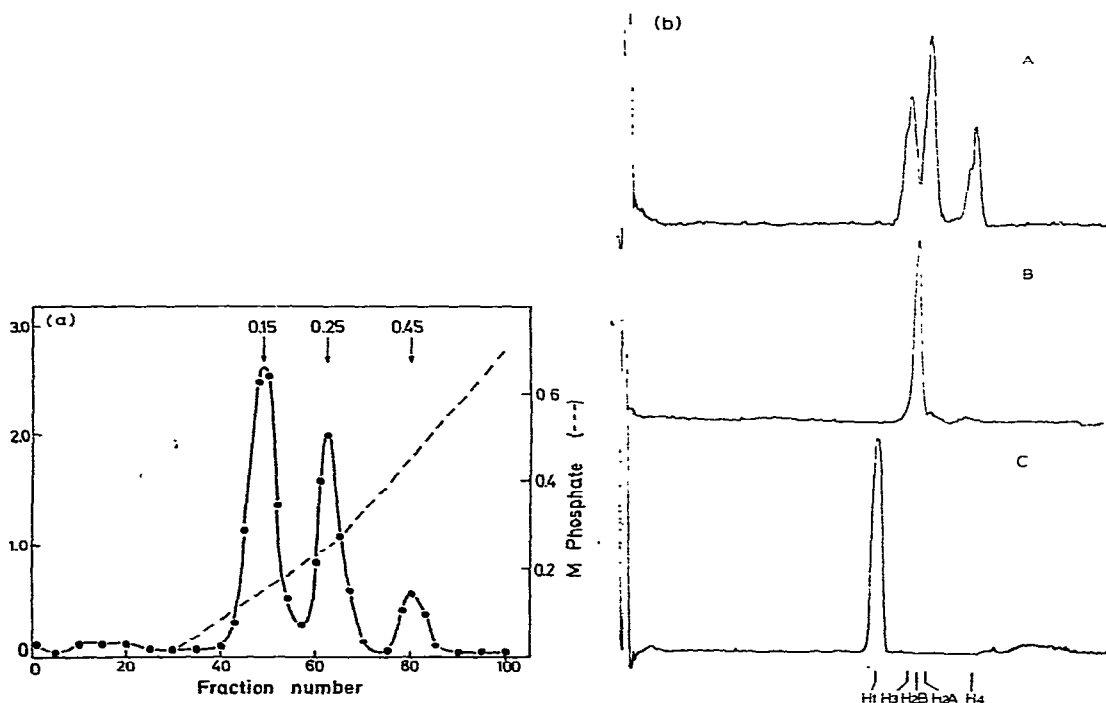

Fig. 1. (a) Chromatography of histones on hydroxyapatite with a gradient of sodium phosphate (pH 6.8) in 5 M urea. Histone (25 mg) was dissolved in 1 mM sodium phosphate (pH 6.8), 5 M urea and loaded on a 20-ml hydroxyapatite column equilibrated with the same buffer. Elution was carried out with two successive sodium phosphate molarity gradients (each 50 + 50 ml), 1 mM–0.3 M and 0.3–0.8 M, both in 5 M urea. Fractions of 2.8 ml were collected. (b) Electrophoresis of hydroxyapatite column fractions in acidic urea–polyacrylamide gels. (A) Histones eluted with 0.15 M, (B) with 0.25 M and (C) with 0.45 M sodium phosphate (pH 6.8) in 5 M urea.

RESULTS AND DISCUSSION

Fig. 1a shows the pattern of elution of bovine histones from hydroxyapatite with a gradient of sodium phosphate (pH 6.8) in 5 M urea. With 0.15 M phosphate, histones H3, H2A and H4 elute together from the column, as revealed by acidic urea–polyacrylamide electrophoresis (Fig. 1b). The two following peaks, with 0.25 and 0.45 M phosphate, consist of histone H2B and H1, respectively, as shown by polyacrylamide gel electrophoresis and amino acid analysis (Table I). The amounts of protein recovered in the peaks were 50, 30 and 8%, respectively.

TABLE I

AMINO ACID ANALYSES OF FRACTIONS FROM BOVINE HISTONES

No corrections have been made for losses during hydrolysis.

Amino acid	Amount in fraction (mole per 100 mole)	
	0.25 M phosphate peak = H2B	0.45 M phosphate peak = H1
Lysine	15.7	25.4
Histidine	3.8	0
Arginine	6.4	1.9
Aspartic acid	5.2	2.4
Threonine	6.6	5.6
Serine	9.3	6.3
Glutamic acid	8.9	3.5
Proline	4.4	9.1
Glycine	5.9	6.6
Alanine	10.8	25.1
Cysteine	0	0
Valine	7.6	5.1
Methionine	Trace	0
Isoleucine	5.4	1.3
Leucine	5.7	4.6
Tyrosine	2.4	0.2
Phenylalanine	1.7	0.4

A similar elution pattern was obtained with a gradient of sodium chloride in 5 M urea, 1 mM sodium phosphate (pH 6.8) (Fig. 2). Again, the arginine-rich histones H3, H4 and the slightly lysine-rich histone H2A appear first in one peak at 0.05 M NaCl. Histone H2A is also found in the second peak at 0.38 M NaCl, together with the other slightly lysine-rich histone H2B. Histone H1, which has the highest lysine content, elutes with 0.85 M NaCl.

A combination of NaCl and phosphate elution is shown in Fig. 3a. The sodium chloride concentration is fixed at 0.6 M so that all of the histones, except histone H1, will flow through the column. Histone H1 is then eluted with a phosphate gradient in 5 M urea, 0.6 M NaCl, and appears in two peaks comprising 2.8% and 7.6% of the material applied, respectively, with similar electrophoretic mobilities in acidic urea gels (Fig. 3b). Whether the chromatographic separation is caused by a microhetero-geneity of histone H1 or is due to modifications of the histone by acetylation or phosphorylation has not been investigated. The strong binding of histone H1 to hydroxyapatite has been shown previously by Bernardi et al.[3], who found that 0.55 M
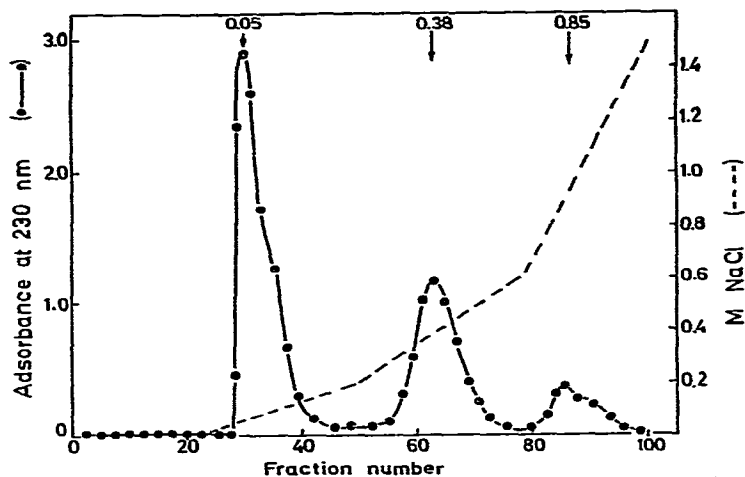
Fig. 2. Chromatography of histones on hydroxyapatite with a gradient of sodium chloride in 5 M urea, 1 mM sodium phosphate (pH 6.8). Histone (67 mg) was dissolved in 1 mM sodium phosphate (pH 6.8), 5 M urea and loaded on an 80-ml hydroxyapatite column. Elution was carried out with three successive sodium chloride gradients (each 80 + 80 ml), 0.0–0.2 M, 0.2–0.6 M and 0.6–1.5 M, all in 5 M urea, 1 mM sodium phosphate (pH 6.8). Fractions of 5.8 ml were collected.
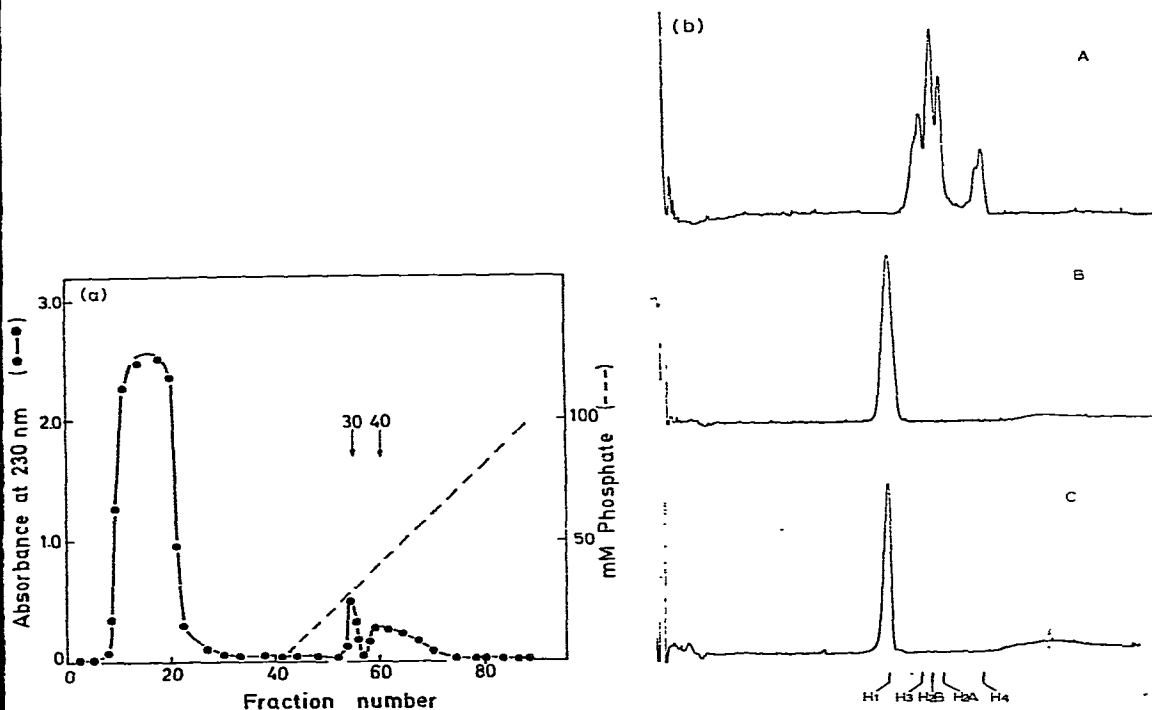
Fig. 3. (a) Chromatography of histones on hydroxyapatite with a gradient of sodium phosphate in 5 M urea, 0.6 M NaCl. Histone (83.9) was dissolved in 5 M urea, 0.6 M NaCl, 1 mM sodium phosphate (pH 6.8) and loaded on a 100-ml hydroxyapatite column. The unadsorbed material was washed out with the same buffer and the column developed with a 1 mM–0.1 M sodium phosphate gradient (130 + 130 ml) in 5 M urea, 0.6 M NaCl. Fractions of 2.9 ml were collected. (b) Electrophoresis of hydroxyapatite column fractions in acidic urea–polyacrylamide gels. (A) Histones, not adsorbed on the column; (B) histone eluted with 30 mM and (C) histone eluted with 40 mM sodium phosphate (pH 6.8) in 5 M urea, 0.6 M NaCl.

sodium phosphate is needed for elution. The lower value found in this study (see Fig. 1) may be explained by the addition of 5 M urea to all buffers.

The basic nature of the histone molecules is due mainly to the amino acids lysine and arginine, which have isoelectric points of 9.74 and 10.76, respectively. Histidine contributes only slightly.

. To investigate the different behaviours of the histones on hydroxyapatite further, the elution molarities of the synthetic polymers poly(L-lysine) and poly(L-arginine) were determined. Table II shows that poly(L-arginine) elutes with 0.12 M NaCl in 5 M urea, 1 mM sodium phosphate, whereas a 10-fold higher NaCl concentration is needed to elute poly(L-lysine). The corresponding sodium phosphate concentrations in 5 M urea are 0.4 and 0.7 M, respectively. These results suggest that it is not the basicity of the molecule that determines the chromatographic behaviour on the column, but rather the ε-NH$_2$ group of lysine binds much more strongly to the phosphate groups of hydroxyapatite than the guanidino group of arginine. This finding fits well with the unexpected observation of Bartley and Chalkley[8] that there is no significant contribution of the hydrogen bonds between arginine and the phosphate groups of DNA towards the strength of DNA–histone binding. The different chromatographic behaviour of lysine and arginine could explain the separation of histones on hydroxyapatite: if the histones are arranged according to the number of lysine residues they contain (Table III), the order obtained is the same as that in which they are eluted from the column.

TABLE II

ELUTION MOLARITIES (mole/l) OF SYNTHETIC POLYPEPTIDES FROM HYDROXY-APATITE COLUMNS

Polypeptide	Eluting solvent	
	NaCl in 5 M urea 1 mM sodium phosphate (pH 6.8)	Sodium phosphate (pH 6.8) in 5 M urea
Poly(L-arginine)	0.12	0.4
Poly(L-lysine)	1.2	0.7

TABLE III

LYSINE CONTENT OF CALF THYMUS HISTONES

Histone	Lysine residues per molecule	Reference
H1 (F1)	59	4
H2B (F2b)	20	9
H2A (F2a2)	14	10
H3 (F3)	13	11
H4 (F2a1)	11	12

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