Volume 6 Number 4 April 1979 Nucleic Acids Research

# Salt-dependent interconversion of inner histone oligomers

Andrew P.Butler , Rodney E.Harrington* and Donald E.Olins

University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, and Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830, USA

Received 2 November 1978

## ABSTRACT

The inner histone complex, extracted from chicken erythrocyte chromatin in 2 M NaCl at pH 7.4, has been characterized by sedimentation equilibrium and sedimentation velocity. High speed sedimentation equilibrium studies indicate that in 2 M NaCl the inner histones are a weakly associating system with contributions from species ranging in molecular weight from dimer to octamer. The appearance of a single boundary (3.8S at 2 M NaCl) in sedimentation velocity studies conducted over a wide range of protein concentrations and ionic conditions indicates that the various histone oligomers present are in rapid equilibrium with one another. At higher salts the equilibrium is shifted to favor higher molecular weight species; in 4 M NaCl essentially all of the histone is octameric at protein concentrations above 0.2 mg/ml. The facile interconversion of histone oligomers suggests that small alterations in histone-histone interactions may be responsible for changes in nucleosome conformations during various biological processes.

## INTRODUCTION

Extraction of histones from chicken erythrocyte chromatin with 2 M NaCl at pH 7.4 yields predominantly two species: a slowly sedimenting (1.4S) fraction containing the lysine-rich histones (H1 and H5) and a rapidly sedimenting (3.8S) fraction containing the "inner histones" (H2A, H2B, H3, and H4) (1). Sedimentation equilibrium studies indicated an average molecular weight of about 51,000 daltons for this inner histone complex (1) — approximately what would be expected for a tetramer containing one subunit of each of the inner histones. This complex was shown to contain equimolar amounts of the inner histones and was denoted a "heterotypic tetramer" (1). Since nucleosome core particles appear to contain two of these heterotypic tetramers associated with 140 base pairs of DNA (2, 3), the heterotypic tetramer was postulated as a fundamental structural component of the nucleosome, with important biological consequences for replication, transcription, and development (4).

Researchers at another laboratory (5), using 2 M NaCl extraction of histones at pH 8, suggested on the basis of chemical cross-linking experiments that the predominant inner histone species in 2 M NaCl is a heterotypic octamer containing two each of the four inner histones. More recently, equilibrium centrifugation results supporting a histone octamer of 107,000 daltons were published (6). In yet another report (7), light-scattering measurements yielded an apparent molecular weight of 56,000 daltons for the inner histones in 2 M NaCl. The chemical nature of the "tetramer" remains in doubt as well. While a great deal of data supports the concept of a heterotypic species (1, 6, 8) others (9) believe the high salt histones are composed of a mixture of homotypic forms (H3-H4 tetramers and H2A-H2B dimers). Molecular weight studies alone cannot distinguish these compositional differences. However, studies of the protein concentration dependence and solvent effects on the molecular weights can be used as a probe of the stability of various classes of oligomers.

Because of the continuing disagreement concerning the aggregation state of salt-extracted inner histones, we have conducted our own molecular weight determinations. Our results are consistent with the presence of a concentration-dependent mixture of histone oligomers in 2 M NaCl. Furthermore, we show that at higher salt concentrations (4 M NaCl) this equilibrium mixture can be shifted almost entirely into the octameric form, with only slight dissociation at very low protein concentrations (<0.2 mg/ml).

## METHODS

### Inner Histone Preparation

Chicken erythrocyte nuclei were prepared from either fresh chicken blood or frozen chicken blood obtained from Pel-Freez (Rogers, AR) (10). The nuclear pellet was extracted with 0.35 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF). All subsequent buffers used in the isolation contained 0.1 mM PMSF to inhibit proteolysis. Nuclei were swollen in 1 mM $Na_2EDTA$ and further swollen and lysed in glass-distilled water.

This crude chromatin was made 10 mM in Tris-HCl (pH 7.4), 10 mM in dithiothreitol (DTT), and 0.1 mM PMSF. Solid NaCl was added slowly with thorough mixing to a final concentration of 2 M, and the material was extracted for 6–24 hr. DNA was removed from this extract by centrifugation in a Beckman Ti-50 rotor at 48,000 rpm for 12 hr at 4°C. The supernatant was collected and concentrated by vacuum dialysis. The

concentrated NaCl extract ($A_{278} \simeq 6.5$) was layered on 5–20% sucrose gradients containing 2 M NaCl, 10 mM Tris-HCl, 0.1 mM DTT (pH 7.2) and centrifuged for 44 hr at 50,000 rpm in an SW 50.1 rotor at 4°C. The 5-ml gradients were fractionated by use of an ISCO Density Gradient Collector (Model 640) equipped with an ISCO UV-5 Absorbance Monitor. The rapidly sedimenting peak (Fig. 1A), containing the inner histones, and the slowly sedimenting peak, containing H5 and H1, were separately pooled; dialyzed against 2.0 M NaCl, 10 mM Tris HCl, 0.1 mM DTT, 0.1 mM PMSF (pH 7.4); concentrated to 10 mg/ml; and stored frozen at -20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11) of the two pools is shown in Fig. 1B. Scans of the stained gel reveal that H3, H2B, H2A, and H4 are present in equimolar quantities (± 10%) in the inner histone pool and that no other protein band contributes more than 1% to the total mass. Furthermore, inner histones isolated in this fashion contain no proteolytic fragments, nor does there appear to be any appreciable contamination with DNA or nucleotides [as shown by negative diphenylamine assay and the following spectral ratios for the inner histones in 2 M NaCl, 10 mM Tris-HCl, 0.1 mM DTT (pH 7.4): $A_{278}/A_{260} = 2.35 \pm 0.05$; $A_{230}/A_{278} = 9.5 \pm 0.1$].

Analytical Ultracentrifugation

Samples of inner histones were prepared for ultracentrifugation by dialysis versus several changes of 10 mM Tris-HCl, 0.1 mM DTT (pH 7.4) containing the desired concentration of NaCl. After extensive dialysis, protein concentration was adjusted to the appropriate value by dilution with dialysis buffer.

All ultracentrifugation experiments were conducted with a Beckman Model E ultracentrifuge equipped with UV absorbance optics and scanner.

For equilibrium centrifugation, standard double-sector cells containing a sample column of ~3 mm were used. The meniscus depletion method (12) was chosen because the distribution of protein at sedimentation equilibrium spans a broad range of concentrations and is thus particularly suitable for analysis of associating systems. Measurements of the partial specific volume ($\bar{v}$) were made in 2 M and 4 M NaCl using the method of Edelstein and Schachman (13). Histones were dialyzed exhaustively against buffers prepared with either $H_2O$ or $D_2O_3$ and the distributions of protein in the two samples at sedimentation equilibrium were compared.

For sedimentation velocity experiments, the dialyzed inner histone samples were made 0.5% in sucrose by addition of 20% sucrose (in 2 M NaCl), then were loaded into

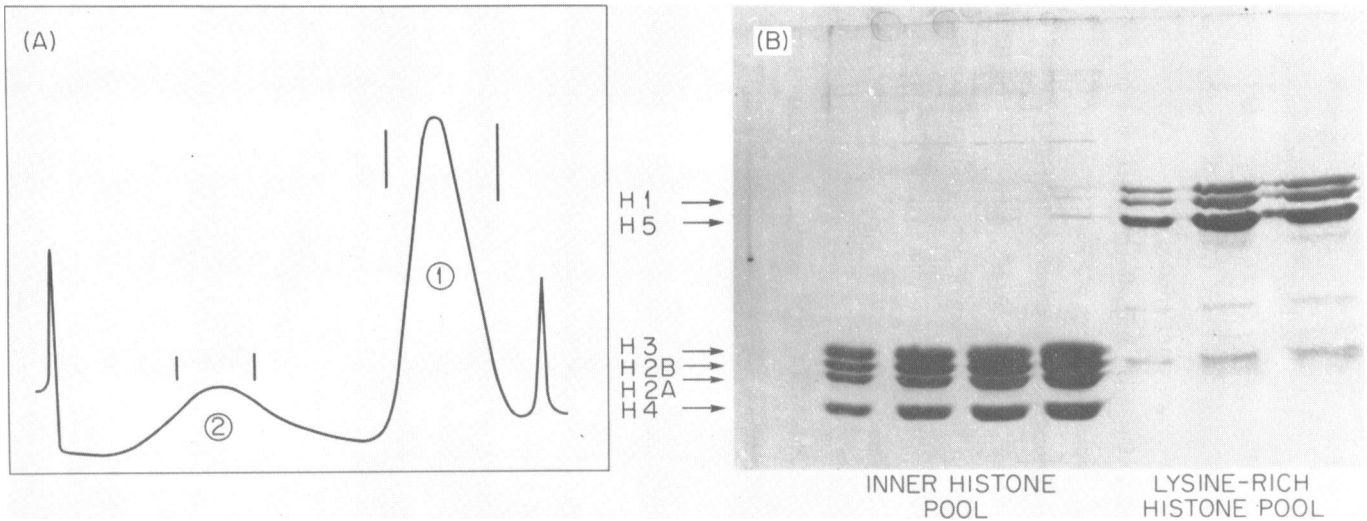


Figure 1. Extraction of inner histones from chicken erythrocytes. Histones were extracted from swollen chromatin by use of 2 M NaCl as described in the text. (A) Optical density profile of sucrose gradient step. The direction of centrifugation was from left to right. Gradients of 5–20% sucrose included 2 M NaCl, 10 mM Tris HCl, 0.1 mM DTT (pH 7.2); centrifugation was for 44 hr at 50,000 rpm. Peak 1 is the inner histone pool and peak 2 contains H1 and H5. (B) Sodium dodecyl sulfate-polyacrylamide gel analysis of pooled histone fractions from (A). The different lanes shown for each pool correspond to different protein loads applied to the gel.

synthetic boundary cells. Centrifugation was at 40,000 rpm. The results were treated by conventional log $r_b$ versus t plots, where $r_b$ is the midpoint of the observed boundary. Corrections for solvent density and viscosity were based on tabulated data (14) to obtain $S_{20,w}$.

## RESULTS AND DISCUSSION

In Fig. 2, we show representative data from equilibrium ultracentrifugation runs in 2, 3, and 4 M NaCl. At 2 and 3 M NaCl the curves of $\ell n$ Y (Y is relative displacement of the scanner trace and is proportional to protein concentration) versus $r^2$ are nonlinear, indicating molecular weight heterogeneity. At 4 M NaCl the data fall sensibly on a single straight line as expected for a single homogeneous species.

Determination of molecular weight from the data in Fig. 2 is complicated by the considerable uncertainty in the partial specific volume, $\bar{v}$. The rather wide range of published $\bar{v}$ values and estimated experimental errors are shown in Table 1. The range of $\bar{v}$ values presently available translates into a molecular weight range of 36,500 daltons, clearly an unacceptable level of uncertainty for the determination of absolute molecular weights. The disagreement in $\bar{v}$ measurements may be due in part to evaporation of the solutions with high salt concentrations or to uncertainty in the histone concentrations.

The relative molecular weights given by the least-square lines in Fig. 2 will be unaffected by the absolute value of $\bar{v}$ provided the quantity remains constant over the salt range considered here. On purely physical grounds, we would expect this to be approximately true. In order to demonstrate that $\bar{v}$ remains constant throughout the salt range studied, $\bar{v}$ was determined in 2 M and 4 M NaCl solutions by analytical centrifugation (13). The results (Table 1) show no salt dependence. However, the relatively large errors inherent in this method (~5–6%) prevent the use of $\bar{v}$ determined in this way for the calculation of accurate molecular weights. The absence of salt effects on $\bar{v}$ has been verified by others (8).

When the upper limit to the range of $\bar{v}$ values ($\bar{v}$ = 0.767) is used, our equilibrium data from Fig. 2 lead to an apparent weight average molecular weight ($M_w$) in 2 M NaCl of 54,000, approximately the molecular weight of an inner histone tetramer. However the curvature of the $\ell n$ Y vs. $r^2$ plot in Fig. 2 clearly indicates a range of molecular weights from about 20,000 daltons to greater than 80,000. At 3 M NaCl the average

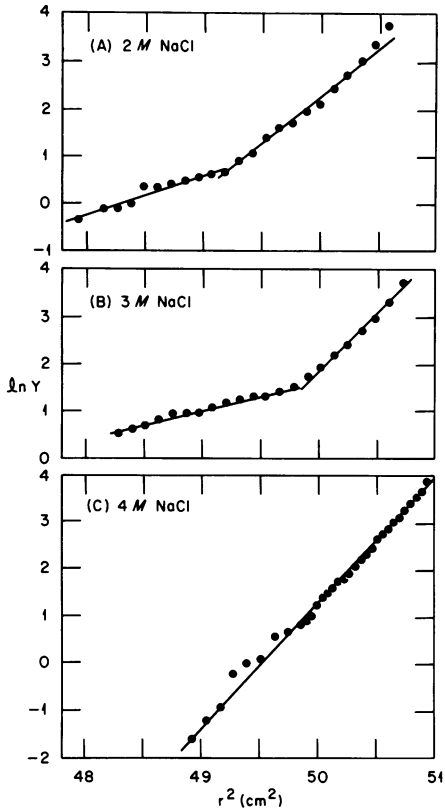


Figure 2. Equilibrium centrifugation patterns of inner histones as a function of salt concentration. T = 18°C; 32,000 rpm; buffer = 10 mM Tris-HCl (pH 7.4), 0.1 mM DTT, and (A) 2 M, (B) 3 M, or (C) 4 M NaCl.

$M_w$ is about 85,000, but again a mixture of histone oligomers is definitely present. At 4 M NaCl, however the protein is essentially monodisperse, with an $M_w$ of 102,000. The ratios of these molecular weights would, therefore, reflect a conversion from tetramer to octamer (with possible contributions from hexamer) as the salt concentration is increased from 2 to 4 M. The margin of error in the data at 2 M NaCl is too large to permit a sensible distinction between homotypic and heterotypic tetramers. These molecular weights have not been corrected for the Donnan effect. The appropriate correction is given by equation 37 of reference 15 and requires a knowledge of the macromolecular charge. If we estimate the maximum expected charge on the inner histones at pH 7 as

TABLE 1. Reported $\bar{v}$ Values

| Source | $\bar{v}$ (ml/g) | Conditions | Apparent $M_w$ from our 4 M NaCl data |
|---|---|---|---|
| Thomas and Butler (6) | 0.767 ± 0.014 | 1.975 M NaCl, 68.5 mM sodium borate (pH 9) | 102,000 |
| Calculated from amino acid composition | 0.746 | — | 73,000 |
| Our measurements [Edelstein-Schachman (13) ultracentrifuge method] | 0.746 ± 0.048 | 2.0 M NaCl, 10 mM Tris-HCl, 0.1 mM DTT (pH 7.4) | |
| | 0.742 ± 0.037 | 4.0 M NaCl, 10 mM Tris-HCl, 0.1 mM DTT (pH 7.4) | |
| Chung et al. (8) | 0.730 ± 0.005 | 2 M NaCl, 10 mM CHES (pH 9) | 65,000 |

$Z \doteq 85$, this equation predicts an increase of the molecular weights reported here by less than ~4% (in 2 M NaCl) or ~2% (in 4 M NaCl). The ratios of the molecular weights would remain essentially uneffected.

Lower limit values for the molecular weights in 4 M NaCl corresponding to $\bar{v} = 0.730$ are also shown in Table 1 for comparison. These range from 40,000 (data not shown) to 65,000 daltons in 2 and 4 M salt, respectively, and do not correspond closely to any known histone oligomer. A molecular weight of 40,000 in 2 M salt would require a mixture of four different histone trimers in order to account for the stoichiometry observed on polyacrylamide gels (Fig. 1B). We conclude, therefore, that the partial specific volume of the core histone complex must be at the upper end of the reported experimental range.

The apparent concentration dependencies which we observe from the distributions of molecular weights across the equilibrium ultracentrifuge cell are shown at 2 M and 4 M NaCl in Fig. 3. These curves were obtained by a floating seven-point linear least-squares analysis of the data given in Fig. 2. At 2 M NaCl, the molecular weight distribution is large, implying a mass action equilibrium among at least three predominant species. At 4 M NaCl, however, the concentration dependence is small above 0.2 mg/ml inner histones. The scatter in the data at low concentrations is so large as to pre-

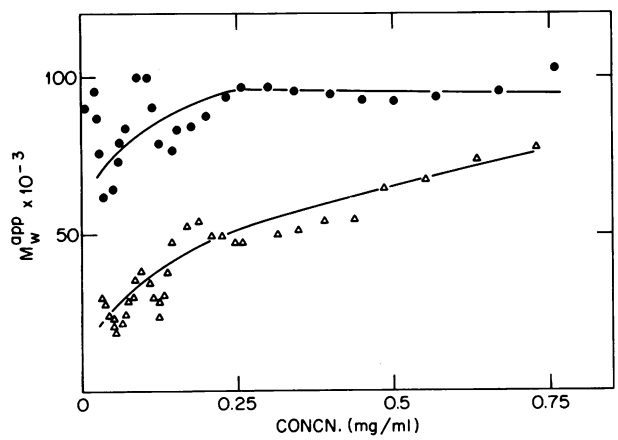


Figure 3. Apparent weight-average molecular weight ($M_{w,r}$) versus protein concentration of inner histones in 2 M (△) and 4 M NaCl (○), 10 mM Tris-HCl, 0.1 mM DTT (pH 7.4). Values of $M_{w,r}$, corresponding to histone concentration c(r), were evaluated for each radial position r by linear least-squares regression of seven data points on the plot of $\ell n\ c(r)$ versus $r^2$. T = 18°C, 32,000 rpm, 48–60 hr.

clude the determination of solution nonideality effects, but the data nevertheless suggest the presence of a single predominant species.

Sedimentation coefficients as a function of NaCl are shown in Fig. 4. Here the sedimentation coefficients are given for a protein concentration of 1 mg/ml, but independent experiments have demonstrated negligible concentration dependence. $S_{20,w}$ is remarkably constant over the salt range 2 to 4 M compared with the relative increase found in $M_w$ over the same range. The most probable explanation of this phenomenon is that inner histone oligomers are in rapid chemical equilibrium so that the observed value of $S_{20,w}$ represents a weighted average of the various species (16). Thus the slight upward trend seen near 4 M NaCl would represent a greater contribution by the octamer. We note that at this histone concentration (1 mg/ml) the equilibrium ultracentrifugation demonstrates that a considerable fraction of the protein has a molecular weight greater than the tetramer value (Fig. 3).

The sedimentation coefficient $S_{20,w} = 3.8$ is in excellent agreement with other reports (1, 7, 8, 17) which attribute this value to a "heterotypic tetramer." Our sedimentation coefficient in 4 M NaCl, $S_{20,w} = 4.2$, is somewhat smaller than Thomas and Butler's (6) value of 4.8 in their 2.0 M buffer, which they attribute to an octamer; and

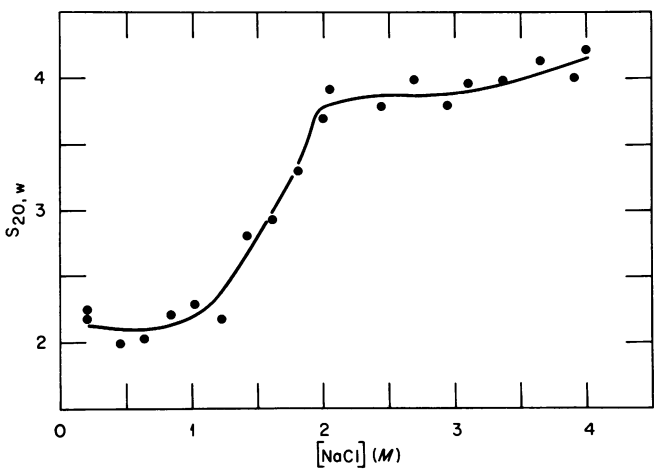


Figure 4. Dependence of inner histone sedimentation coefficient ($S_{20,w}$) on salt concentration. Samples were prepared by dilution of a 10 mg/ml stock solution of histones in 2 M NaCl to a final concentration of 1 mg/ml at the desired NaCl concentration. Centrifugation was at 20.0°C and 40,000 rpm.

our value in 2 M NaCl is smaller yet. We are unable to account for these discrepancies, particularly in comparing our 4 M value with Thomas and Butler's 2 M value, since little evidence for polydispersity exists in either case. We also note that this sedimentation coefficient is higher than the value (2.54S) reported for pure H3-H4 tetramer (18), though the latter value was determined under much different ionic conditions than any of the other sedimentation coefficients quoted here.

The uncertainty in the size and frictional coefficient of the histone octamer, as manifested by the range of values reported for the molecular weight and sedimentation coefficient of the inner histones, is very likely due to the strong concentration dependence (in 2 M NaCl), salt and temperature dependence (9), and pH effects (1, 9) which have been observed for inner histone associations. Nonetheless, both our data and the results of others (8, 9) strongly suggest that inner histones in solution are an equilibrium mixture which under favorable conditions (e.g. 4 M NaCl) can be driven into an octameric form. This octamer is presumably analogous to the "octamer" of histones (2 each of H2A, H2B, H3 and H4) found in nucleosomes.

Thus the inner histone complex can be viewed as a reversibly dissociating octamer although the dissociation mechanism is not fully specified by the sedimentation equilibrium method. Dissociation is favored by low protein concentration (Fig. 3; refs. 8 and 9) or low salt concentration (Figs. 3 and 4; refs. 9 and 19). Low temperature also shifts the equilibrium towards the octamer (9). Acidic conditions (pH $<6$) tend to cause the complex to disproportionate into a mixture of homotypic species (9). Despite this plasticity of subunit organization, there appears to be remarkably little alteration in histone secondary or tertiary structure accompanying these transitions. For example, the $\alpha$-helicity of the inner histones is constant (51 $\pm$ 5%) between 1 M and 4.6 M NaCl (20), despite the significant alterations in quaternary structure which occur between these salt concentrations. Furthermore, the intensity of tyrosine fluorescence of the inner histones is also unchanged between 0.6 M and 4 M NaCl although large changes in fluorescence intensity occur as a result of limited unfolding (i.e., partial loss of $\alpha$-helix) below pH 5 (21). These observations, plus the rapid establishment of chemical equilibrium among the oligomeric species present in the ultracentrifuge, imply that a variety of histone-histone interactions can occur with little or no change in the conformation of individual subunits; thus relatively small free energy differences exist between different association states. In fact, the free energy change calculated assuming a heterotypic tetramer $\rightleftarrows$ octamer

equilibrium in 2 M NaCl is less than 6 Kcal mole$^{-1}$ (8) suggesting that only 2–3 weak interactions are involved.

The extremely delicate equilibria between these various histone oligomers suggests that the nucleosome particle may itself be in a state of dynamic balance between a number of conformational states. Very recently electron microscopic analysis of nucleosome crystals (22) has led to the same conclusion. We envision changes in histone-histone interactions (as manifested by salt, pH, and temperature effects on the subunit associations of free histones) causing subtle alterations in the shape of the nucleosome which may in fact be necessary for gene activation or replication.

Studies of nucleosome diffusion and sedimentation coefficients as a function of ionic strength (23) indicate the presence of highly cooperative conformational changes in nucleosomes; similar conformational changes brought about by salt or pH have been studied by spectroscopic methods (24). Shifts to low pH (~5), for example, may reflect a relative strengthening of homotypic H3-H4 interactions at the expense of heterotypic interactions, with a consequent alteration of particle shape or stability. Such a change may mimic similar structural changes brought about in vivo by histone modification or binding of regulatory factors to the nucleosome.

## ACKNOWLEDGEMENTS

We are grateful to E. B. Wright and M. Hsu-Hsie for valuable technical assistance. This research was sponsored by the Division of Biomedical and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation, and by an NIH research grant to DEO (GM 19334). APB is a postdoctoral investigator supported by subcontract No. 3322 from the Biology Division of Oak Ridge National Laboratory to The University of Tennessee. Portions of this work were presented in preliminary form in the abstracts of the 22nd Annual Meeting of the Biophysical Society (21).

*On leave, 1976-77 from the Department of Chemistry, University of Nevada, Reno, NV 89507, USA

## REFERENCES


1. Weintraub, H., Palter, K. and Van Lente, F. (1975) Cell 6, 85–110
2. Van Holde, K. E., Shaw, B. R., Lohr, D., Herman, T. M. and Kovacic, R. T. (1975) Proc. FEBS 10th Meet., 57–72
3. Noll, M. (1976) Cell 8, 349–355
4. Alberts, B., Worcel, A. and Weintraub, H. (1977) in International Symposium on the Organization and Expression of the Eukaryotic Genome. Tehran, 1976 (Bradbury, E. M. and Javaherian, K., eds.), pp. 165–191, Academic Press, New York
5. Thomas, J. O. and Kornberg, R. D. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2626–2630
6. Thomas, J. O. and Butler, P. J. G. (1977) J. Mol. Biol. 116, 769–781
7. Campbell, A. M. and Cotter, R. I. (1976) FEBS Lett. 70, 209–211
8. Chung, S-Y., Hill, W. E. and Doty, P. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1680–1684
9. Eikbush, T. H. and Moudrianakis, E. N. (1978) Biochemistry 17, 4955–4964
10. Olins, A. L., Carlson, R. D., Wright, E. B., and Olins, D. E. (1976) Nucleic Acids Res. 3, 3271–3291
11. Laemmli, U. K. (1970) Nature 227, 680–685
12. Yphantis, D. A. (1964) Biochemistry 3, 297–317
13. Edelstein, S. J. and Schachmen, H. K. (1967) J. Biol. Chem. 242, 306–311
14. Svedberg, T. and Pederson, K. O. (1940) The Ultracentrifuge, Oxford University Press (Clarendon), London
15. Roark, D. E. and Yphantis, D. A. (1971) Biochemistry 10, 3241–3249
16. Gilbert, G. A. (1959) Proc. Roy. Soc. A250, 377
17. Lilley, D. M. J., Pardon, J. F. and Richards, B. M. (1977) Biochemistry 16, 2853–3859
18. Moss, T., Cary, P. D., Crane-Robinson, C. and Bradbury, E. M. (1976) Biochemistry 15, 2261–2267
19. Roark, D. E., Geohegan, T. E., Keller, G. H., Matter, K. V. and Engle, R. L. (1976) Biochemistry 15, 3019–3025
20. Thomas, G. J., Prescott, B. and Olins, D. E. (1977) Science 197, 385–388
21. Butler, A. P., Olins, D. E., Harrington, R. E. and Hill, W. E. (1978) Biophys. J. 21, 66a
22. Dubochet, J. and Noll, M. (1978) Science 202, 280–286
23. Gordon, V. C., Knobler, C. M., Olins, D. E. and Schumaker, V. N. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 660–663
24. Zama, M., Thomas, G., Prescott, B. and Olins, D. E. (1977) Cold Spring Harbor Symp., Quant. Biol. 42, 31–41; Biophys. J. 21, 66a