A rapid and gentle method for the salt extraction of chromatin core histones H2A, H2B, H3 and H4 from rat liver nuclei

M. JAMALUDD1N, MOHANAN PHILIP and H. SHARAT CHANDRA

Microbiology and Cell Biology Laboratory, ICMR Unit for Genetics and Cell Biology and Centre for Theoretical Studies, Indian Institute of Science, Bangalore-560 012

Abstract. A complex of histones H2A, H2B, H3 and H4 has been isolated from purified rat liver nuclei by a method which is both gentle and rapid. Nuclei were homogenised in 0·25 M sucrose and the residual nuclear material obtained after centrifligation was adsorbed on calcium phosphate gel. After removing histone H1 from the adsorbed material by washing with 1M NaCl in 25 mM sodium phosphate buffer, pH 6·0, histones H2A, H2B, H3 and H4 were eluted together, with 2 M NaCl in 25 mM sodium phosphate buffer, pH 7·0. The core histones so obtained migrated as a single sharp band on polyacrylamide gel electrophoresis under non-denaturing conditions. Fractionation of the freshly prepared core histones on a Sephadex G-100 column yielded two major protein peaks. The peak having the larger elution volume contained histones H2A and H2B in equal amounts while the peak with the smaller elution volume contained all the four histones. Histones H3 and H4 were present in larger proportions in the second peak.

Introduction

The conformational properties of the inner histones, H2A, H2B, H3 and H4, displaced from chromatin and brought into solution at high ionic strength (2M NaCl) and at neutral pH, appear to be similar to those assumed by these histones in intact chromatin (Weintraub *et al.,* 1975; Thomas *et al.,* 1977). However, there is disagreement as to the nature of the complexes formed by the histones under these conditions. Thomas and Butler (1977) have recently provided hydrodynamic and cross-linking evidence for an octameric species of the above histones at pH 7.0 as well as at pH 9.0. This is consistent with the earlier interpretation of Thomas and Kornberg (1975) about the nature of the histone complex in 2M NaCl. On the other hand the data of Weintraub *et al.* (1975) and others (Campbell and Cotter, 1976; Lilley *et al.,* 1977) have led them to conclude that the stable histone species in 2M NaCl is a heterotypic tetramer made up of one each of the inner histones. Recent hydrodynamic data of Chung *et al.* (1978) suggest an

equilibrium between a heterotypic tetramer, (H2A.H2B.H3.H4), and an octamer, (H2A.H2B.H3.H4)$_2$.

When histones H2A, H2B, H3 and H4 are simultaneously present in solution, in the absence of DNA, they constitute interacting systems of heterocomplexes (Skandrani *et al.*, 1972; D'Anna and Isenberg, 1974a; Kelley, 1973; Roark *et al.*, 1974; 1976; Kornberg and Thomas 1974; Sperling and Bustin 1975; 1976; Hyde and Walker, 1975). The equilibria in such a system are governed by pH, ionic strength, temperature and protein concentration (Sperling and Bustin, 1975). At low ionic strength and neutral pH, the strength of interaction of the different histone pairs varies in the order H3/H4 > H2B/H4 ≃ H2A/H2B > H3/H2A (D'Anna and Isenberg, 1974b). The H3/H4 pair forms an (H3)$_2$.(H4)$_2$ tetramer (Kornberg and Thomas, 1974; D'Anna and Isenberg, 1974b). The tetramer may be in a concentration-dependent equilibrium with the (H3.H4) dimer (Roark *et al.*, 1974). The complex of histones at high ionic strength appears to be different from that at low ionic strength. Therefore, additional reactions among the histones should ensue from an increase in ionic strength. Neither the volume changes accompanying such reactions nor their rates are known at present. Such of those reactions which involve volume changes may be exaggerated. by the strong pressure gradients generated during the prolonged ultracentrifugation (Kegeies *et al.*, 1967; Ten Eyck and Kauzmann, 1967) usually used in the separation of the salt-displaced histones from DNA. The disagreement about the nature—octameric, tetrameric or otherwise—of the histone complex in 2M NaCl is very likely due to the differences in the conditions under which histones are isolated. In order to investigate this possibility and other problems of histone interactions at high ionic strength there is a need for a method of isolating histones which is rapid and which does not sacrifice the gentleness of the salt-extraction procedure. In this paper, we report such a method. A preliminary report of this work has appeared (Philip *et al.*, 1978).

Materials and methods

Preparation of pure nuclei from rat liver

All operations were carried out at 0 to 4° C. Nuclei were prepared by a modification of the Chauveau procedure (Chauveau *et al.*, 1956). Freshly collected liver tissue (from two animals) was minced in 0.9% NaCl and homogenised in 0.35M sucrose-3 mM CaCl$_2$ containing 0.1 mM phenylmethylsulphonylfluoride. The homogenate was passed through four layers of cheese cloth and centrifuged at 600 g for 20 min. All solutions contained 0.1 mM phenylmethylsulphonylfluoride. The pellet obtained was suspended in 0.25 M sucrose-3 mM CaCl$_2$ containing 0.1% Triton X-100. The suspension was centrifuged at 600 g for 15 min and the residue was washed with the same solution but without Triton X-100. The nuclei thus obtained were suspended in 2.2 M sucrose-3 mM CaCl$_2$ and centrifuged at 50,000 g for 90 min using the SW 25.1 rotor in a Spinco Model L 2-50 ultracentrifuge. The pelleted nuclei were homogenised in 0.25 M sucrose -3 mM CaCl$_2$ and centrifuged at 600 g for 10 min. The residue of pure nuclei was homogenised in 0.25 M sucrose and the pellet was collected in a preweighed centrifuge tube by centrifugation at 15,000 g for 15 min, and the weight of the pellet determined.

Adsorption of the nuclear pellet on calcium phosphate gel and removal of histone H1

To the pellet obtained in the previous step, a calculated volume of calcium phosphate gel was added at a gel to pellet (wet wt) ratio of 0.45. The pellet was carefully removed from the centrifuge tube and stirred gently with the gel for 15 min. The gel was subsequently collected by centrifugation and washed with 5 ml of 25 mM sodium phosphate buffer, pH 6.0. Histone H1 was removed by eluting the gel twice with 5 ml portions of the same buffer containing 1M NaCl, by stirring for 10 min each time.

Elution of histones H2A, H2B, H3 and H4

The gel, now free of histone H1, was eluted with 5 ml of 2 M NaCl in 25 mM sodium phosphate buffer, pH 7.0, by stirring for 10 min and the eluate was obtained by centrifugation at 12,000 g for 5 min.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis under non-denaturing conditions was performed at 4° C in 0.2 M sodium phosphate buffer, pH 7.0, as described by Lewis (1976). Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed at room temperature according to Weber *et al.* (1972). The method of Alfageme *et al.* (1974) was followed for urea-acetic acid-Triton X-100 polyacrylamide gel electrophoresis using 4M urea and 0.37% Triton X-100. Gels were stained for 1 h with Amido black (0.4% in acetic acid: methanol: water:: 1:5: 15, v/v) and destained by diffusion in the same solvent.

Sephadex G-100 and Biogel P-100 column chromatography

Columns of Sephadex G-100 (2 × 93 cm) and Biogel P-100 (1.7 × 75 cm) were equilibrated with 2 M NaCl in 25 mM sodium phosphate buffer, pH 7.0, and eluted with the same buffer. The columns were calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen and ribonuclease.

Other methods

Calcium phosphate gel was prepared as described by Dixon and Webb (1964). Concentration of histones in solution was estimated spectrophotometrically using the relationship A_{230} 3.3 = 1 mg/ml in 1 cm path-length cuvettes (Hnilica, 1975).

Results and discussion

Washing the calcium phosphate gel with 25 mM sodium phosphate buffer, pH 6.0, containing 1M NaCl resulted in the elution of a protein which migrated as a broad band on sodium dodecylsulphate polyacrylamide gel electrophoresis (figure 1). Its electrophoretic mobility was comparable to that of an authentic sample of histone H1 under the same conditions. Furthermore, the protein and authentic histone H1 migrated as doublets on urea-acetic acid-Triton X-100 polyacrylamide gel electrophoresis (data not shown) confirming its identity as histone H1.

Elution of the histone H1-depleted gel with 2M NaCl in 25 mM sodium phosphate buffer, pH 7.0, resulted in an eluate, which upon urea-acetic acid-Triton X-100 polyacrylamide gel electrophoresis revealed the presence of histones H2A, H2B, H3 and H4 (figure 2, left). The minor bands observed on the gels have been identified earlier as variants of histones (Franklin and Zweidler, 1977). Since histones H2A, H2B, H3 and H4 are present in equimolar amounts in chromatin (Joffe *et al.*, 1977), we assume that the four histones are present in equimolar ratio in our preparation. Polyacrylamide gel electrophoresis under non-denaturing conditions at pH 7.0 gave only one sharp protein band (figure 2, right). This suggests that the four histones are present as a specific complex rather than as a random aggregate. The nature of this complex is being investigated. DNA could not be detected in the preparation by the diphenylamine method (Burton, 1956).

Gel filtration of the 2 M NaCl eluate from calcium phosphate gel on a Sephadex G-100 column gave two major protein peaks and a minor UV-absorbing material which appeared in the void volume (figure 3A). The apparent molecular weights of the included peaks were 74,000 and 45,000, respectively, when compared with the elution volumes of marker proteins. Since histones behave abnormally on Sephadex columns (Roark *et al.*, 1976), we carried out gel filtration experiments in a Biogel P-100 column under similar conditions. In this case, only two peaks were observed corresponding to apparent molecular weights of 64,000 and 38,000 respectively. Analysis of the components of the smaller molecular weight peak from the Sephadex G-100 column by urea-acetic acid-Triton X-100 polyacrylamide gel electrophoresis revealed the presence of histones H2A and H2B in equal amounts (figure 3B, right), suggesting that it could be the heterodimer, (H2A. H2B), reported
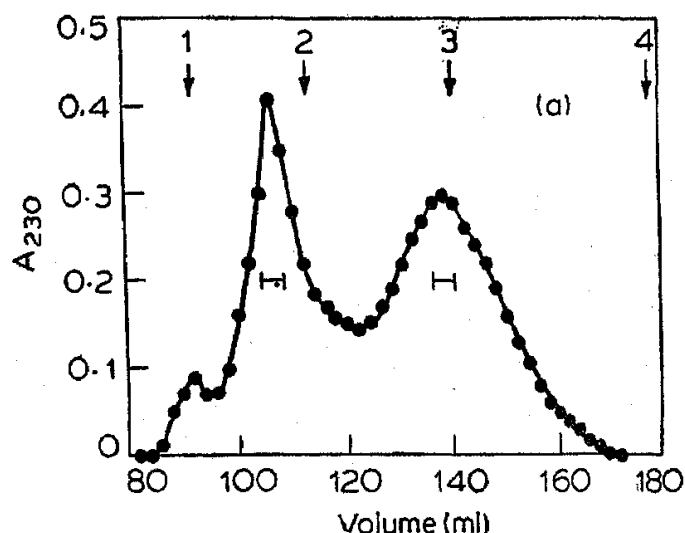

Figure 3A. Gel-filtration of the 2 M NaCl eluate (pH7·0) from rat liver chromatin adsorbed on to calcium phosphate gel, on a Sephadex G-100 column.

The column (2 × 93 cm) was equilibrated with 2 M NaCl in 25 mM sodium phosphate buffer, pH 7·0. A freshly prepared histone sample (1·8 ml, 3 mg/ml) was applied and eluted with the equilibration buffer at a flow rate of 18 ml per hour at 4°C. Fractions (1·8 ml) were collected and absorbance at 230 nm was measured. Arrows 1,2,3 and 4 are, respectively, the void volume, elution volumes of bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000) and chymotryosinogen (M_r 25,000).
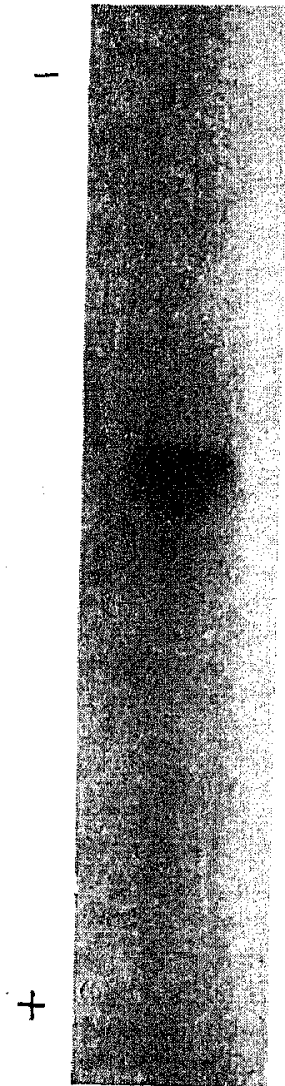
Figure 1. Sodium dodecyl sulphate polyacrylamide gel electrophoretic pattern. The 1 M NaCl eluate (pH 6·0) from rat liver chromatin adsorbed on calcium phosphate gel was used for electrophoresis in 0·1% sodium dodecyl sulphate-10% polyacrylamide gels. About 50 μg of protein was applied and a current of 8 mA per tube was maintained. Direction of migration was from top to bottom.
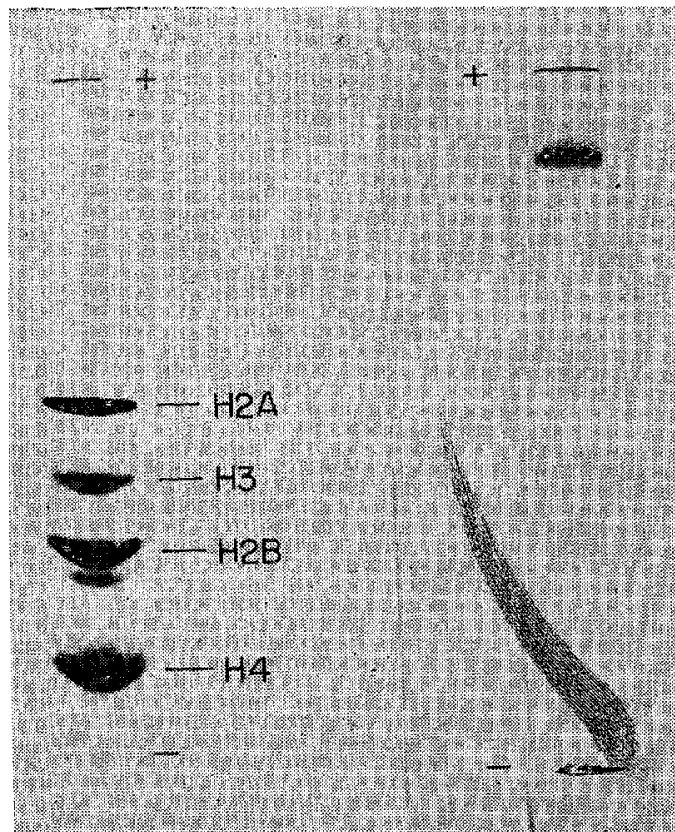
Figure 2. Electrophoresis of the 2 M NaCl eluate under denaturing and non-denaturing conditions.

The method of Alfageme *et al.* (1974) was used with 4M-urea and 0·37% Triton X-100. About 150 µg of protein was applied and electrophoresis was performed for about 4h (left panel). Non-denaturing polyacrylamide gel electrophoresis of the eluate was according to the method of Lewis (1976). 0·2 M sodium phosphate buffer, pH 7·0, was used in 10% polyacrylamide gels. About 60 µg of protein in 2 M NaCl and 10% glycerol was applied (right panel). Electrophoresis was for 20 h at 4° C and 100 volts. Direction of migration was from top to bottom.
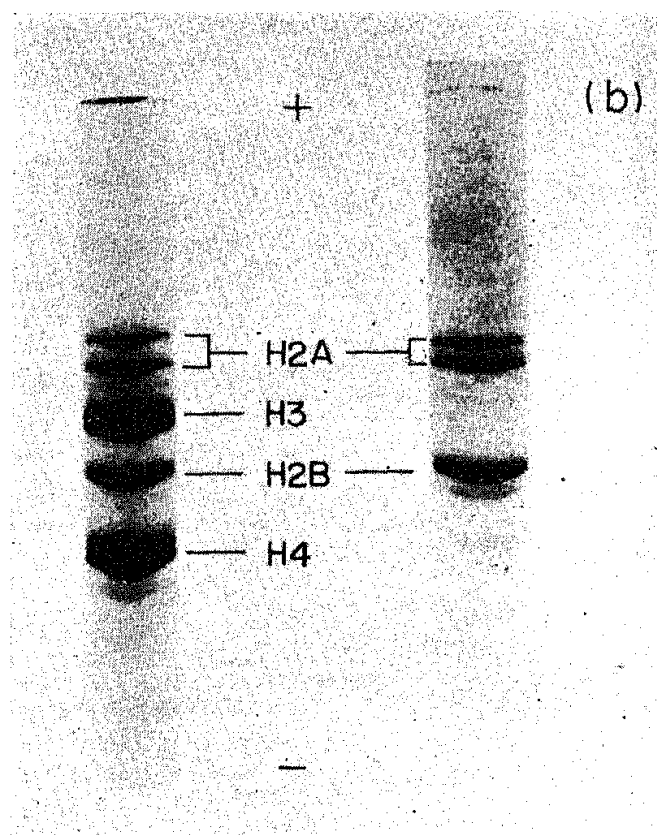
Figure 3B Urea-acetic acid-Triton X-100 gel electrophoretic pattern of the histones in the second peak (left) and the third peak (right).

Fractions in the two peaks indicated by horizontal bars in figure 3A were separately concentrated by dialysis against Aquacide. The concentrated protein was dialysed against the same buffer as that used in the subsequent electrophoresis.

earlier (Skandrani *et al.,* 1972; D'Anna and Isenberg, 1974a; Kelley, 1973; Roark *et al.,* 1974). But its apparent molecular weight under our experimental conditions (38,000), is a little higher than that reported previously for the free dimer whether studied under similar ionic conditions (M_r 32,000, Weintraub *et al.,* 1975) or under different conditions (M_r 33,500, Roark *et al.,* 1976).

Electrophoretic analysis of the larger molecular species from the Sephadex column (after concentration) showed the presence of all the four histones, but histones, H3 and H4 were present in relatively larger amounts than either H2A or H2B (figure 3B, left). The ratio of H2B: H3 as estimated by their staining intensity compared to that of whole histones (figure 2, left) was 1: 1.7. After concentrating the pooled fractions indicated in figure 3A (middle peak) by dialysis against solid Sephadex, the protein was subjected to a synthetic boundary sedimentation velocity experiment in the analytical ultracentrifuge (7.9° C, 52,000 rpm, 1 mg per ml protein). Only one boundary was observed and a plot of the logarithm of the distance of the boundary from the rotor centre against time was linear. From the gradient, after the usual corrections, and using an apparent partial specific volume of 0.73 ml per g, an $s_{20,w}$ of 2.6S was calculated. The sedimentation coefficient differs very little from the value (2.54S) reported for the $(H3)_2.(H4)_2$ tetramer (Kelley, 1973). The apparent molecular weights estimated using Biogel and Sephadex columns, 64,000 and 74,000, respectively, however, are different from those expected for the tetramer (values of 42,000 using Biogel column and 100,000 using Sephadex column were reported by Roark *et al.,* 1976). Because of these reasons, and in view of the known failure of (H2A.H2B) dimer to form higher oligomers (Kelley, 1973), we consider it unlikely that the species is a mixture of $(H3)_2.(H4)_2$ and $(H2A)_2.(H2B)_2$ tetramers. We are inclined to think that the histone species isolated by us is the hexamer, $(H3)_2.(H4)_2.H2A.H2B$. As mentioned earlier, the apparent molecular weight of this species as obtained from gel filtration experiments is lower than expected. The reason for this lower estimate may be that the hexamer is in dissociation equilibrium with the stable $(H3)_2.(H4)_2$ tetramer and (H2A.H2B) dimer. The existence of such a dissociation equilibrium would also explain the low sedimentation coefficient. The hexamer, $(H3)_2.(H4)_2.H2A.H2B$, has been indicated in the cross-linking experiments of Thomas and Kornberg (1975). The present data do not unambiguously define the nature of the histone complex in 2 M NaCl. The presence of one sharp band on polyacrylamide gel electrophoresis under non-denaturing conditions and the apparent dissociation pattern observed on gel filtration, however, are consistent with an octameric $(H2A.H2B.H3.H4)_2$, species dissociating primarily into a heterotypic hexamer, $(H3)_2.(H4)_2.H2A.H2B$, and a lysine-rich dimer, (H2A.H2B).

The proteins present in the void volume of the column could not be ascertained because of their presence in a very low concentration.

Histone H2A, after chromatography and processing for electrophoresis was found to be split into two bands on urea-acetic acid-Triton X-100 polyacrylamide gel electrophoresis (figure 3B). This may be because of the presence of a protease, specific for histone H2A. Eickbush *et al.* (1976) reported the presence of such a protease in chromatin from calf thymus and liver. This enzyme cleaves histone H2A specifically at high ionic strength, between Val 114 and Leu 115, removing a 15-amino-acid segment from the C-terminal end. The presence of such a protease

in rat liver chromatin has so far not been reported. This possibility is being investigated.

The method described in this paper enables the rapid isolation of histones H2A, H2B, H3 and H4 without exposing them to extremes of pH and without subjecting them to high centrifugal forces. In addition, histone H1 can be obtained in a relatively pure form. The isolation of histones can be completed in less than 2 h after the preparation of nuclei. Only about 20 min elapse between dissociation of histones from DNA and their isolation. This should afford an opportunity to study the properties of the histones soon after their dissociation from DNA and to investigate any reactions they undergo with time and in response to various environmental parameters. Such studies are in progress in our laboratory.

By employing this method, we have isolated the histones from chicken erythrocyte nuclei also. Our preliminary results suggest that the behaviour of chicken erythrocyte histones during Sephadex column chromatography is very similar to that of rat-liver histones. However, the H2A band was not split suggesting the possible absence of the H2A-specific protease in this system. This result also suggests that the proteolytic cleavage of H2A may not be the primary cause for the behaviour observed in Sephadex columns. Eickbush and Moudrianakis (1978) observed that in the calf thymus system, the cleavage of H2A by its specific protease resulted in the dissociation of the histone octamer in 2 M NaCl into $(H3)_2 \cdot (H4)_2$ tetramer and dimers composed of H2B and residues 1-114 of H2A. We have attributed the apparent dissociation occurring in the column primarily to dilution by axial dispersion, but we have not ruled out the possibility of enzymatic effects.

Acknowledgements

References

Alfageme, C. R., Zweidler, A., Mahowald, A. and Cohen, L. H. (1974) *J. Biol. Chem.,* **249,** 3729.
Burton, K. (1956) *Biochem, J.,* **62,** 315.
Campbell, A. M. and Cotter, R. I. (1976) *FEBS Lett.,* **70,** 209.
Chauveau, J., Moule, Y. and Rouiller, G. H. (1956) *Exp. Cell. Res.,* **11,** 317.
Chung, S.-Y., Hill, W. E. and Doty, P. (1978) *Proc. Natl. Acad. Sci. USA,* **75,** 1680.
D'Anna, Jr. J. A. and Isenberg, I. (1974a) *Biochemistry,* **14,** 2098.
D'Anna, Jr. J. A. and Isenberg, I. (1974b) *Biochemistry,* **13,** 4992.
Dixon, M. and Webb, E. C. (1964) *Enzymes* 2nd ed. (London: Longman Green) p 42.
Eickbush, T. H., Watson, D. K. and Moudrianakis, E. N. (1976) *Cell,* **9,** 785.
Eickbush, T. H. and Moudrianakis, E. N. (1978) *Fed. Proc,* **37,** 1463.
Franklin, S. G. and Zweidler, A. (1977) *Nature* (*London*), **266,** 273.
Hnilica, L. S. (1975) *Methods Enzymol.,* **E40,** 102.

Hyde, J. E. and Walker, I. O. (1975) *FEBS Lett., 50,* 150.

Joffe, J., Keene, M. and Weintraub, H. (1977) *Biochemistry, 16.* 1236.

Kegeles, G., Rhodes, L. and Bethune, J. L. (1967) *Proc. Natl. Acad. Sci. USA, 58,* 45.

Kelley, R. I. (1973) *Biochem. Biophys. Res. Commun., 54,* 1588.

Kornberg, R. D. and Thomas J. O. (1974) *Science, 184,* 865.

Lewis, P. N. (1976) *Can. J. Biochem., 54,* 641.

Lilley, D.M. J., Pardon, J. F. and Richards, B. M. (1977) *Biochemistry, 16,* 2853.

Philip, M., Jamaluddin, M. and Sharat Chandra, H. (1978) *II Meeting of the Indian Society of Cell Biology,* Delhi Jan. 7-11 (Abstract)

Roark, D. E., Geoghegan, T. E. and Keller, G. H. (1974) *Biochem. Biophys. Res. Commun., 59,* 542.

Roark, D.E., Geoghegan, T.E., Keller, G. H., Matter, K. V. and Engle, R. L. (1976) *Biochemistry, 15,* 3019.

Skandrani, E., Mizon, J., Sautiere, P. and Biserte, G. (1972) *Biochimie, 54,* 1267.

Sperling, R. and Bustin, M. (1975) *Biochemistry, 14,* 3322.

Sperling, R. and Bustin, M. (1976) *Nucleic Acids Res., 3,* 1263.

TenEyck, L. F. and Kauzmann, W. (1967) *Proc. Natl. Acad. Sci. USA, 58,* 888.

Thomas, Jr. G· J., Prescot, B. and Olins, D. E. (1977) *Science, 197,* 385.

Thomas, J.O. and Kornberg, R. D. (1975) *FEBS Lett., 58,* 353.

Thomas, J.O. and Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. USA, 72,* 2626.

Thomas, J.O. and Butler, P. J. G. (1977) *J. Mol. Biol., 116,* 769.

Weber,K., Pringle, J. R. and Osborn, M. (1972) *Methods Enzymol., C26* 327.

Weintraub, H., Palter, K. and Van Lente, F. (1975) *Cell 6,* 85.