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Nucleosome core histone complex isolated gently and rapidly in 2 M NaCl is octameric

(sedimentation coefficient/diffusion coefficient/molecular weight/pressure effect)

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ABSTRACT **A relatively stable specific complex of the chromatin core histones H2A, H2B, H3, and H4 has been obtained in 2 M NaCl/25 mM sodium phosphate buffer, pH 7.0. The histone core complex has an apparent specific volume of 0.73 ml/g. Its sedimentation coefficient was dependent on rotor speed (angular velocity, ω) and attained different stable values at low and high rotor speeds. The drop in sedimentation coefficient occurred sharply between ω^2 values of about 9×10^6 and 1.1×10^7 (radians/sec)^2. The $s^0_{20,w}$ corresponding to zero angular velocity (1 atmosphere pressure) was 6.6 S ± (SEM) 0.1 S. At high rotor speeds the value decreased to 3.8 S ± 0.1 S. The core complex has a diffusion coefficient, $D_{20,w}$, of 5.4×10^{-7} cm^2/sec and a molecular weight of 108,000 ± (SD) 2500.**

Biochemical, x-ray, neutron-scattering, and electron microscopic evidence supports the view that the primary structural unit of eukaryotic chromatin is the nucleosome (for reviews see refs. 1 and 2). Each nucleosome appears to involve a stretch of 140 base pairs of DNA wound around a protein core consisting of two each of the "inner" histones, H2A, H2B, H3, and H4. Such nucleosome core particles are linked together by a variable length of DNA to which one of the other major histones, H1, or, in special cases H5 or H6, could bind.

One of the major unsolved problems of nucleosome structure is the manner in which the four histones, H2A, H2B, H3, and H4, are arranged among themselves within the nucleosome core. There seems to be general agreement that the conformational properties of the core histones in 2 M NaCl are similar to those obtained in intact chromatin (3–8). There is no agreement, however, about the nature of the histone complex present and hence about the structure of the histone core in chromatin. Thomas and Kornberg (4) on the basis of crosslinking experiments inferred that both in chromatin and in 2 M NaCl the histones occur as octamers, in conformity with the earlier proposal of Kornberg (9). Thomas and Butler (10, 11) by using hydrodynamic and crosslinking methods have corroborated the octamer hypothesis by determining the molecular weight of the oligomer of histones prepared by them in 2 M NaCl to be about 107,000. The internal makeup of the octamer is based on the $(H3)_2$·$(H4)_2$ tetramer and (H2A·H2B) dimer, arranged as dimer–tetramer–dimer (4). Eickbush and Moudrianakis (12) have shown that the bonds linking the dimers to the tetramer are relatively weak.

On the other hand, Weintraub *et al.* (3) and others (7, 13) have published results showing the molecular weight of the histone oligomer in 2 M NaCl to be about half that expected of an octamer. Weintraub *et al.* (3) suggested that the stable histone species present in 2 M NaCl is a heterotypic tetramer (H2A·H2B·H3·H4). The relative orientation of the individual histones in the tetramer is undetermined. Two heterotypic tetramers are envisaged to be paired to form the nucleosome core protein (3, 6, 14). Chung *et al.* (15) presented hydrodynamic and chemical data on the histone core complex in 2 M NaCl that support such an association of heterotypic tetramers to form the octamer.

When histones H2A, H2B, H3, and H4 are present simultaneously in solution, in the absence of DNA, they constitute interacting systems of heterocomplexes (16, 17). The strength of interactions among the various histone pairs, at low ionic strength and neutral pH, is unequal and varies in the order H3/H4 > H2B/H4 ≈ H2A/H2B > H3/H2A (18). The equilibria among histones in solution are governed by pH, ionic strength, temperature, protein concentration, and possibly also by the nature of the buffer ions present (16). When all four histones are simultaneously present in equimolar proportions at low ionic strength it appears that predominantly heterodimers are formed (17). Higher oligomeric structures of histones are present in 2 M NaCl. In general, the formation of higher molecular weight oligomers in aqueous protein systems involves the formation of hydrophobic and ionic bonds. The formation of these bonds requires a decrease in ordered water structure about the groups participating in the bonding. This leads to a positive volume change of about 10–20 ml/mol of bond (19–22). It has been shown that in interacting systems even extremely small changes in the specific volume can lead to marked effects during high-speed centrifugation as a result of the strong pressure gradients generated in the ultracentrifuge cell or tube (23–25). Ultracentrifugation has so far been the method of choice to separate histones extracted in 2 M NaCl from the residual chromatin material (see, for example, refs. 3, 10, 12, 13, and 15). It appeared to us that the disagreement about the nature—octameric, tetrameric, or otherwise—of the basic histone unit in 2 M NaCl, and hence in chromatin, is very likely due to the different association–dissociation equilibria resulting from differences in conditions under which histones are isolated.

In order to investigate this possibility we have recently worked out a gentle but rapid method of isolating the core histones in 2 M NaCl at neutral pH (26). This method does not employ high-speed ultracentrifugation. We have examined the properties of the core histones isolated in this manner. In this paper we show that the histone complex so isolated has the sedimentation coefficient, diffusion coefficient, and molecular weight expected of an octamer. We also show that the sedimentation coefficient is dependent on rotor speed and that it undergoes an abrupt transition from a stable higher value at low rotor speeds to a stable lower value at high rotor speeds. The lower value of the sedimentation coefficient is the same as that reported in the literature for the heterotypic tetramer.

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MATERIALS AND METHODS

Preparation of Core Protein. The core protein was prepared as described before (26) with a slight modification. Briefly, the modified method consists in adsorbing and lysing purified rat liver nuclei on calcium phosphate gel, removing histone H1 by repeated washing with 0.8 M NaCl/25 mM sodium phosphate buffer, pH 6.0, and eluting the core histones, H2A, H2B, H3, and H4, with 2 M NaCl in 25 mM sodium phosphate buffer, pH 7.0.

Analytical Ultracentrifugation. A Spinco model E analytical ultracentrifuge was used with the An-D rotor. The rotor temperature was maintained at 4°C. A scribed capillary-type centerpiece was used for synthetic boundary sedimentation velocity experiments in a 12-mm double-sector cell. The histone samples were dialysed against 2 M NaCl/25 mM sodium phosphate buffer, pH 7.0, for 6 hr and the dialysate was used in the solvent sector (0.45 ml) and the protein was used in the solution sector (0.15 ml). After boundary formation at 8000 rpm the rotor speed was increased to the desired value and photographs were taken at 4-min intervals by using schlieren optics and a bar angle of 60°. Boundary positions were measured on a microcomparator and the sedimentation coefficient was calculated from the gradient of the plot of ln r (r being the distance of the boundary from rotor center) against time.

Determination of the Apparent Specific Volume. The apparent specific volume (φ') of the core protein in 2 M NaCl/25 mM sodium phosphate buffer, pH 7.0, at 4°C was calculated from the measured densities of the dialysate (ρ_0) and the solution (ρ) by using the relationship

$$\varphi' = \frac{1 - \frac{\rho - \rho_0}{c}}{\rho_0}$$

Densities were measured with a Digital Density Meter DMA-60 (Anton Paar, Graz, Austria) calibrated with air and distilled water. The temperature was maintained at 4°C. The protein concentration (c) was obtained from dry weight determination.

Calculation of Sedimentation Coefficient at Atmospheric Pressure and $s_{20,w}$. The sedimentation coefficient, s_p, of a pressure-dependent system at pressure p atmospheres is related to that at 1 atmosphere pressure (s_1) by the equation (27):

$$s_p = s_1(1 - Kp) = s_1\left[1 - \frac{K\rho\omega^2}{2}(r^2 - r_m^2)\right]$$

In this expression r and r_m are the distances of the boundary and the meniscus, respectively, from the rotor center; ρ is the density; and K is the dependence of the density, viscosity, and specific volume on pressure. s_p was obtained as a function of rotor speed in synthetic-boundary sedimentation velocity experiments after formation of the boundary at nearly the same position in the cell in all cases. The values were then plotted against the square of the angular velocity, ω^2, and extrapolated to zero ω^2 to obtain the sedimentation coefficient at 1 atmospheric pressure. $s_{20,w}$ was calculated by using the standard procedure (28).

Determination of Diffusion Coefficient. The diffusion coefficient of the protein species was obtained from the synthetic-boundary sedimentation velocity experiment at 16,000 rpm. The squares of the ratios of the area to maximum height, $(A/H_{max})^2$, of the schlieren patterns were plotted against time and the diffusion coefficient, D, was calculated from the slope ($4\pi D$).

Estimation of Histone Concentration. Histone concentrations were estimated from the number of fringes in interference photographs, taking four fringes as 1 mg/ml (29).

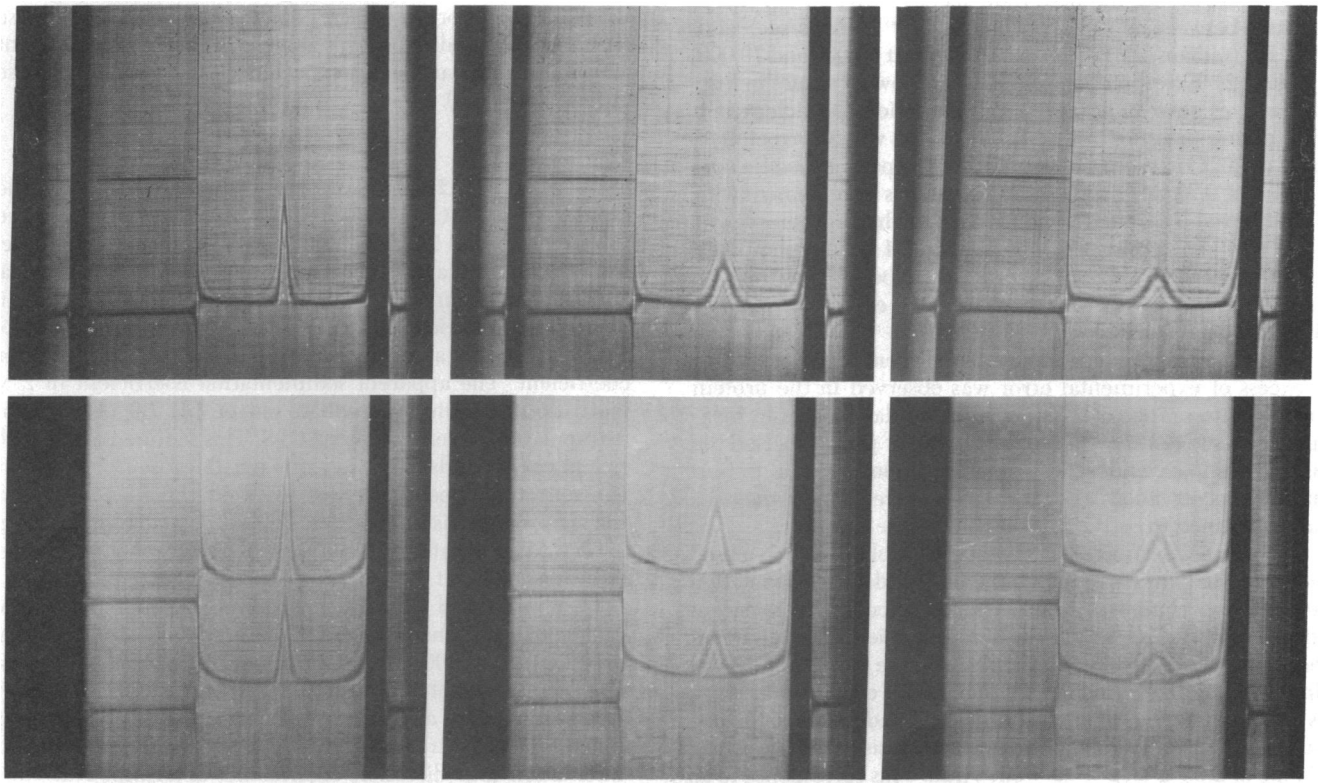

FIG. 1. Sedimentation pattern of the histone core complex in synthetic-boundary sedimentation velocity experiments at low and high rotor speeds. The top panel shows photographs of the schlieren patterns at 4, 40, and 72 min after attainment of operational speed (16,000 rpm). Protein concentration was 5.4 mg/ml. The bottom panel shows photographs of the schlieren patterns at 4, 24, and 52 min after attainment of 40,000 rpm. In this experiment, two cells, one with a positive wedge window and the other with a plain window, were used simultaneously. Protein concentration in the wedge cell (top pattern) was 6 mg/ml and in the plain cell was 4 mg/ml. Experimental details are given in the text.

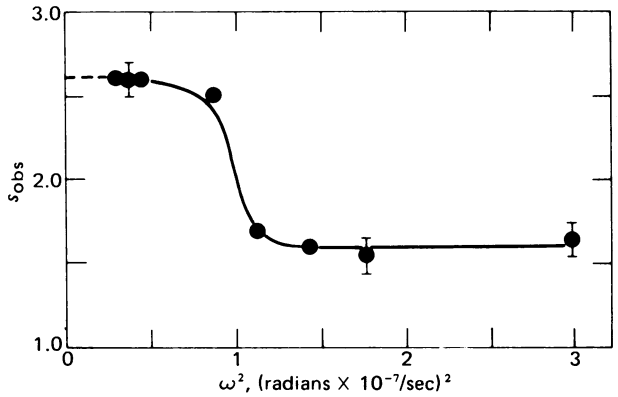

FIG. 2. Variation of the apparent sedimentation coefficient (s_{obs}) of the core complex with angular velocity of centrifuge rotor. The bars indicate SEM of the s_{obs} in three independent determinations.

RESULTS

Sedimentation Coefficient of Core Protein Varies with Rotor Speed and Attains Stable Values at Low and High Rotor Speeds. In a previous paper (26) we presented polyacrylamide gel electrophoretic data that suggested that the core protein prepared by our method consisted of a specific complex of approximately equimolar amounts of histones H2A, H2B, H3, and H4. Joffe *et al.* (30) and Rall *et al.* (31) showed earlier that in chromatin the core histones are present in equimolar proportions. Examination of the sedimentation behavior of the histone complex in the analytical ultracentrifuge showed that it moved as one symmetrical boundary at several rotor speeds (Fig. 1). Plots of ln r against time were linear at high and low rotor speeds but the sedimentation coefficient attained different stable values at low and high rotor speeds with a rather sharp transition between ω^2 values of about 9×10^6 (radians/sec)^2 and 1.1×10^7 (radians/sec)^2—i.e., between about 63,000 and 79,000 $\times g$ (Fig. 2). Extrapolation of the data at low ω^2 values in Fig. 2 to zero ω^2 gave an apparent sedimentation coefficient at 1 atmospheric pressure of 2.63 S. This value when corrected to water at 20°C gave an $s_{20,w}$ of 6.6 S. An apparent specific volume, φ', of 0.73 ml/g was used. This value is in agreement with that reported by Chung *et al.* (15) for the histone complex in 2 M NaCl. The density of 2 M NaCl/25 mM sodium phosphate buffer, pH 7.0, at 4°C was determined to be 1.083 g/ml. The viscosity of the solvent was taken as 1.233 centipoise at 20°C (32) (1 poise = 0.1 Pa·sec).

No concentration dependence of sedimentation coefficient in excess of experimental error was observed in the protein concentration range of 2–6 mg/ml. Circular dichroism measurements suggest that below about 2 mg of protein per ml the histone complex undergoes a conformational transition (unpublished observations). For these reasons we consider the $s_{20,w}$ value obtained to be the $s^0_{20,w}$ of the histone complex.

Diffusion Coefficient of Histone Complex. We have used the schlieren patterns of the synthetic-boundary sedimentation velocity experiment at 16,000 rpm to calculate an apparent diffusion coefficient, D, for the histone species occurring under the experimental conditions. Fig. 3 shows the plot of ln r against time used to determine the sedimentation coefficient as well as a plot of $(A/H_{max})^2$ against time used to determine the diffusion coefficient. A linear regression analysis of the latter gave a slope of $2.00 \pm 0.02 \times 10^{-4}$ cm^2/min. This corresponds to an apparent diffusion coefficient of 2.65×10^{-7} cm^2/sec. The same value was obtained if a D_{app} was calculated from each of the schlieren patterns and extrapolated to infinite time as suggested by Schachman (28). When corrected to water at 20°C

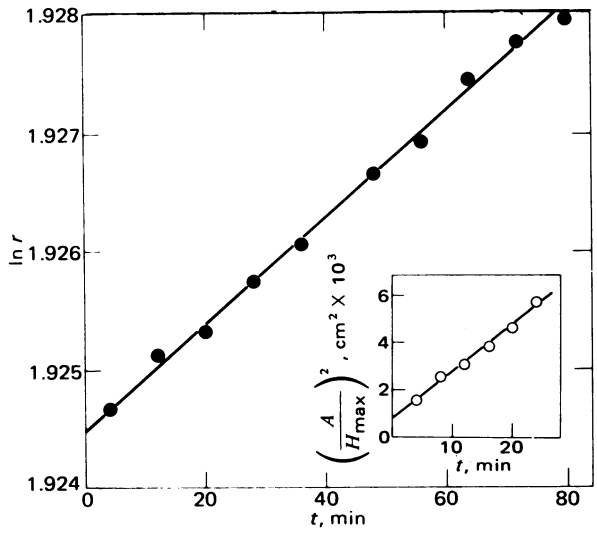

FIG. 3. Plot of ln r against time of the sedimentation velocity data at 16,000 rpm and the plot of $(A/H_{max})^2$ of the schlieren patterns against time. (*Inset*) Each schlieren pattern was assumed to be an isosceles triangle and A/H_{max} was obtained by measuring the width at half the maximum height on a microcomparator.

this value is equivalent to a $D_{20,w}$ of 5.4×10^{-7} cm^2/sec, which is reported here. This value is in the range expected of a species of the size of an octamer of histones.

Molecular Weight of Histone Complex. After a linear regression analysis of the sedimentation data in Fig. 2, an apparent sedimentation coefficient of 2.6 S was calculated for the histone complex at 16,000 rpm. Combination of the values of the apparent sedimentation and diffusion coefficients in the equation, $M_r = s/D \cdot RT/(1 - \varphi' \rho_0)$ (33) gave a molecular weight of 108,000 ± 2500 for the histone complex occurring under these experimental conditions. This value is in excellent agreement with the molecular weight expected of an octamer of these histones (108,700).

DISCUSSION

Histones H2A, H2B, H3, and H4 when isolated gently and rapidly in 2 M NaCl at neutral pH from rat liver nuclei moved as a single species in nondenaturing polyacrylamide gel electrophoresis (26) and sedimented as a single symmetrical boundary in the analytical ultracentrifuge (Fig. 1). The sedimentation coefficient, however, was found to vary with rotor speed, suggesting a pressure dependence of the sedimentation coefficient. The apparent sedimentation coefficient in 2 M NaCl/25 mM sodium phosphate buffer, pH 7.0, obtained by extrapolation to zero ω^2, was 2.63 S. This sedimentation coefficient corresponds to an $s_{20,w}$ of 6.6 S. Because no significant concentration dependence was observed, this $s_{20,w}$ is in effect the $s^0_{20,w}$ for the histone species. This $s^0_{20,w}$ value is in the range expected of a solvated globular protein of the size of an octamer of histones (see ref. 34).

Combination of the apparent sedimentation and diffusion coefficients obtained in a synthetic-boundary sedimentation experiment (16,000 rpm) in the Eisenberg equation (33) gave a molecular weight of 108,000 ± 2500 for the histone complex. Good estimates of anhydrous molecular weights of fairly large macromolecules are obtained (33) by the use of apparent sedimentation and diffusion coefficients determined at finite concentrations (28, 35), provided their determination is carried out under the same conditions (35), as has been done here. Furthermore, we have not observed significant concentration dependence in our system. For these reasons it is fair to conclude

that the molecular weight determined by us represents the true value of the histone species. The molecular weight taken together with the histone composition (26) is strong evidence that the histone complex isolated by our method is the octamer consisting of two molecules each of histones H2A, H2B, H3, and H4.

It is obvious from the above results that the chromatin core histones can be isolated in a relatively stable octameric state without the use of any crosslinking agent. Furthermore, because the method employed by us for the preparation of the histone octamer is quite gentle and because the octamer is obtained soon after dissociation from chromatin, it is reasonable to assume that its native conformation has been preserved to a large extent.

At this stage it is not possible to say anything specific about the shape of the octameric complex. From the molecular weight, the sedimentation coefficient, and the apparent specific volume of the histone octamer a frictional coefficient (f/f_0) of 1.23 ± 0.02 was obtained. This value suggests that the shape of the histone octamer is not too different from that of a solvated globular protein.

Thomas and Butler (10, 11) reported a molecular weight of $107{,}500 \pm 7700$ for the octamer of histones isolated by them in 2 M NaCl, but they determined a much higher apparent specific volume (0.767 ml/g) compared to our value of 0.73 ml/g. Even so, the sedimentation coefficient obtained by them (4.8 S), as they themselves pointed out, is much too low for its molecular weight unless one assumes a very high degree of hydration or asymmetry or both. They used a rotor speed of 59,000 rpm. Our results show that the octamer of histones in 2 M NaCl undergoes a sharp conformational transition near about 28,000 rpm (about $60{,}000 \times g$). If we use the apparent specific volume given by Thomas and Butler (10) and our sedimentation data at 52,000 rpm we too obtain an $s_{20,w}$ of 4.8 S, but we did not observe the appreciable concentration dependence of the sedimentation coefficient observed by Thomas and Butler. Our preparation differs from that of Thomas and Butler (10) in yet another property. Thomas and Butler (10) state that their preparation is stable at 4°C and 37°C. Our preparation is unstable at and above about 10°C, as judged by gel-filtration experiments, and precipitates at 37°C (unpublished results). A temperature-dependent dissociation was observed also by Eickbush and Moudrianakis (12) during gel filtration and sedimentation experiments on an octameric complex isolated by them.

Thomas and Butler (10, 11) reported the sedimentation coefficient of the crosslinked octamer to be 5.3 S, a value higher than that of the untreated complex (4.8 S). Thomas and Butler attribute this higher value to a tightening of the structure by the crosslinking and to a probable contribution to the mass by the linking agent. But even their higher value is lower than that obtained by us for the untreated core protein at atmospheric pressure. It is possible that even the crosslinked material could undergo a conformational change at high centrifugal forces. The intriguing possibility that the crosslinked octamer had an altered conformation to begin with cannot also be ruled out. It may be noted that Thomas and Butler (10) subjected the histones in solution in 2 M NaCl to a high centrifugal force for several hours before crosslinking.

The question whether the histone species with the sedimentation coefficient of 3.8 S formed at high rotor speeds is the same as the heterotypic tetramer cannot be answered at present. It is also not clear whether the octamer can be reformed to its native state after the centrifugal field is removed. The sharpness of the conformational transition, however, is suggestive of an irreversible process. The experiments of Chung *et al.* (15) suggest that the rate of reformation of the octamer is extremely low. Thomas and Butler (11) also state that the dissociation of the histone octamer is not wholely reversible. The results of Eickbush and Moudrianakis (12) indicate the contrary. The reason for this discrepancy is not clear. It is possible that in the experiments of Eickbush and Moudrianakis the high protein concentration (8 mg/ml) and the Sephadex G-100 gel filtration facilitated reassociation. A possible aggregating influence of Sephadex gels on histone complexes has been reported (36). Eickbush and Moudrianakis (12) observed that their preparation of an octameric histone complex eluted from a Sephadex G-100 column as if its molecular weight were 135,000 whereas in a sucrose density gradient the same complex sedimented as though its molecular weight were only 55,000. They attributed this radically different behavior of the complex in the two transport methods to its nonglobular nature. These results could also mean a pressure-dependent dissociation of the complex in the centrifugal field. In any case, it is apparent from the foregoing that pressure effects should be taken into account when investigating histone complexes by ultracentrifugal methods.

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