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A new h.p.l.c. isolation procedure for chicken and goose erythrocyte histones

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Total chicken erythrocyte histones were separated by reversed-phase h.p.l.c. using a multi-step acetonitrile gradient in a very short time (35 min). The proteins were eluted in the following order: H1, H5, H2B, H2A.2, H4, H2A.1 and H3.2. Applying a special gradient system adapted for the separation of very-lysine-rich histones, chicken erythrocyte H5 was resolved into two subfractions. Their electrophoretic mobilities were identical in both SDS and acetic acid/urea/Triton polyacrylamide-gel electrophoresis, but different in free-flow electrophoresis. Amino-acid-sequence analyses revealed that the two components only differ with respect to position 15, one having glutamine in that position and the other arginine. A separation of histones prepared from goose erythrocytes disclosed no H5 subfractionation. Furthermore, histones obtained from anaemic-chicken blood were analysed by the above-mentioned h.p.l.c. conditions. An alteration in the relation of H1 to H5 was detected, but no further differences in the number and quantity of the histones and histone variants were observed as compared with the corresponding proteins processed from normal-chicken blood.

INTRODUCTION

Histones are the basic proteins that are complexed with DNA to form the nucleosome, the fundamental subunit of chromatin in eukaryotes [1]. The nucleosomal core region contains the histone octamer (H2A, H2B, H3, $H4)_2$, whereas the nucleosomal linker region is associated with H1[2]. Four of the five histone classes are composed of several variants, differing slightly in their sequence [3,4]. Nucleated erythrocytes of birds, reptiles, amphibians and fish contain a very basic histone H5 that, to a large extent, replaces histone H1 in the mature erythrocytes [5–9]. H5 may also consist of two variants [10]. $H1^0$, a subfraction of the class of H1 histones, and H5 might have similar functions in their inhibition of DNA synthesis and DNA transcription [11,12]. $H1^0$, H5 and the main class-H1 histones all belong to the same family of very-lysine-rich chromosomal proteins that are all involved in the higher-order chromatin organization [13–15].

The fractionation of the six major histones present in avian erythrocytes by a combination of techniques including selective extraction, oxidation, gel filtration, and ion-exchange chromatography is a complicated and time-consuming procedure [16]. The further purification and separation of H5 into both its variants by conventional ion-exchange chromatography can take several days [10,17]. Recently, we have described rapid and simple methods for separating calf thymus H1 and core histones by reversed-phase h.p.l.c. [18,19].

The present paper demonstrates a rapid h.p.l.c. separation of total histones, of the very-lysine-rich histones and of two H5 subfractions of both normal- and anaemic-chicken blood. In separating the very-lysine-rich histones from goose erythrocytes, no H5 subfractionation could be observed. Furthermore, histones and histone variants of mature and immature erythrocytes were compared.

MATERIALS AND METHODS

Chemicals

H.p.l.c. grade acetonitrile (type S) was purchased from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.), the water was from Promochem (Wesel, Germany). The trifluoroacetic acid (TFA), formic acid, CNBr and phenylhydrazine hydrochloride were obtained from Sigma Chemie (Munich, Germany).

Preparation of histones

Adult White Leghorn chickens (about 1 kg body wt.) were made anaemic by subcutaneous injection of 8 mg of phenylhydrazine hydrochloride (dissolved in 0.15 M-NaCl, pH 6.7) on each of five consecutive days as described [20]. The course of the induced anaemia was monitored by the haematocrit. The anaemic chickens were killed on day 6 and their blood was collected by cardiac puncture. Clotting was prevented by the presence of trisodium citrate [10 ml of 3.8% (w/v) trisodium citrate/100 ml of blood]. Nuclei were prepared by the method of Weintraub [21], with minor modifications. After centrifugation at 800 *g* for 5 min, the cells were washed three times in buffer A [0.14 M-NaCl/0.01 M-Tris/HCl (pH 7.1)/0.015 M-trisodium citrate/0.1 mM-phenylmethanesulphonyl fluoride/1 mM-2-mercaptoethanol] and then lysed in five times their volume of buffer B [0.5% Nonidet P40/0.01 M-NaCl/0.005 M-$MgCl_2$/0.01 M-Tris/HCl (pH 7.4)/0.1 mM-phenylmethanesulphonyl fluoride/1 mM-2-mercaptoethanol]. Nuclei thus obtained were collected by centrifugation at 1800 *g* for 10 min, and the nuclear pellet was washed

Abbreviations used: TFA, trifluoroacetic acid; PAGE, polyacrylamide-gel electrophoresis; AUT, acetic acid/urea/Triton; AU, acetic acid/urea; a.u.f.s., absorbance units full scale.

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once with buffer B and twice with buffer B without Nonidet P40. The pellet was centrifuged and thereupon treated with 0.35 M-NaCl in order to remove the high-mobility-group proteins. After being stirred for 30 min at 4 °C, it was centrifuged for 30 min at 25000 *g* at 4 °C.

Whole histones were then extracted from the pellet with 5 vol. of 0.2 M-H_2SO_4 for 1 h and the histones obtained as described by Gurley *et al.* [22], except that the protein precipitation was performed with 20% (w/v) trichloroacetic acid. For the preparation of the very-lysine-rich histones (H1 + H5), instead of the 0.2 M-H_2SO_4 treatment the pellet was extracted twice with 5% (w/v) $HClO_4$ for 30 min, precipitated with 20% (w/v) trichloroacetic acid and washed twice with cold acidified acetone and three times with pure acetone. Blood of normal White Leghorn chickens and of geese was obtained by withdrawal from the wing vein. Cells, nuclei and histones were prepared as outlined above.

H.p.l.c.

All h.p.l.c. experiments were performed on a Beckman h.p.l.c. gradient system using two 114 M pumps and a 421 A system controller. The eluent was monitored by the absorbance at 210 nm with a model 165 variable-wavelength u.v./visible-light detector. The detector signal was documented on a Shimadzu C-R3A integrator utilizing an automatic baseline correction.

All histone separations were performed on a Beckman Ultrapore C_8 column (4.6 internal diameter × 75 mm; 5 μm particle size; 30 nm pore size; end-capped). The freeze-dried histones were dissolved in water containing 0.1% TFA, and samples of 2.5–15 μg were injected for analytical runs. For semi-preparative runs, 40–150 μg of histones were applied to the column.

The total histones were chromatographed within 35 min at room temperature and at a constant flow of 0.5 ml/min, using a multi-step acetonitrile gradient starting at 73% (v/v) A/27% B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile). The concentration of solvent B was increased linearly in the following order: from 27 to 32% B (during 6 min), from 32 to 38% (2 min), from 38 to 39% (15 min) and from 39 to 50% B (8 min). The isocratic conditions (50% B) were continued for an additional 4 min. The very-lysine-rich histones were separated within 35 min at room temperature and at a constant flow of 0.5 ml/min, running a linear gradient from 74% A/26% B to 66% A/34% B over 45 min.

Gel electrophoresis

Histone fractions from the h.p.l.c. runs were collected, freeze-dried and stored at −20 °C. The histones were identified by using both SDS/PAGE by the method of Laemmli [23] (15% polyacrylamide/0.1% SDS) and AUT/PAGE as described by Zweidler [24] [12% (w/v) polyacrylamide/0.9 M-acetic acid/8 M-urea/0.37% Triton X-100]. The H2A CNBr-cleavage fragments were analysed by AU/PAGE as described by Panyim & Chalkley [25] (15% polyacrylamide/0.9 M-acetic acid/6 M-urea). The gels were stained for 1 h with 0.1% Serva Blue R in 40% (v/v) ethanol/5% (v/v) acetic acid and destained overnight in 20% (v/v) ethanol/5% (v/v) acetic acid.

Free-flow electrophoresis

This was performed as described by Hannig *et al.* [26]. The histones were dissolved in 1 M-acetic acid and 5 μl of a 0.2% solution was injected at a field strength of about 140 $V \cdot cm^{-1}$. The histone bands were detected by their u.v. absorption at 225 nm.

CNBr cleavage

Histone fractions prepared from reversed-phase h.p.l.c. peaks were cleaved by CNBr by the method of Urban *et al.* [3]. The histones (40 μg) were dissolved with a few crystals of CNBr in 55 μl of 99% (v/v) formic acid. After solution the reaction mixture was diluted with 25 μl of doubly distilled water, incubated at room temperature for 24 h, freeze-dried, redissolved in 80 μl of water and freeze-dried again.

RESULTS AND DISCUSSION

To separate avian erythrocyte histones, classical extraction methods [16] and different types of gel electrophoresis have been applied [24]. Both procedures are, however, laborious and, moreover, time-consuming.

As we have already reported, reversed-phase h.p.l.c. may also be used for a rapid separation of calf thymus histones [18]. Several histones and histone variants were fractionated, with the sole exception of H4 from an H2A variant. Under changed analytical conditions, the problem of the H4/H2A separation was successfully resolved [19]. Moreover, these techniques even allow the resolution of certain isoproteins of H1. For the purpose of examining histones of chicken mature and immature erythrocytes, which also contain H2A variants, and in addition to H1 a further very-lysine-rich histone (H5), it appeared promising, therefore, to develop a rapid and sensitive h.p.l.c. separation method.

Fig. 1 demonstrates the separation of the total histones prepared from nuclei of chicken mature erythrocytes

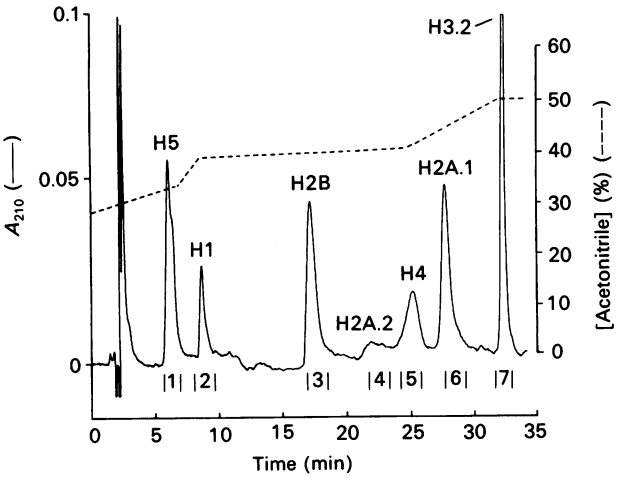

Fig. 1. Separation of histones (15 μg) from chicken mature erythrocytes by reversed-phase h.p.l.c.

The column (4.6 mm × 75 mm) contained Beckman Ultrapore C_8 and the flow rate was 0.5 ml/min using the multi-step acetonitrile gradient as described in the Materials and methods sections. Absorbance was monitored at 210 nm (0.1 a.u.f.s.). In order to obtain fractions 1–7, 150 μg of histones were injected on to the h.p.l.c. column. Numbered fractions were freeze-dried and subjected to SDS/PAGE (Fig. 2*a*) or AUT/PAGE (Fig. 2*b*) analysis.

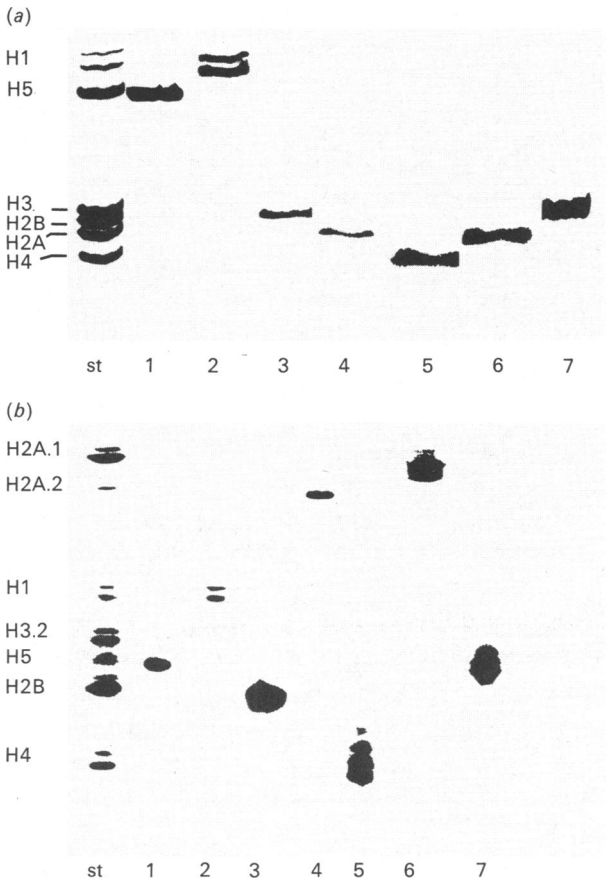

Fig. 2. Gel electrophoresis of fractions 1–7 from the separation described in Fig. 1

(*a*) SDS/PAGE; (*b*) AUT/PAGE; st, total histones (40 μg) from chicken mature erythrocytes. The protein load was 1.5–5 μg (lanes 1–7).

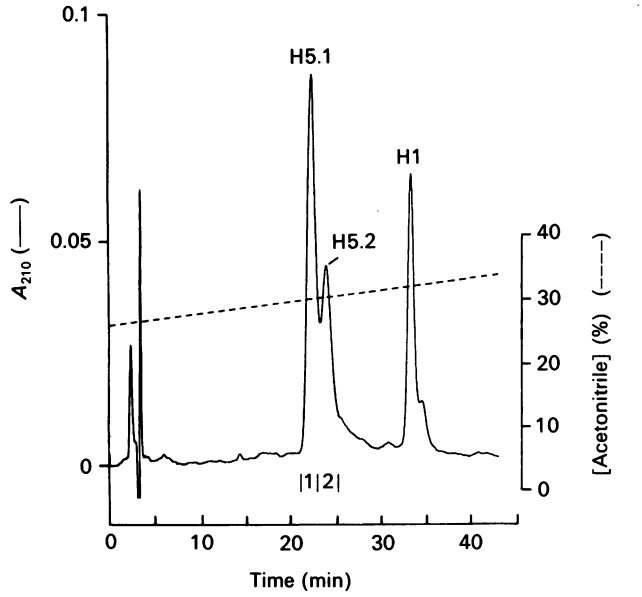

Fig. 3. Separation of the very-lysine-rich histones (7.5 μg) from chicken mature erythrocytes by reversed-phase h.p.l.c.

The column (4.6 mm × 75 mm) contained Beckman Ultrapore C_8. The flow rate was 0.5 ml/min, with a linear gradient from 74% A/26% B (solvent B, 0.1% TFA in acetonitrile) to 66% A/34% B over 45 min. Absorbance was monitored at 210 nm (0.1 a.u.f.s.). In order to obtain fractions 1 and 2, 40 μg of histones were injected on to the reversed-phase h.p.l.c. column. Numbered fractions were freeze-dried and subjected to SDS/PAGE (Fig. 4*a*), AUT/PAGE (Fig. 4*b*) and free-flow electrophoresis (Figs. 6*a* and 6*b* respectively).

using an Ultrapore C_8 column. The seven peaks, obtained within 35 min, were identified by gel electrophoreses and CNBr cleavage.

Electrophoretic analysis of each individual fraction by SDS/PAGE (Fig. 2*a*) has led to the following assignment: fraction no. 1, histone H5; no. 2, H1; no. 3, H2B; no. 4, H2A; no. 5, H4; no. 6, H2A; no. 7, H3. To characterize the histones with regard to variants, the fractions were further analysed by AUT/PAGE (Fig. 2*b*) with the following result: fraction 4 most likely contains H2A.2; fraction 6, H2A.1; and fraction 7, H3.2. The correct assignment of H2A.1 and H2A.2 was also proved by CNBr cleavage, since only H2A.2 contains methionine [3]. As expected, fraction 6 (H2A.1) was resistant, whereas fraction 4 (H2A.2) was cleaved by CNBr. This was examined by AU/PAGE (gel not shown). Other well-known histone variants (H3.1 and H3.3) were not observed in our h.p.l.c. system. Gel-electrophoretic studies by other authors [3] have shown that H3.1 is totally absent, and H3.3 is found in negligible amounts in chicken erythrocytes. In accordance with this, in our histone preparation we could not even detect traces of either H3.1 or H3.3.

It has been proposed that H5 may play an essential part in repression of transcription [27,28]. Moreover, H5 is one of the histones which has been most thoroughly examined. In addition to known sequences [29,30], there are several studies on the secondary and tertiary structures of these proteins and conformational changes [31–33]. A smaller, but nevertheless clearly discernible, shoulder in the H5 peak (shown in Fig. 1) prompted us therefore to examine the very-lysine-rich histones by a gradient system specifically developed for this purpose. Under these conditions separation of H5 into two subfractions, H5.1 and H5.2, was possible (Fig. 3). In order to exclude artificial h.p.l.c. effects, the separation also was performed by applying different eluent systems and column types. Moreover, rechromatography showed, in spite of some inevitable cross-contamination, that the two components retained their chromatographic identities (results not shown). Furthermore, both H5 subfractions were analysed by SDS/PAGE (Fig. 4*a*) and AUT/PAGE (Fig. 4*b*); however, no differences in their electrophoretic mobilities could be observed. With considerable experimental effort and in a time-consuming procedure, Greenaway & Murray [10] were able to separate, albeit incompletely, two chicken erythrocyte H5 histones designated 'HVa' and 'HVb', which differ from one another only by one amino acid out of a total of 189. In position 15 glutamine is replaced by arginine. In order to ascertain whether the fractions in question concerned HVa and HVb, the very-lysine-rich histones from goose erythrocytes, whose H5 reveals no polymorphism [29], were examined first by h.p.l.c. (Fig. 5). Indeed, only a single peak was obtained in this case. The sequence of H5 from goose erythrocytes is only partially comparable with that of chicken; therefore,

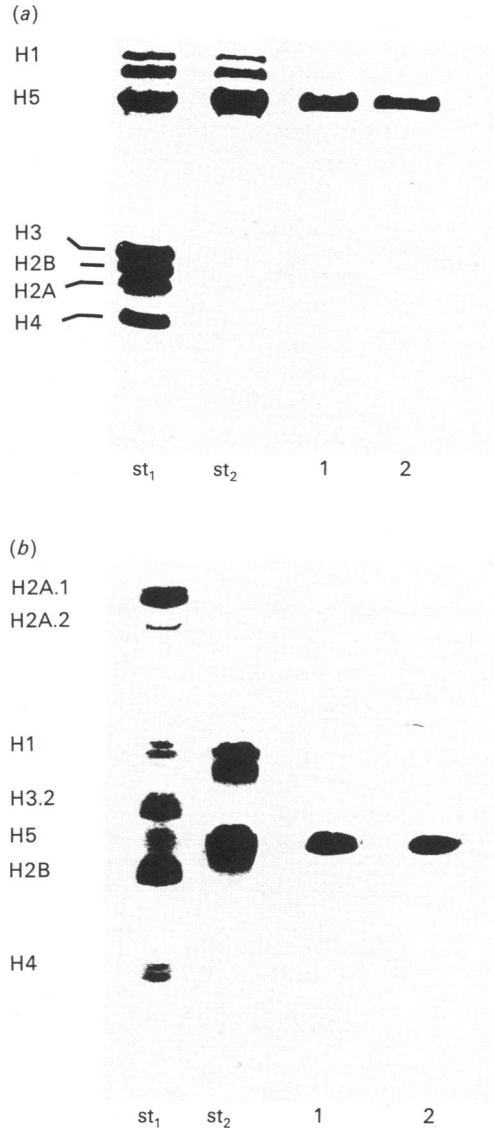

Fig. 4. Gel electrophoresis of the fractions 1 and 2 separated with the h.p.l.c. system used in Fig. 3

(*a*) SDS/PAGE; (*b*) AUT/PAGE; st_1, total histone (40 μg) from chicken mature erythrocytes; st_2, very-lysine-rich histones (7.5 μg) from chicken mature erythrocytes. The protein load was 2 μg (lanes 1 and 2).

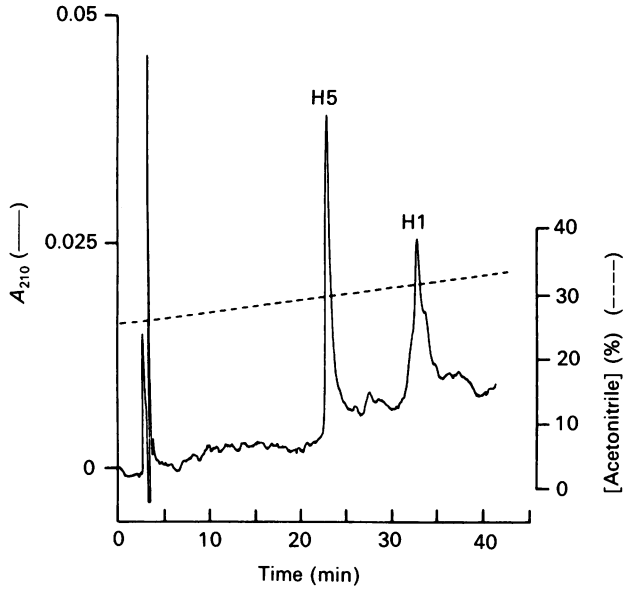

Fig. 5. Separation of the very-lysine-rich histones (2.5 μg) from goose mature erythrocytes by reversed-phase h.p.l.c.

The experimental conditions were the same as for Fig. 3.

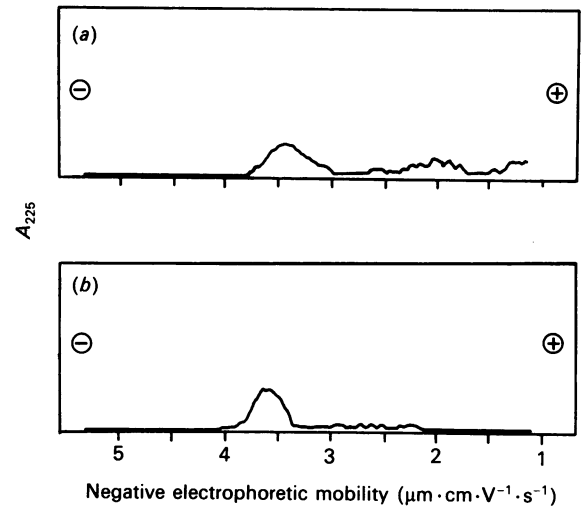

Fig. 6. Free-flow electrophoresis of fractions 1 (*a*) and 2 (*b*) from the separation described in Fig. 3

The freeze-dried histone fractions were dissolved in 1 M-acetic acid, and 5 μl of a 0.2% solution was injected at a field strength of 140 $V \cdot cm^{-1}$. The proteins were detected by u.v. absorption at 225 nm.

H5.1 and H5.2 were further analysed by free-flow electrophoresis. The single charge difference between the two proteins resulting from this amino acid substitution may suggest dissimilar electrophoretic mobilities. In fact H5.1 (Fig. 6*a*) reveals a negative electrophoretic mobility of 3.48 as compared with 3.67 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$ in case of H5.2 (Fig. 6*b*). In view of the higher negative electrophoretic mobility (according to a higher positive charge of the protein), it is assumed that fraction H5.2 should contain arginine at position 15 and H5.1 glutamine respectively. The sequence analyses of rechromatographed proteins derived from the fractions H5.1 and H5.2 (the former containing roughly 90% glutamine, whereas the latter largely consists of arginine at position 15) confirmed this result.

On the one hand it is difficult to envisage that the sole replacement of glutamine by arginine could by itself constitute an adequate ground for the fractionation of this protein mixture by reversed-phase h.p.l.c. On the other hand, a separation of different conformations possibly conditioned by the substitution of glutamine could well be envisaged.

Using this method we sought to compare histones from anaemic-chicken blood (Fig. 7) with histones from chicken mature erythrocytes (Fig. 1). In the region of the core histones, no particular differences were observed, whereas the mutual relation of H1 to H5 changed (Table 1). However, the relation of H5.1 to H5.2 from chicken

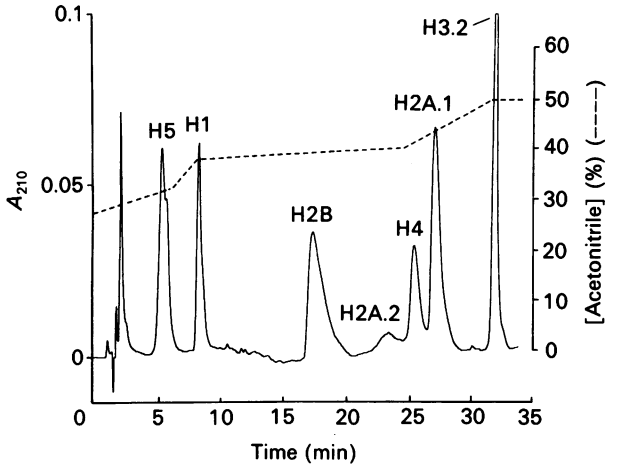

Fig. 7. Separation of histones (15 μg) from chicken immature erythrocytes by reversed-phase h.p.l.c.

H.p.l.c. conditions same as Fig. 1.

Table 1. Peak area proportion (% of total) of 210 nm of H1 and H5 histones from chicken mature and immature erythrocytes

Results are given as means ± S.E.M.

H.p.l.c. peak	Peak area proportion (%)	
	Mature erythrocytes ($n = 7$)	Immature erythrocytes ($n = 4$)
H1	23.65 ± 0.64	37.44 ± 0.33
H5	76.35 ± 0.64	62.56 ± 0.35

mature (Fig. 3) and immature erythrocytes (chromatogram not shown) remained unchanged.

We were able to demonstrate that avian erythrocyte histones could be separated by reversed-phase h.p.l.c. not only in an extremely short time, but also very efficiently as compared with the methods so far used. Furthermore, two H5 fractions were obtained. The amino-acid-sequence analyses permit the conclusion that, under the conditions given, two proteins of high purity (90%) could be isolated, whose content differs only with regard to one single amino acid.

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