A Modified Procedure for Fractionating Histones

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A method is described, which is capable of fractionating histones obtained from any animal source into five major groups. Although the method is based on procedures initially developed by E. W. Johns (1964), involving differential solubility in solutions of acids and of ethanol, it gives a cleaner separation and possesses the considerable advantage that the starting material is whole histone rather than a nucleoprotein preparation.

Various chromatographic or electrophoretic procedures have been devised to produce large amounts of a specific fraction of histone (Kinkade & Cole, 1966; Fambrough & Bonner, 1969; Iwai & Senshu, 1969; Mauritzen *et al.*, 1966), but application of the operations to the separation of all the histone fractions from a given creature or tissue would be time-consuming. The most popular method for large-scale fractionation of all the histone groups is that devised by E. W. Johns (Method 1 of Johns, 1964; Phillips & Johns, 1965). This approach is based on differences in the solubility of the various histone fractions in different aqueous organic environments. The main drawbacks of this method are that it is based on the wet weight of starting tissue (Johns, 1964), which gives very variable yields of histones, that it is best applied to rather large amounts of tissue, and finally that the various fractions are sometimes appreciably cross-contaminated (Panyim & Chalkley, 1969; McGillivray, 1968; Laurence *et al.*, 1966).

We have modified the Johns (1964) method so that it can be applied to isolated whole histone samples (which would permit us to stockpile histones from creatures that give only small yields of nucleoprotein, such as *Drosophila*). Our scheme is applicable to small or large quantities of histone, and fractions can be obtained in a high degree of purity (98%) as measured by microdensitometer analysis of the electrophoretic band patterns.

The results presented show that the five major fractions of histone from a number of vertebrates and from an invertebrate (*Drosophila melanogaster*) were efficiently separated by the modified method. The method was unsuccessful when applied to histones isolated from one plant source, the pea.

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Materials and Methods

Histone was isolated from chromatin by methods previously described by Panyim *et al.* (1971). Ethanol-HCl refers to a solution containing 10ml of conc. HCl (sp.gr. 1.18), 90ml of water and 400ml of anhydrous ethanol.

The following method (outlined in Scheme 1) utilizes volumes and quantities appropriate for processing 500mg of whole histone. The method can be scaled down directly to as little as 10–15mg of histone as described below for *Drosophila*. All manipulations are performed at 0°C unless otherwise stated. This is of prime importance in minimizing cross-contamination of the various fractions.

Isolation of histone fractions

Histone fraction F1. Whole histone (500mg) was dissolved in 1mM-HCl (100ml), the solution was centrifuged at 12000g for 10min and the supernatant containing the histone collected. Sufficient concentrated HClO₄ was added (dropwise with shaking) to make the final solution 0.5M in HClO₄. After standing for 15min, the solution was centrifuged at 12000g for 5min and the pellet was kept. The supernatant, containing histone F1, was treated with 3M-H₂SO₄ to a final concn. of 0.2M, followed by precipitation with a 6vol. excess of acetone. The solution was stored overnight at –14°C before collection of the histone F1 pellet. If the starting material was 30mg or less, the first pellet was washed with 0.25vol. of 0.5M-HClO₄ and combined with the initial histone F1 supernatant.

Histone fraction F2b. The histone insoluble in 0.5M-HClO₄ was collected and dissolved in 1mM-HCl (100ml). Conc. HClO₄ was added to a final concn. of 0.5M and after 15min the solution was centrifuged at 12000g for 5min. The supernatant was rejected. The pellet containing histones F2b, F3

Scheme 1. Summary of method for the extraction of histone groups from whole histone

For details see the text. Abbreviations are: ethanol-HCl, 80% ethanol-0.26M-HCl; ethanol, anhydrous ethanol; acetone-H₂SO₄ precipitation or ethanol-H₂SO₄ precipitation refers to a solution being dialysed against 50 vol. of 0.2M-H₂SO₄ and then precipitated by 4 vol. of acetone or ethanol, respectively; recycled refers to the following series of steps: the ethanol-HCl pellet is dissolved in 1 mM-HCl, precipitated with a final concn. of 0.5 M-HClO₄ and the pellet extracted in ethanol-HCl.

and F2a (F2a refers to the combined histone fractions F2a1 and F2a2) was washed once with 50 ml of 0.5M-HClO₄ and then extracted into 60 ml of ethanol-HCl (with a glass homogenizer) and left for 10 min. After centrifugation, the supernatant containing histones F3+F2a was kept and the pellet again dissolved in 1 mM-HCl and treated with 0.5M-HClO₄ as described above. The pellet was extracted into 40 ml of ethanol-HCl. The aim at this stage is to extract as much of histones F2a and F3, while extracting as little histone F2b, as possible. The key factors for obtaining pure histone F2b and histone fractions F2a+F3, with as little cross-contamination as possible, are to extract at 0°C, to use small volumes of ethanol-HCl and to keep the time of contact of histone F2b and ethanol-HCl to a minimum. The histone F2b pellet after six cycles was washed with acetone and dried *in vacuo*.

Histone fractions F2a+F3. The supernatants from the first three histone F2b washings with ethanol-HCl contained histones F2a+F3 contaminated with a variable amount of histone F2b. It is highly advantageous to remove all traces of histone F2b before fractionating histones F2a+F3. To do this, the histones F2a+F3 were recycled by precipitation from the ethanol-HCl extracts and by extracting the pellet into fresh ethanol-HCl as described in the flow chart (Scheme 1). Pure histones F2a+F3 were then obtained by precipitation with acetone-H₂SO₄ (i.e. treatment of the supernatant with 3M-H₂SO₄ to give a final concn. of 0.2M, followed by precipitation with 4 vol. of acetone), washing with acetone and drying *in vacuo*. It is essential that the pellet is dried before the next step, otherwise the presence of acetone will give artificially high E_{230} readings.

Histone fraction F3. Histone F2a+F3 was dissolved in 60 ml of ethanol-HCl (if it did not dissolve completely, it was redissolved in 1 mM-HCl, precipitated by 0.5M-HClO₄ and the pellet extracted in an additional 20 ml of ethanol-HCl). The E_{230} of the solution was recorded and adjusted to $E_{230} = 6$ (1.8 mg/ml) with ethanol-HCl. The solution was dialysed against 7 vol. of anhydrous ethanol for 5 h with vigorous stirring. The contents of the dialysis bag were removed, centrifuged at 12000g for 5 min and the pellet (histone F3) was washed once with the supernatant of the dialysis (96.7% ethanol), four times with acetone and dried.

Histone fraction F2a. The supernatant from the dialysis precipitation above was taken and dialysed overnight against 7 vol. of ethanol. If a small precipitate developed, it could be discarded. Fractions F2a1 and F2a2 were precipitated in acetone-H₂SO₄, washed once with acetone and then dried.

Histone fractions F2a1 and F2a2. The method described previously (Johns, 1964) requires considerable recycling to attain the required purity (98%), with an attendant loss in yield. A direct and complete

separation of histone F2a into its two components can be effected by gel-filtration techniques. We have found a column length of 38 cm to be adequate; the diameter of the column should be adjusted to accommodate an appropriate amount of gel, computed from the requirement that 10 g of dry gel are used for every 8 mg of total histone F2a. The methods we use for separating 12.5 mg of histone F2a are as follows.

A batch (15 g) of Bio-Gel P-100 (100–200 mesh, Bio-Rad Laboratories, Richmond, California, U.S.A.), swollen overnight in 375 ml of 10 mM-HCl, was packed in a column (2.5 cm × 38 cm) and equilibrated with 250 ml of 10 mM-HCl. The histone was applied to the top of the column in a total vol. of 0.5–1.0 ml in 10 mM-HCl. The flow rate was adjusted to 6.5 ml/h under a pressure head of 40 cm of water. The column was run at 4°C. Fractions (1.5 ml) were collected and the histone assayed by its E_{230} . Appropriate fractions were pooled (one is guided in this decision by analysis of various peak fractions on polyacrylamide gels), made 0.2M in H₂SO₄ and after standing for 30 min at 0°C were precipitated in 95% (v/v) ethanol and centrifuged at 12000g for 10 min. The pellets were washed in acetone before being dried *in vacuo*.

Problems and remedies

If the fractionation described above is not satisfactory in a given system, then, typically, certain cross-contamination patterns are more frequently observed than others. These are presented in Table 1 with the appropriate course of action to purify the histone fraction in question.

Application to fractionation of small amounts of histone

This method has been applied to as little as 15 mg of total histone. If the amount of histone is limited, the volumes of all solutions should be scaled down accordingly. It is also advantageous to use glass centrifuge tubes and to homogenize with a microprobe sonicator. Because of the demand for high concentrations in the separation of histone F2a into fractions F2a1 and F2a2 by the classical method of Phillips & Johns (1965), it is particularly advantageous to exploit the gel-filtration method at the terminal stage of separation if one starts with less than 25 mg of whole histone.

Results and Discussion

The results of applying the technique described to histones from human placenta, calf thymus, fish liver, and the fruit fly, *Drosophila melanogaster*, are shown in Plate 1. The fractions are electrophoretically pure and the extent of cross-contamination is less than 2%

Table 1. *Problems and remedies in histone fractionation*

Fraction	Problem	Remedy
F1	Although this is the easiest fraction to purify, sometimes indistinct electrophoretic bands can be seen usually moving faster than histone F1; these are due to proteolysis	If proteolysis unavoidable, the histone F1 is purified on the carboxylic resin Amberlite CG-50. Proteolysis products are eluted ahead of histone F1
F2b	Contamination with histones F2a Contamination with several minor bands of slightly higher mobility than histone F2b, also caused by proteolysis.	Recycling as described in the text is used If proteolysis unavoidable, the histone is purified on Bio-Gel P-100 (eluted in 1 mM-HCl)
F3	Contamination with histone F2a2 Contamination with histone F2b	Solid is dissolved in final solution of 80% ethanol-0.25 M-HCl (0.5 mg/ml) and dialysed against 7 vol. of ethanol. Pure histone F3 is precipitated Dried material is extracted into ice-cold 80% ethanol-HCl (10 mg/ml). Pure histone F3 is dissolved
F2a1	Contamination with histone F2a2 Contamination with histone F3	More Bio-Gel is used or amount of histone applied to column is decreased Higher concentrations are used at dialysis step to precipitate histone F3 more effectively
F2a2	Contamination with histone F2a1	More Bio-Gel is used or less histone is applied to column

as judged by quantitative microdensitometer analysis of the gels. That similarly isolated fractions from different creatures are chemically comparable has been shown by amino acid analysis (Panyim *et al.*, 1971).

That the various recycling operations described in the Materials and Methods section are really necessary is documented in Plate 2, which shows specific fractions at various key stages during the purification. Thus, as has long been known, histone F1 is easily isolated pure from either whole histone (see Plate 2a) or from nucleohistone-starting material (Johns, 1964). Histone F2b (see Plate 2b) is not obtained pure simply by washing with ethanol-HCl; exhaustive washing without redissolving merely results in a constant extent of contamination with histones F2a+F3. Presumably, once histones F2a+F3 have been dissolved from the surface of a histone F2b-enriched solid particle, then the rest of histones F2a+F3 within the interior of the particle is trapped in an insoluble histone F2b matrix. Thus it is necessary to redissolve and reprecipitate after each washing of the histone F2b pellet. The multiple fraction (histones F2a+F3) is invariably initially contaminated with about 10–15% of histone F2b as shown in Plate 2(c). However, the double recycling rapidly and totally removes histone F2b. In other methods of separation, histone F2b was frequently found as a contaminant of histones F3 and F2a1, presumably because some of them had remained at this stage.

The advantage of adjusting the concentration of

Fig. 1. *Separation of the histone F2a fraction on Bio-Gel P-100*

Column chromatography was performed as described in the Materials and Methods section. The fraction vol. was 1.5 ml.

histones F3+F2a before dialysis is that too high a concentration leads to contamination of histone F3 with histone F2a2, whereas too low a concentration leads to pure histone F3 but contamination of histone F2a with histone F3. The important factors in the precipitation of histone F3 are: (1) the concentration of histones F3 and F2a2; histone F3 should not be below 600 µg/ml and histone F2a2 should not be above 700 µg/ml, as the precipitation of both is

EXPLANATION OF PLATE I

Separation of histone fractions from various organisms

For details see the text. The sources used were: (a) *Drosophila melanogaster*; (b) human placenta; (c) calf thymus; and (d) fish liver. Abbreviations are: ox., oxidized form of the histone F3; red., reduced form of histone fraction F3.

EXPLANATION OF PLATE 2

Analysis of various steps in the fractionation

For details see Scheme 1. (a) Isolation of histone F1 from whole histone; (b) isolation of histone F2b from the histones F2b, F2a and F3; (c) purification of combined histone fraction F2a+F3; (d) isolation of histones F3, F2a1 and F2a2 from the combined histone fraction F2a+F3. 1st, 2nd, 3rd and 5th refer to the number of times of recycling.

concentration-dependent; (2) the temperature should be approx. 2°C; and (3) the concentration of ethanol and HCl should be that obtained by dialysis of 1 vol. of 80% (v/v) ethanol–0.25M-HCl against 7 vol. of anhydrous ethanol (i.e., 96.7% ethanol–0.027M-HCl). The ethanol concentration range in which histone F3 is completely precipitated and histone F2a2 barely begins to precipitate is small. The separation of the histone F3+F2a fraction into histones F3 and F2a is shown in Plate 2(d). Histone F2a, consisting of two fractions F2a1 and F2a2, can be separated efficiently by exclusion chromatography as shown in Fig. 1. The final fractionation of histone F2a is also shown in Plate 2(d).

The basis for the separation of histone fractions lies essentially in the differential solubility of the various histone perchlorates in aqueous acid and of the histone chlorides in ethanol. The order of solubility of the perchlorates is $F1 \gg F2b = F2a = F3$ and of the chlorides is $F1 < F2b \ll F3 < F2a2 < F2a1$. The fact that the arginine-rich histones are less soluble in $HClO_4$ than the lysine-rich histone F1, despite the reverse order of solubility for the chlorides in aqueous ethanol, may be related to the greater polarizability of the charge spread over the guanidino groups than of the charge on the ϵ -amino groups of lysine, and to a consequently greater affinity of arginine residues for the polarizable ClO_4^- ion.

The method described works well with all animal histones that we have tried, including many not reported in the present paper. However, when we attempted to apply this approach to a plant tissue we were unsuccessful. $HClO_4$ did not give the clean separation of histone F1 with plant histones that one finds with animal histones. Substantial amounts of histones F2b and F2a2 were extracted into 0.5M- $HClO_4$ from whole pea histone. Since these two fractions from peas migrate substantially more slowly

than the comparable animal histone fractions, one might question their assignment as histones F2b and F2a2. However, they are eluted from cation-exchange resins by guanidine hydrochloride gradients at approximately the same position as their animal counterparts (Fambrough & Bonner, 1966). The chemical reason for the failure of the separation system in the pea remains to be resolved. For the animal kingdom, however, the approach described has been satisfactory with either large or small amounts of whole histone.

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