A NEW PROCEDURE FOR THE ISOLATION AND FRACTIONATION OF HISTONES

Deneys R. VAN DER WESTHUYZEN and Claus VON HOLT
Department of Biochemistry, University of Cape Town, South Africa

Received 15 March 1971

1. Introduction

The usual method for the isolation of histones, involving the extraction of histones from chromatin with dilute sulphuric or hydrochloric acid, may result in the denaturation of various chromosomal components due to the extreme pH conditions. The ability of histones to regenerate the super-coil configuration of native nucleohistone is sometimes reduced [1, 2] by the acid extraction technique. Evidence has been presented [3] that histones to some extend bind to DNA during acid treatment and form part of the so-called non-histones. The present investigation was undertaken to develop a procedure for the isolation and fractionation of histones under mild pH conditions.

Nucleoproteins dissociated at high ionic strength can be separated into protein and DNA by ultracentrifugation [4–9] or gel filtration [8–10], but we have found these procedures unsuitable for the large scale isolation of histones. The method for histone isolation presented here is based on the finding of Mirsky and Ris [11] that protamine added in vitro to nucleoprotein directly displaces histones.

A procedure which avoids extreme pH conditions is described for the large scale isolation and partial fractionation of calf thymus histones. Histones, virtually completely displaced from deoxyribonucleoprotein by protamine, and isolated by exclusion chromatography, appear to be almost identical on disc electrophoresis to histones prepared by acid extraction of deoxyribonucleoprotein. Histones are fractionated by gel filtration and ammonium sulphate precipitation into lysine-rich histones (F1), arginine-rich histones (F3 + F2a1), and slightly lysine-rich histones F2a2 and F2b.

2. Materials and methods

Protamine sulphate, grade 1 (Sigma), contaminated with histones, was purified by cooling a 2% protamine solution from room temperature 4°. The bulk of protamine, which settled out as a sticky precipitate, was freeze-dried and taken as purified protamine. Calf thymus deoxyribonucleoprotein was prepared by direct extraction of cells with dilute saline solutions according to the method of Busch [12] except that diisopropyl-fluorophosphate was replaced with 0.05 M sodium bisulphite as a protease inhibitor [13, 14] throughout all steps. All procedures for the isolation and fractionation of histones were carried out at 4° unless otherwise stated.

Histones were displaced from deoxyribonucleoprotein by protamine and isolated as follows: Deoxyribonucleoprotein was dissolved at a concentration of 4 mg/ml in 2.0 M NaCl, 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0). Protamine was dissolved at a concentration of 20 mg/ml in a separate volume of the same buffer at room temperature. Equal volumes of the two solutions were mixed together and dialyzed against 0.15 M NaCl, 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0) until the DNA was completely precipitated as a deoxyribonucleoprotamine complex. The precipitation of DNA was considered to be complete when no typical nucleic acid absorption in the UV spectrum could be detected in the supernatant. The suspension was centrifuged and the supernatant, containing histone and the excess protamine, was concentrated by ultrafiltration through a UM10 membrane (Amicon). The concentrate was then frac-

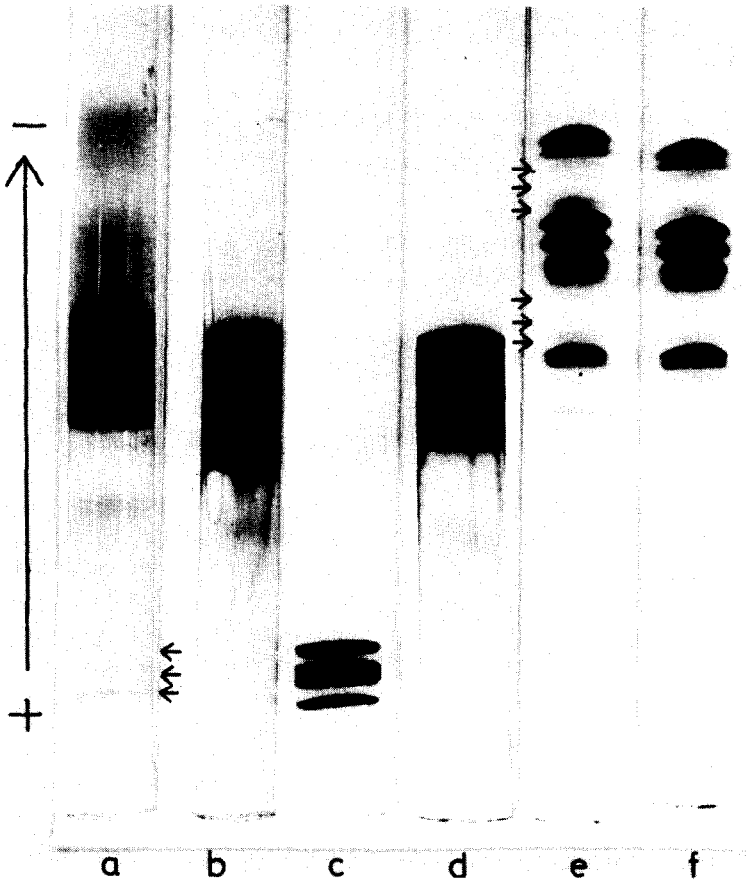

Fig. 1. Polyacrylamide gel electrophoretic patterns of histones (20 μg) and protamine (800 μg). 1 hr electrophoresis: (a) protamine sulphate, grade 1 (Sigma), (b) purified protamine, (c) histone fraction from Sephadex G-50, (d) protamine fraction from Sephadex G-50. 3.5 hr electrophoresis: (e) histone fraction Sephadex G-50, (f) 0.4 N H_2SO_4 extracted histones.

tionated into histone and protamine by gel filtration on a column of Sephadex G-50, equilibrated with 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0).

For reference purpose histones were also extracted from deoxyribonucleoprotein with 0.4 N H_2SO_4, dialyzed against deionized water and freeze-dried.

Proteins were analyzed by disc electrophoresis on polyacrylamide gel (15% acrylamide) in 2.5 M urea at pH 2.5 as described by Panyim and Chalkley [15]. All samples were dissolved in 8 M urea–0.5 M β-mercaptoethanol and incubated at room temperature for several hours before electrophoresis. The sample volume was 10–40 μl and 10–20 μg histone or 800 μg protamine were applied. The duration of electrophoresis was 1 hr or 3.5 hr. The nomenclature of Phillips and Johns [16] was used to describe the histone fractions.

Fractionation of whole histone was effected on columns of Sephadex G-100 equilibrated with the same solutions as used to dissolve the sample. The column dimensions used were 2.5 × 100 cm and the flow rate was 15 ml/hr. Ammonium sulphate fractionation of histones was achieved by adding ammonium sulphate to 70% saturation to a solution containing 5 mg/ml protein in 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0) at 4°.

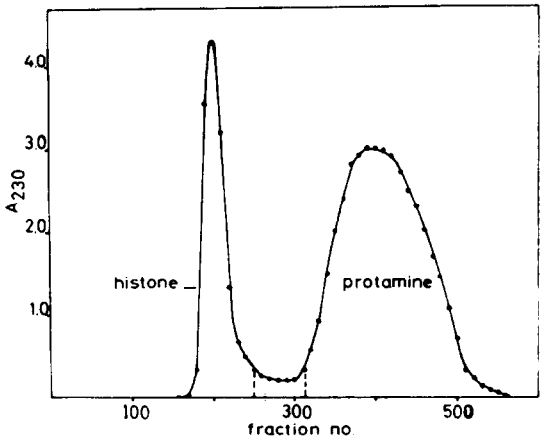

Fig. 2. Gel filtration on Sephadex G-50 of a mixture of histones and protamine obtained after precipitation of DNA by protamine. A 250 ml sample containing 900 mg histone was applied to Sephadex G-50 equilibrated with 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0). Bed dimensions: 14 X 70 cm. Flow rate: 700 ml/hr. Fraction volume: 20 ml.

3. Results and discussion

The electrophoretic pattern of commercial protamine sulphate, grade 1 (Sigma), showed very small amounts of histone-like material (see arrows), while the protamine which precipitated at 4° was found to be electrophoretically pure (fig. 1.)

Histones were completely separated by gel filtration on Sephadex G-100 from the histone and protamine mixture obtained after the precipitation of DNA with protamine (see fig. 2). Up to 3 g of histones could be separated on a 14 X 70 cm bed. On an analytical scale 78% of the protein in deoxyribonucleoprotein was recovered in the histone fraction. The electrophoretic patterns of the two protein fractions eluted from the Sephadex G-50 column are shown in fig. 1. There is no sign of cross-contamination of the peaks and the pattern of the histone fraction after electrophoresis for 3.5 hr is almost identical to that of histone extracted with 0.4 N H_2SO_4 from deoxyribonucleoprotein. Only faint bands (see arrows), indicating slight degradation [14], are visible in the 1 hr and 3.5 hr patterns of the histones isolated by this method. There are no clearly distinguishable non-histones present, neither as slower moving bands nor as protein staining at the origin of the gel. Histones were similarly isolated from chicken erythrocyte deoxyribonucleoprotein prepared by the method of Murray et al. [17], and were shown to be identical to acid-extracted histones from this source. The procedure may therefore be applicable to the isolation of histones from various tissues.

The residual amount of histones which co-precipitated with the DNA and protamine during dialysis was checked by acid extracting the precipitates with 0.4 N H_2SO_4. It was estimated electrophoretically that only a few percent of the most strongly bound histone fractions, F2a1 and F3 [7], and virtually none of the fractions F2a2, F2b and F1 precipitated with the DNA. The separation of histones from DNA were therefore almost complete. If lower protamine concentrations of 3 mg/ml and 1 mg/ml protamine in the final mixture were used, substantially greater amounts of histones, especially F2a1 and F3, co-precipitated with DNA. Increasing the final deoxyribonucleoprotein concentration to 8 mg/ml, at a 10 mg/ml protamine concentration, did not cause a significant increase in non-displaced histone. The displacement of histones from a suspension of chicken erythrocyte deoxyribonucleoprotein has recently been reported [18]. We found however, that the

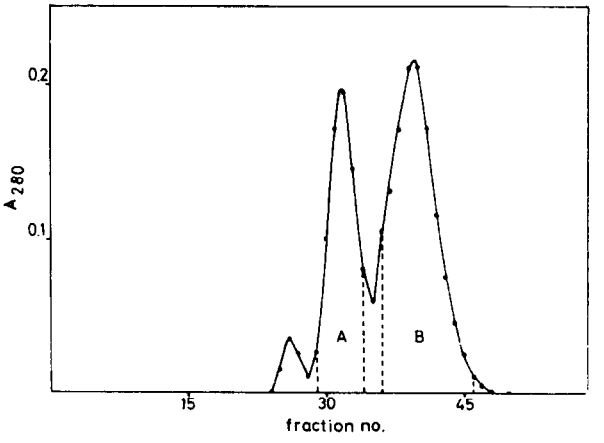

Fig. 3. Gel filtration on Sephadex G-100 of histones from calf thymus. 3 ml 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0) containing 50 mg histone were applied to Sephadex G-100 equilibrated with 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0). Bed dimensions 2.5 X95 cm. Flow rate 15 ml/hr. Fraction volume: 6 ml.

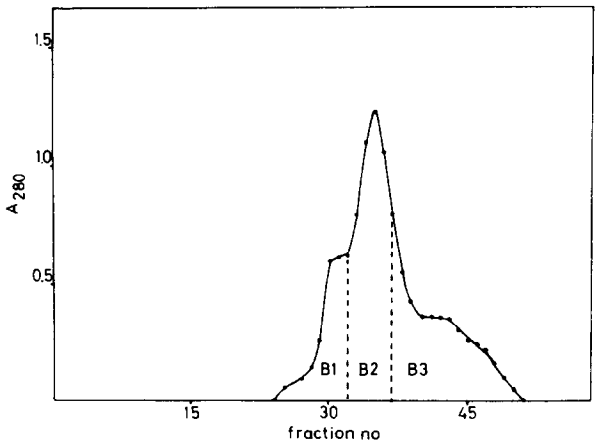

Fig. 4. Gel filtration on Sephadex G-100 of histone fraction B. 30 ml of 0.05 M sodium bisulphite, 0.05 M sodium citrate (pH 4.0) containing 20 mg fraction B were supplied to Sephadex G-100 equilibrated with 0.05 M sodium bisulphite, 0.05 M sodium citrate (pH 4.0). Bed dimensions: 2.5 × 95 cm. Flow rate 15 ml/hr. Fraction volume: 6 ml.

amount of histone displaced by protamine from a fine suspension of calf thymus deoxyribonucleoprotein in 0.10 M NaCl, 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0) during a three hour incubation at 4° was considerably less, especially of histones F2a1 and F3, than that displaced using the method described here.

Chromatography on Sephadex G-100 equilibrated with 0.05 M sodium bisulphite, 0.05 M sodium acetate (pH 5.0), resolved calf thymus histones into two fractions, A and B (See fig. 3). Peak A contained histones F2a1, F3 and F1 and could be further fractionated with ammonium sulphate at 70% saturation. Histones F3 and F2a1 precipitated and histone F1 remained in the supernatant. Histones F2a2 and F2b, which comprised fraction B, could be partially resolved into fractions B1, B2 and B3 by gel filtration on Sephadex G-100 equilibrated with 0.05 M sodium bisulphite, 0.05 M sodium citrate (pH 4.0) (fig. 4).

The electrophoretic patterns of the fractionated histones are shown in fig. 5. The procedure appears to be highly efficient for the separation of almost uncontaminated arginine-rich (F3 + F2a1), slightly lysine-rich (F2a2 + F2b) and lysine-rich (F1) histones. Histones F2b and to a lesser degree F2a2 can be obtained as reasonably pure fractions. This procedure, which fractionates acid extracted and protamine displaced histones similarly, appears to compare quite favourably to the often employed and more complicated fractionation on Amberlite CG-50 using a quanidinium chloride gradient [19].

The separation of histones by gel filtration is complicated by their tendency to form aggregates of varying molecular weights [20]. Minimum aggregation is observed in the extreme pH range between pH 1.0 and pH 3.0 [21–23] and at very low ionic

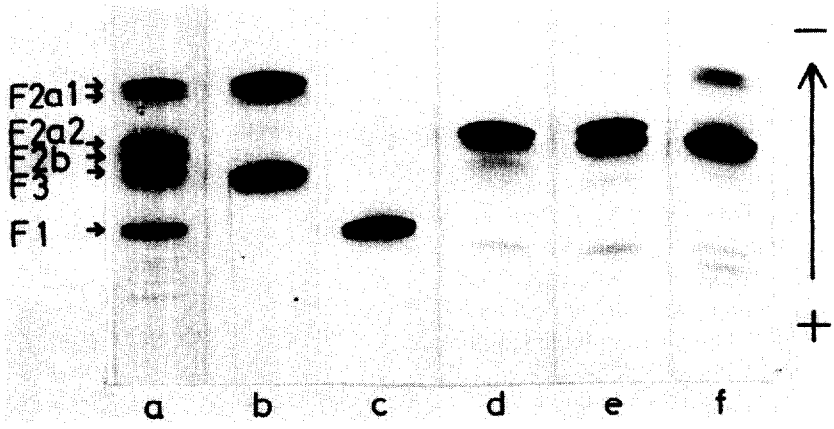

Fig. 5. Polyacrylamide gel electrophoresis of the products of fractionation of calf thymus histones. 3.5 hr electrophoresis: (a) 0.4 N H_2SO_4 extracted histones, (b) precipitate from fraction A, at ammonium sulphate saturation 0.7 (F2a1 + F3), (c) supernatant (F1), (d) fraction B1 (F2a2), (e) fraction B3 (F2a2 + F2b), (f) fraction B3 (F2b).

concentrations [24]. Although considerable aggregation of certain fractions exists between pH 4–5, a satisfactory group separation of histones has been achieved avoiding conditions which may lead to irreversible conformational changes of the histones.

Acknowledgements

We thank the Council for Scientific and Industrial Research (South Africa) for their support, and gratefully acknowledge grants from the University of Cape Town Staff Research Fund and the Fourcade Bequest. One of us (D.R. v.d. W.) thanks the C.S.I.R. for a postgraduate bursary.

References

[1] J. Bonner and B.M. Richards, unpublished observation referred to by J. Bonner and D.Y.H. Tuan in: Structural Chemistry and Molecular Biology, eds. A. Richard and N. Davidson (Freeman, San Francisco, London, 1968) p. 417.
[2] B.M. Richards, J.F. Pardon and E. Hirst, Biochem. J. 117 (1970) 59 P.
[3] J. Sonnenbichler and P. Nobis, European J. Biochem. 16 (1970) 60.
[4] J. Bonner, R.C. Huang and N. Maheshwari, Proc. Natl. Acad. Sci. U.S. 47 (1962) 1548.
[5] G. Giannoni and A.R. Peacocke, Biochim. Biophys. Acta 68 (1963) 157.
[6] G.P. Georgiev, L.N. Ananieva and J.V. Kozlov, J. Mol. Biol. 22 (1966) 363.
[7] H.H. Ohlenbusch, B.M. Olivera, D. Tuan and N. Davidson, J. Mol. Biol. 25 (1967) 299.
[8] R.D. Bauer and R. Johanson, Biochim. Biophys. Acta 199 (1966) 48.
[9] J.E. Loeb, Biochim. Biophys. Acta 145 (1967) 427.
[10] J.E. Loeb, Biochim. Biophys. Acta 182 (1969) 225.
[11] A.E. Mirsky and H. Ris, J. Gen. Physiol. 34 (1951) 475.
[12] H. Busch in: Methods in Enzymology, Vol. 128, ed. H. Grossman (Academic Press, New York, 1968) p. 68.
[13] S. Panyim, R.H. Jensen and R. Chalkley, Biochim. Biophys. Acta 160 (1968) 252.
[14] J. Bartley and R. Chalkley, J. Biol. Chem. 245 (1970) 4286.
[15] S. Panyim and R. Chalkley, Arch. Biochem. Biophys. 130 (1969) 337.
[16] D.M.P. Phillips and E.W. Johns, Biochem. J. 94 (1965) 127.
[17] G. Murray, G. Vidali and J.M. Neelin, Biochem. J. 107 (1968) 208.
[18] K. Evans, P. Konigsberg and R.D. Cole, Arch. Biochem. Biophys. 141 (1970) 389.
[19] P. Rasmussen, K. Murray and J. Luck, Biochemistry 1 (1962) 79.
[20] P.F. Davison, D.W.F. James, K.V. Shooter and J.A.V. Butler, Biochim. Biophys. Acta 15 (1954) 415.
[21] P.F. Davison and K.V. Shooter, Bull. Soc. Chim. Belg. 65 (1956) 85.
[22] E.W. Johns, J. Chromatog. 33 (1968) 563.
[23] J.H. Diggle and A.R. Peacocke, FEBS Letters 5 (1968) 329.
[24] P.A. Edwards and K.V. Shooter, Biochem. J. 114 (1969) 227.