THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 8, Issue of April 25, pp. 3629–3637, 1980
Printed in U.S.A.

Core Histone Associations in Solutions of High Salt

AN OSMOTIC PRESSURE STUDY*

(Received for publication, October 19, 1979)

Arnold Stein and David Page‡

From the Developmental Biochemistry Section, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland 20205

We have measured the concentration dependence of the osmotic pressure for solutions of core histones in 2.0 M NaCl, pH 8.0, over the concentration range from 1 to 20 mg/ml at 25°C. The ratio of the osmotic pressure to the protein concentration is nearly constant over this range, with a value corresponding to an apparent number average molecular weight of 75,000. Gel filtration of a typical core histone preparation at 5°C at an initial concentration of 4.6 mg/ml resulted in the elution of 10% of the sample by weight as low molecular weight material consisting only of histones H2A + H2B, likely a dimer complex; 90% of the sample eluted at a volume close to that for a cross-linked histone octamer marker. The presence of this amount of low molecular weight material in the sample requires that the remaining material have a number average molecular weight of at least 92,500 to yield a measured value of $\bar{M}_n$, over the whole sample, of 75,000. The existence of an appreciable amount of histone tetramers is therefore excluded for core histone concentrations greater than about 2 mg/ml. $\bar{M}_n$ for the purified high molecular weight fraction, however, was only 80,000, indicating that histones H2A + H2B interact with a larger complex in an association equilibrium. At 35°C, the concentration dependence of the osmotic pressure clearly indicated that an association equilibrium occurred. We show that the concentration dependence of the osmotic pressure for a solution of total core histones at 25°C can be described well by a dimer + hexamer ⇌ octamer equilibrium with $K = (1.5 \pm 0.5) \times 10^5$ M^{-1} and a second osmotic viral coefficient, $B = (1.8 \pm 0.17) \times 10^{-4}$ cm^3 mol g^{-2}, where the dimer and hexamer have the compositions (H2A H2B) and (H3 H4)$_2$ (H2A H2B), respectively. We also found $s^\circ_{20,w}$ for the equilibrium mixture was 4.5 and have performed sedimentation equilibrium measurements on the same material as used in the osmometry for comparison with the results of others.

In chromatin, histones H2A, H2B, H3, and H4 exist as octameric complexes of two each of these species (1); DNA is wrapped around these octamers to form compact particles called nucleosomes (2–4). Differential susceptibilities of active and repressed genes to nucleases suggest that the structure of nucleosomes differs for these two types of chromatin regions (5, 6). Indeed, there is evidence which suggests that in transcriptionally active regions of chromatin, nucleosomes are in an extended conformation (see Ref. 7 and references contained therein), and specific models have been suggested for how nucleosomes may extend (8, 9). Because interactions between the histones likely contribute to the stability of the nucleosome, it is of interest to understand how the core histones interact with each other. In solution, each of the core histones can interact with each other (10); strong interactions can occur in the pairs H2A + H2B, H3 + H4, and H2B + H4 (10–13). Moreover, the arginine-rich histones form a very stable (H3 H4)$_2$ tetramer (12, 13). However, which interactions are preferred in equimolar mixtures of all four core histones has proven difficult to determine and it is still a matter of controversy.

The interactions that occur in 2 M NaCl, pH 7 to 9, have been of particular interest, mainly due to initial observations that under these conditions salt-extracted core histones appeared to form a nearly homogeneous complex as assessed by sedimentation and gel filtration (14), and by cross-linking with dimethylsuberimidate (1). Additionally, high salt concentrations possibly provide an environment similar to that provided by DNA in chromatin by reducing the electrostatic repulsion between the highly charged histones (14, 15).

Controversy has arisen over the value of the molecular weight of this high salt histone complex and, consequently, which histone-histone interactions are preferred. Weintraub *et al.* (14) reported a molecular weight of 51,000 based upon low speed sedimentation equilibrium measurements, and 60,000 by gel filtration in combination with sedimentation velocity data. They suggested that the histone complex was predominantly a "heterotypic tetramer" consisting of one each of the core histones. In contrast, the cross-linking studies of Thomas and Kornberg (1) indicated that histones exist as octamers in solution, under essentially the same conditions. A tetramer molecular weight was then supported by light- (16) and neutron-scattering (17) studies. However, Thomas and Butler (18) obtained an octamer molecular weight (107,500) using the low speed sedimentation equilibrium method, in a study which included the required measurement of the solute density increment. Using the same method, Chung *et al.* (19) reported that the histone complex in 2 M NaCl was, in fact, not homogeneous, but that histones participated in an association equilibrium. They measured apparent molecular weights ranging from 60,000 at a sample concentration of 0.5 mg/ml to a maximum value of 80,000 at about 5 mg/ml; at higher concentrations the apparent molecular weight decreased due to thermodynamic nonideality. They suggested that heterotypic tetramers associated rather weakly to form octamers. Recently, Eickbush and Moudrianakis (20) also reported a concentration-dependent molecular weight using the high speed sedimentation equilibrium method. However, they observed a maximum value for the apparent weight average molecular weight of about 95,000 at a concentration

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‡ Portions of this work were submitted as part of a Senior Thesis in the Swarthmore College Honors Program.

of 1.5 mg/ml, thus differing from the measurements of Chung *et al.* (19). These investigators suggested that the concentration-dependent molecular weight reflects the assembly of the histone octamer from the $(H3\ H4)_2$ tetramer and 2 (H2A H2B) dimers. Most recently, two additional high speed sedimentation equilibrium studies were reported, both over approximately the same concentration range (<1 mg/ml); one reported a weight average molecular weight of 98,000 (21), and the other, 54,000 for a heterogeneous mixture (22).

Thus, the molecular weights obtained by sedimentation equilibrium are in poor agreement with each other, while those obtained by two other methods (light and neutron scattering) were consistent with a tetramer. In order to help clarify this problem, we have used one of the most reliable methods for measuring protein molecular weights (23), high precision osmometry. Osmometry is very well suited to this problem: 1) the auxiliary measurement of the protein density increment is not required; measurement of this quantity for samples in high salt solutions is difficult (22), and disagreement in the value of the density increment is a major source of uncertainty in the sedimentation equilibrium studies; 2) the overall number average molecular weight and second osmotic virial coefficient, which also contains useful information, are directly obtained from the osmotic pressure measurements; in all other studies reported, the weight average molecular weight was obtained directly which, of course, is slanted toward the largest species present; 3) the measurements are made at atmospheric pressure; the high hydrostatic pressure in the case of high speed sedimentation equilibrium measurements could possibly perturb the chemical equilibrium (24); and 4) small amounts of DNA contaminating the histone preparation have a negligible influence on the osmotic pressure in contrast to sedimentation equilibrium measurements using the photoelectric scanner; for example, 1% (by weight) DNA contamination of a histone preparation will contribute more than 20% to the absorbance at 280 nm.

MATERIALS AND METHODS

Preparation of Histones—Chicken erythrocyte nuclei were prepared as described by Hymer and Kuff (25). Nuclei (100 ml) at 100 A_{260} units/ml in 0.25 M sucrose, 10 mM Tris/HCl, pH 8.0, 1 mM $CaCl_2$ were digested with micrococcal nuclease (Worthington) at 200 units/ml for 15 min at 37°C. The nuclei were pelleted by centrifugation, suspended in about 40 ml of 0.25 mM Na_3EDTA, pH 8.0, and dialyzed overnight against 5 liters of 0.25 mM Na_3EDTA, pH. 8.0, 0.1 mM phenylmethylsulfonyl fluoride (a protease inhibitor). The sample was centrifuged at 6,000 × *g* for 15 min; aliquots of the supernatant were adjusted to 0.6 M NaCl, 10 mM Tris/HCl, pH 8.0, by addition of buffered 5 M NaCl, and the material was fractionated on SW 27 sucrose gradients (2.5 ml/tube) containing 0.6 M NaCl, 10 mM Tris/HCl, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride at 5°C. The salt-washed chromatin was then adjusted to 2.5 M NaCl (by addition of solid NaCl and gentle stirring), 0.05 M sodium phosphate, pH 7.0, 0.1 mM phenylmethylsulfonyl fluoride, and the DNA was removed by batch extraction twice with hydroxylapatite (Bio-Rad, DNA grade) using gentle shaking for 15 min at 5°C. Histones were concentrated at 5°C using an Amicon apparatus with a YM 10 membrane; the histones were then dialyzed against 2.0 M NaCl, 10 mM Tris/HCl, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride. A small portion of this material was dialyzed extensively against the same buffer minus the phenylmethylsulfonyl fluoride for spectral analysis. The A_{230}/A_{260} ratio (measured using a Cary 219 spectrophotometer) for the histone solution *versus* the dialysate was 18 ± 0.5; A_{280}/A_{260} was 1.89 ± 0.05 and A_{278}/A_{260} = 2.00 ± 0.05 for all preparations. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (26) showed that the core histones were present in equal amounts, by comparison with sodium dodecyl sulfate-extracted histones from chromatin core particles loaded directly on the gel, and that no H1 or H5 histone or histone degradation products were present (Fig. 1).

Cross-linked histone octamers were prepared by cross-linking chromatin that had been sedimented through 0.6 M NaCl, as described above, with dimethylsuberimidate in 2.0 M NaCl, 0.1 M sodium borate, pH 9.0 (1). DNA was removed by hydroxylapatite batch extraction, as above, and octamers were purified by sedimentation on SW 41 sucrose gradients containing 0.1 M NaCl, 0.01 M sodium phosphate, pH 7.0.

Core Histone Concentration Determination—The concentration of the sample was determined from the absorbance at 275.5 nm in 0.1

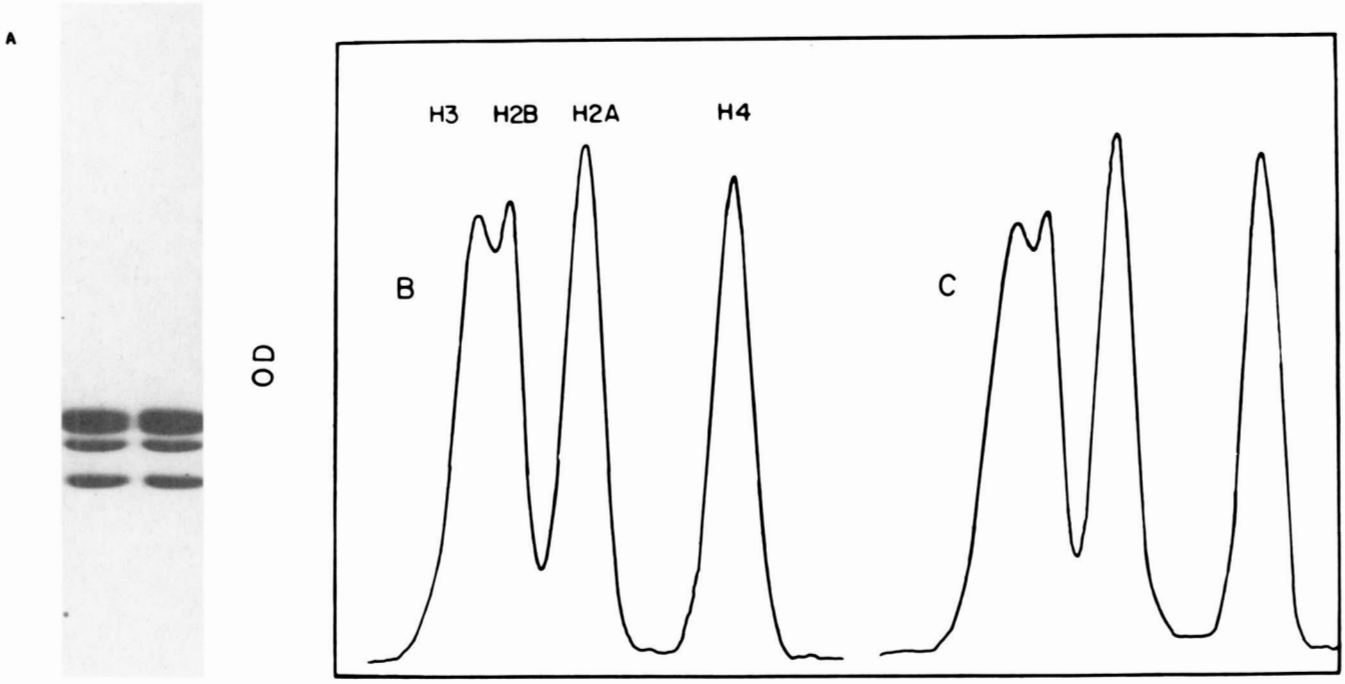

FIG. 1. **Sodium dodecyl sulfate polyacrylamide gel electrophoresis of a typical histone preparation and chromatin core particle histones.** Samples were prepared for electrophoresis by dissolving 2 M salt core histones or core particles directly into sample buffer. *A*, salt-extracted histones used in measurements (*left*), core particle histones (*right*); *B*, densitometer tracing of core particle histones; *C*, tracing of salt-extracted histones.

N HCl, 0.1 M NaCl by comparison with a standard solution of acid-extracted core histones, having an identical UV spectrum, prepared by weight. Acid-extracted histones were prepared from nuclei washed several times with 0.3 M NaCl, then suspended in water; the histones were extracted with 0.25 N HCl, precipitated with acetone, and dried under vacuum. The histones were then dissolved in water, and adjusted to 5% perchloric acid to precipitate only the core histones, thereby removing H1 and H5 (27); core histones were washed several times with 5% perchloric acid, redissolved in 0.25 N HCl, precipitated with acetone, and dried extensively under vacuum. Standard histone solutions were prepared in triplicate; 40 to 50 mg of dried core histones were weighed and dissolved in 20.0 ml of 0.1 N HCl, 0.1 M NaCl. The histones dissolved readily and completely, and the UV absorption spectrum was measured. The absorption coefficient, $A^{1\%}_{275.5}$, was determined to be 3.67 ± 0.02, in close agreement with the value of D'Anna and Isenberg (10), 3.7, for calf thymus histones in water; the spectrum in 0.1 N HCl and in water is the same.[1] Consistent with the large spectral changes observed upon ionization of the phenolic chromophore of tyrosine (28), the UV spectrum of histones in 2.0 M NaCl, pH 8.0, differs appreciably from that in acid or water. Therefore, to relate the absorption coefficient of salt-extracted histones in 2.0 M NaCl, 10 mM Tris/HCl, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride to that in 0.1 N HCl, a concentrated 2 M salt histone sample (25 mg/ml) was diluted 20-fold with 0.1 N HCl or the 2 M salt buffer and the spectra compared; each sample was prepared in triplicate. We found that $A^{1\%}_{280}$ (2.0 M NaCl, pH 8.0)/$A^{1\%}_{275.5}$ (0.1 M HCl) = 1.06 ± 0.03. Additionally, the spectrum for the 2 M salt-extracted histones in acid was identical with that of the acid-extracted standard histone solution. Therefore, if contaminating DNA contributed to the absorbance, the extent of DNA contamination in both samples should be the same. Since the maximal amount of DNA contamination possible is negligible on a weight basis, this method is valid even if small amounts of DNA are present. Thus, $A^{1\%}_{280}$ for salt-extracted histone chlorides in 2.0 M NaCl, pH 8.0, is 1.06 × 3.67 = 3.9 ± 0.1.

Based upon the amino acid contents of the core histones (29), the absorption coefficient of unionized histones should be greater than that for histone chlorides by a factor of 1.08 (giving a value of 4.2) if only the lysyl and arginyl residues were protonated and thus neutralized by Cl^- ions when the histones were precipitated from HCl. It is likely that some additional Cl^- ions were also bound, and therefore we have used a larger absorption coefficient of 4.4 ± 0.1 here. Use of the lower value (4.2) would result in a 5% increase in the value of the molecular weight determined by osmotic pressure.

Our value of $A^{1\%}_{280}$ (4.4 ± 0.1) corresponds to $A^{1\%}_{275}$ = 4.4 ± 0.1, in agreement with the reported value (4.3) of Ruiz-Carrillo and Jorcano (21) determined by both nitrogen determination and amino acid analysis, and to an extinction coefficient at 230 nm of 4.2 ± 0.1 liters g^{-1} cm^{-1}, in agreement with the value (4.2 ± 0.1) of Chung *et al.* (19) by both amino acid analysis and dry weight determination.

Osmotic Pressure Measurements—Osmotic pressure measurements were made with a Mechrolab model 503 membrane osmometer (Hewlett Packard). Briefly, the instrument is based on the dynamic method and operates to null changes in volume (less than 10^{-9} liter solvent flow) on the solvent side of the membrane by continuously adjusting the hydrostatic pressure on the solvent; the servo signal derives from a photocell which senses light refracted by the motion of a bubble in a capillary tube on the solvent side. The instrument is thermostatted, and the temperature of the sample block was measured with a YSI Telethermometer. Membranes were punched from Spectrapor No. 1 membrane tubing (50 mm diameter, M_r = 6,000 to 8,000 cutoff). The osmotic pressure of the protein solution was the pressure reading relative to that obtained for buffer alone; pressures were measured with a precision of ±0.02-cm buffer. The sample compartment (about 0.3 ml) was rinsed at least three times with 0.1 ml of sample solution and then 0.3 ml or more was siphoned in; this procedure was found to provide satisfactory rinsing. Approximately 1 h was required to reach equilibrium. Buffer *versus* buffer readings were checked several times throughout a run, and were often found to be stable to ± 0.02 cm for more than 10 h at 25 and 35°C. We found that erratic readings occurred at 5°C, likely due to the inherent properties of the solvent, membrane, and this osmometer design; thus, measurements could not be made at low temperatures. A molecular weight of 72,000 was obtained in a test run with bovine serum albumin (bovine albumin crystallized, Miles Pentex) for a solution prepared by weight with material directly from the supplier; this is a reasonable value for such a sample which likely contained dimers (30).

The concentration dependence of the osmotic pressure is given most generally by the expression (31)

$$\frac{1}{RT}\frac{\pi}{c} = \frac{1}{\bar{M}_n} + Bc \qquad (1)$$

where R is the gas constant, 78.52 liter-cm buffer deg^{-1} mol^{-1} at 25°C, using a buffer density of 1.079 g/cm^3 (32), T is the absolute temperature, c is the protein concentration in milligrams per ml, π is the osmotic pressure, and B is the second osmotic virial coefficient. $\bar{M}_n$ is the number average molecular weight, defined by

$$\bar{M}_n = \frac{\Sigma_i n_i M_i}{\Sigma_i n_i} \qquad (2)$$

where n_i is the number of moles of the species with molecular weight M_i. In terms of weight concentrations, c_i,

$$\bar{M}_n = \frac{c}{\Sigma_i \frac{c_i}{M_i}} \qquad (3)$$

Thus, according to Equation 1, a linear extrapolation of $1/RT\ \pi/c$ to $c = 0$ gives $1/\bar{M}_n$. However, in an associating system, $\bar{M}_n$ is a function of c and this must also be taken into account.

An apparent number average molecular weight, $\bar{M}_n^{app}$, can be defined as $RT/(\pi/c)$; clearly $\bar{M}_n^{app}$ must be lower than the true $\bar{M}_n$ for a positive value of B. This disparity will increase greatly at higher concentrations.

Analytical Sedimentation Measurements—Sedimentation velocity measurements were made with a Beckman model E analytical ultracentrifuge using the Schlieren optical system; the An H rotor was used at 60,000 rpm, 20°C. Plates were analyzed using a Nikon Microcomparator at × 10 magnification.

Sedimentation equilibrium measurements were made with the model E using the photoelectric scanner; the AnG Ti rotor was used at 8,000 rpm and equilibrium scans were taken after 48 h. The temperature and wavelength were as stated. $\bar{M}_w^{app}$ was calculated by the relation:

$$\bar{M}_w^{app} = \frac{2RT\Delta c/c_0}{\omega^2(1 - \phi'\rho)(r_b^2 - r_m^2)} \qquad (4)$$

where R is the gas constant, T is the absolute temperature, Δc is the concentration change across the cell, c_0 is the initial concentration, ω is the angular velocity, ϕ' is the apparent partial specific volume of the solute, ρ is the density, and r_b and r_m are the radial positions of the cell bottom (oil layer) and meniscus, respectively.

RESULTS

Concentration Dependence of the Osmotic Pressure—The osmotic pressure (π) of a histone solution as a function of concentration (c) at 25°C is shown in Fig. 2, where π/c is plotted *versus* c; three independent runs on three different

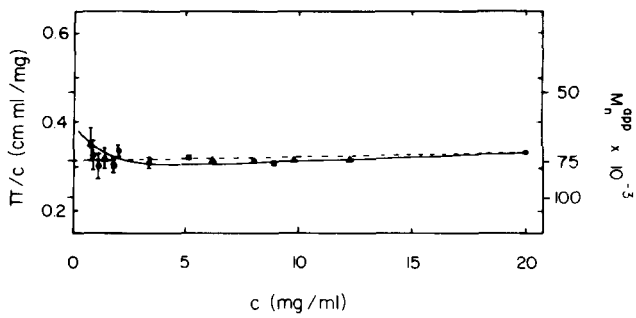

FIG. 2. **Concentration dependence of the osmotic pressure at 25°C.** RT = 2.34 × 10^4 liter-cm buffer mol^{-1}. The symbols ■, ▲, and ● refer to independent runs, 1, 2, and 3, respectively, on different core histone preparations. $\bar{M}_n^{App}$ (outer tick marks) is defined as $RT/(\pi/c)$ (see "Materials and Methods"). The *broken line* is an empirical straight line through the points; the *solid curve* is given by Equation 12 with K = 1.5 × 10^5 M^{-1} and B = 1.77 × 10^{-4} cm^3 mol g^{-2}.

[1] A. Stein and D. Page, unpublished observations.

core histone preparations are in close agreement. Error bars reflect the precision of the pressure measurements. Linear extrapolation of π/c to zero concentration (*broken line*) gives the value 0.312 cm ml mg^{-1} corresponding to a number average molecular weight for the whole sample, $\bar{M}_n$, of 75,000 by Equation 1 if no association equilibrium occurs at concentrations greater than 1 mg/ml. For an association equilibrium, the data require a more detailed analysis (see below). In any event, these measurements indicate that at a concentration of about 2 mg/ml, where the virial correction in Equation 1 is still small, the number average molecular weight is about 75,000. The second osmotic virial coefficient, B, estimated from the slope of the broken line is 1.7×10^{-5} cm^3 mol g^{-2}, a rather small value (see "Discussion"). Clearly, 75,000 can not correspond to a homogeneous histone complex since the sample contained equimolar amounts of the four histone species, each with molecular weights in the range of 11,300 to 15,300 (29). Consequently, the weight average molecular weight, $\bar{M}_w$, must exceed the value of $\bar{M}_n$ (33).

The degree by which $\bar{M}_w$ exceeds $\bar{M}_n$ depends strongly on the protein complexes present in the sample. $\bar{M}_n$ is quite sensitive to the presence of low molecular weight proteins in the sample. For example, for a sample containing 90% histone octamers by weight and 10% monomers, $\bar{M}_w$ would be 100,000, whereas $\bar{M}_n$ would be only 65,000. Therefore, it is important here to examine our histone preparations for the presence of low molecular weight proteins. The good agreement of the three independent runs indicates that the histone preparations are very reproducible. Such agreement with different preparations would not be expected if the samples contained varying amounts of low molecular weight contaminants; this is evident from the above example. Also, sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 1) indicated that the amounts of H1 + H5 histones, non-histone proteins, and histone degradation products possibly contaminating the samples were too low to be detected. Thus, to determine what fraction of the core histones exist as low molecular weight complexes, we subjected a standard preparation to gel filtration, keeping the histones at a fairly high concentration. Fig. 3*A* shows that 10% of the core histone preparation eluted as low molecular weight material, whereas, 90% eluted as a complex of substantially higher molecular weight at a volume very close to that for a purified cross-linked histone octamer marker (not shown). Consistent with observations by others (14, 20, 21), the low molecular weight fraction contained only histones H2A and H2B (Fig. 3*A*, inset) and the high molecular weight fraction was correspondingly depleted in H2A and H2B, as determined by densitometry (not shown).

A similar result was obtained upon fractionating the histones by sucrose gradient centrifugation at 25°C; again about 20% of the H2A + H2B was separated from the other histones (not shown). In contrast to the observation of Weintraub *et al.* (14), we found, by washing the emptied gradient tube with sodium dodecyl sulfate, that only about 1% of the histones pelleted; thus the dissociation of H2A + H2B from the complex was not matched by a corresponding dissociation of H3 + H4 to form aggregated material. Upon sedimenting the column fractionated material (main peak, Fig. 3*A*), some slow sedimenting material was observed (Fig. 3*B*, trailing edge of the band), suggesting that the purified histone complex dissociated to a small extent. These experiments indicate that our histone preparations do contain low molecular weight histone species, and therefore the remainder of the material must have a number average molecular weight greater than 75,000.

We investigated the nature of this sample heterogeneity further by measuring $\bar{M}_n$ for the high molecular weight material which eluted from the gel filtration column (Fig. 3*A*). If the low molecular weight histone species (10% of the total histone by weight) were, for example, noninteracting histone monomers and dimers arising from unequal extraction of histones from the chromatin, $\bar{M}_n$ for the fractionated material should increase measurably. The value of $\bar{M}_n$ expected for this case was calculated by averaging only over the remaining material (90% by weight) such that $\bar{M}_n$ for the whole sample was 75,000, the measure value. This average, $\bar{M}'_n$, is given by

$$\bar{M}'_n = \frac{1 - w_0}{\frac{1}{\bar{M}_n} - \frac{w_0}{M_0}} \qquad (5)$$

where w_0 is the weight fraction of the low molecular weight material of molecular weight, M_0. Here, $w_0 = 0.1$, and we assume $M_0 = 27{,}228$, the value for an (H2A H2B) dimer (29). With these values, $\bar{M}'_n = 92{,}500$. If a portion of the low molecular weight material were histone monomers, $\bar{M}'_n$ would be greater than 92,500. On the other hand, if the H2A + H2B histones participate in an association equilibrium with a larger complex, the removal of H2A + H2B by gel filtration would lead to a value of $\bar{M}'_n$ lower than 92,500 because the system will re-equilibrate by further dissociation, as suggested by Fig. 3*B*. We therefore measured $\bar{M}'_n$, for the purified high molec-

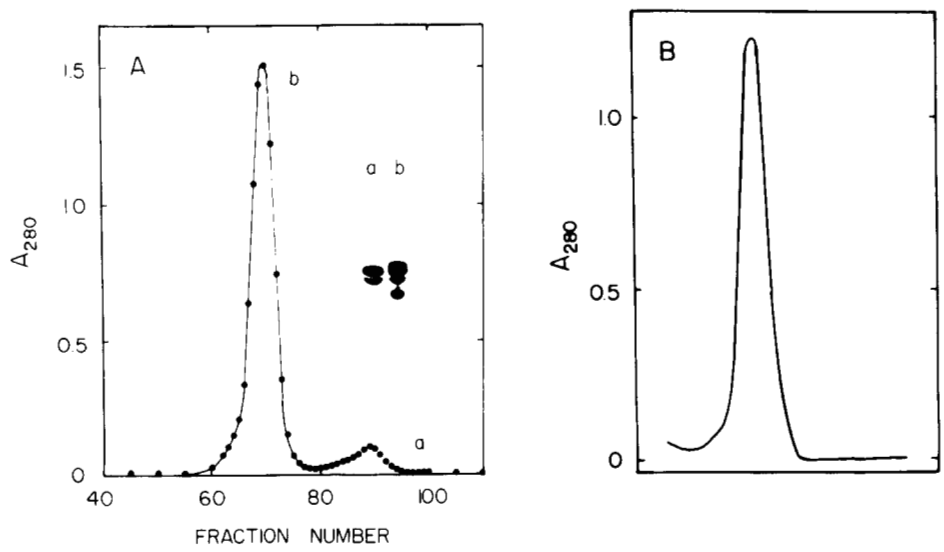

FIG. 3. **Fractionation of histones by gel filtration and sucrose gradient centrifugation.** *A*, 10 ml of a core histone preparation at 4.6 mg/ml were loaded on a Sephacryl S200 column (2.5 × 84 cm) equilibrated with 2.5 M NaCl, 10 mM Tris/HCl, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride at 5°C; the flow rate was 25 ml/h, and 7-min fractions were collected. Fractions 62 through 73 were pooled (*b*), concentrated, and used in the measurements; *Pool a* consisted of Fractions 85 through 93. The *inset* shows sodium dodecyl sulfate polyacrylamide gels on histone *Pools a* and *b*. *B*, histones from *Pool b* were sedimented on an SW 41 sucrose gradient in the same buffer at 25°C for 26 h at 38,000 rpm. Sedimentation was from left to right.

ular weight histone fraction eluted from the column. Fig. 4*A* shows that $\bar{M}'_n$ is not substantially greater than $\bar{M}_n$, for total core histones. The value of $\bar{M}'_n$, obtained by linear extrapolation of π/c to $c = 0$ is about 80,000, measurably below 92,500. This experiment indicates that H2A + H2B participate in an association equilibrium with a larger histone complex.

For concentrated protein solutions, an increase in π/c with concentration due to thermodynamic nonideality may oppose the decrease in π/c expected for an association reaction such that the negative slope characteristic of an associating system is not apparent. We had observed a decrease in the stability of the 2.0 M salt core histone complex with increasing temperature in gel filtration experiments at low histone concentrations (data not shown), consistent with the findings of Eickbush and Moudrinakis (20). Therefore, if the position of equilibrium is temperature-dependent, the association reaction may be more apparent at temperatures higher than 25°C. With this rationale, we measured the concentration dependence of the osmotic pressure at 35°C. Fig. 4*B* shows that π/c decreases with increasing c, as expected for an associating system. The value of π/c increases sharply at concentrations below 2 mg/ml indicating a substantial dissociation of the complex. At 1 mg/ml the apparent number average molecular weight, $\bar{M}_n^{app}$ is 56,600, while at 10 mg/ml $\bar{M}_n^{app}$ is 72,400. Histones recovered from the osmometer after the 35°C run were still completely intact as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and sedimented with a single symmetrical boundary at 20°C, identically as a control sample.

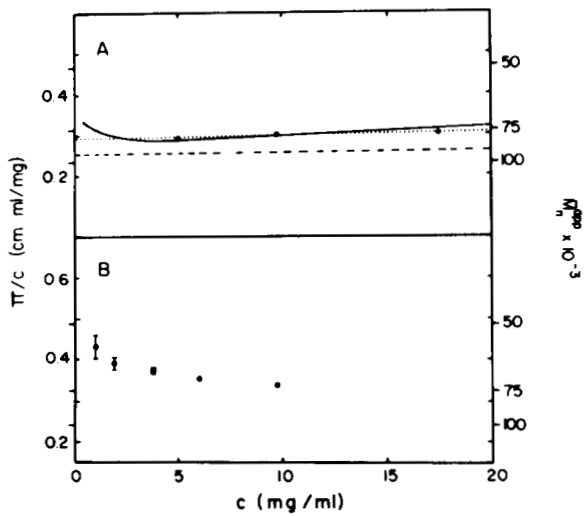

FIG. 4. **Concentration dependence of the osmotic pressure for fractionated core histones at 25°C (*A*) and core histones at 35°C (*B*).** *A*, osmotic pressure measurements were made on the histones from *Pool b*, Fig. 3, under the same conditions as for total core histones (Fig. 2). The *dotted line* is an empirical straight line through the points. The *broken line*, calculated as described in the text, indicates the values of π/c expected for a fractionated nonequilibrating system. The *solid curve*, calculated as described in the text, gives the values of π/c expected for a dimer + hexamer $\rightleftharpoons$ octamer equilibrium depleted in H2A + H2B 10% by weight, with $K = 1.5 \times 10^5$ M^{-1} and $B = 1.77 \times 10^4$ cm^3 mol g^{-2}, the best fit parameters to Equation 12 for the data in Fig. 2. *B*, core histones at 35°C; $RT = 2.43 \times 10^4$ liter-cm buffer mol^{-1}.

Sedimentation Equilibrium Measurements—We also examined one of the histone samples used in the 25°C osmotic pressure measurements (Run 1) by low speed sedimentation equilibrium. The sedimentation measurements were made at 10°C concurrent with the osmotic pressure measurements. Fig. 5*A* shows that the ln c *versus* r^2 plot clearly has upward curvature, showing that the sample was heterogeneous; this is consistent with the value of $\bar{M}_n$ from the osmotic pressure, and the gel filtration data. The value of $\bar{M}_{w,r}^{app}$ near the meniscus is about 70,000, while $\bar{M}_{w,r}^{app}$ near the bottom of the cell is 118,000, using an apparent partial specific volume, ϕ', of 0.75 ml/g, the average of the reported values (18–21). The slope of the straight line corresponds to a molecular weight of 94,000. We also measured $\bar{M}_w^{app}$ at 25°C for comparison with $\bar{M}_n$ from the osmotic pressure. A value of 91,000 was obtained using Equation 4. As described above, Equation 5 predicts that if 10% of the sample has a molecular weight of 27,800 (dimer value) then the remaining 90% must have a number average molecular weight of 92,500 for an overall average of 75,000. Thus, the weight average molecular weight should be slightly greater than (because 92,500 is a number average): $0.1 \times 27{,}800 + 0.9 \times 92{,}500 = 86{,}030$, in reasonable agreement with the sedimentation equilibrium data ($\bar{M}_w^{app} = 91{,}000$).

We have also examined many other histone preparations by low speed sedimentation equilibrium using the UV scanner at initial concentrations of about 1 mg/ml, and have always obtained results very similar to those described here.

We next attempted to determine why some laboratories have obtained somewhat different results using this method. Although our histone sample was of high purity (see "Materials and Methods"), the extinction coefficient of DNA at 280 nm (11.5 liters g^{-1} cm^{-1}) is about 26 times greater than that of histones, and the presence of very small amounts of DNA which co-isolate with the histone complex could therefore be

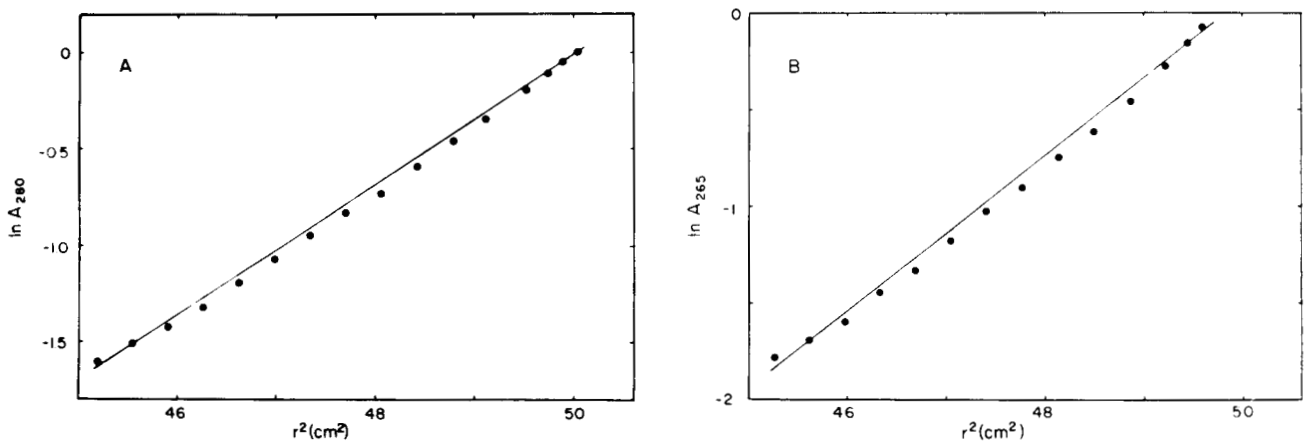

FIG. 5. **Sedimentation equilibrium plots in ln absorbance *versus* radius squared for core histones used in the osmotic pressure measurements.** The straight lines serve to illustrate the curvature of the points. *A*, scanned at 280 nm; *B*, scanned at 265 nm; here, the absorbance exceeded the value of 1.0 (off scale) before the cell bottom was reached.

a source of error. The fraction of the absorbance signal which could be due to DNA contamination is about 2½ times greater at 265 nm than at 280 nm. Therefore, after sedimentation equilibrium had been achieved in the run at 280 nm, shown in Fig. 5*A*, we changed the wavelength to 265 nm and rescanned the cell. Base-lines were then recorded at both 280 nm and 265 nm. Also, we had verified that the absorbance at both wavelengths did not vary with radial position at the start of the run, before redistribution had occurred. Fig. 5*B* shows that the apparent weight average molecular weights across the cell do differ substantially at 265 nm and 280 nm (compare with Fig. 5*A*). At 265 nm, sample heterogeneity is more apparent, and $\bar{M}_{w,r}^{app}$ is more than 150,000 near the cell bottom. The slope of the straight line here corresponds to a molecular weight of 112,000. Thus, it appears that contaminating DNA contributes substantially to the signal at 265 nm. At 280 nm the contribution due to contaminating DNA should be about 2½ times smaller, but not negligible. Therefore, in this case, contaminating DNA appears to increase the true value of $\bar{M}_w^{app}$ and we conclude that the use of the UV scanner may not be completely reliable for the analysis of histone samples.

Sedimentation Velocity Measurements—We also measured the sedimentation coefficient of our histones in 2 M NaCl, using the Schlieren optical system to exclude any contributions to the signal from possible DNA contamination. The dependence of the value of $s^0_{20,w}$ on concentration is shown in Fig. 6; a value for the partial specific volume of 0.75 ml/g was used in correcting the sedimentation coefficient to standard conditions. $s^\circ_{20,w}$ is 4.48 ± 0.03; a value of 4.36 is obtained for $\bar{v} = 0.73$ ml/g, and 4.65 for $\bar{v} = 0.77$ ml/g. The histone complex sedimented with a single approximately symmetrical boundary, as shown in the inset of the figure. No slow sedimenting peak was observed, as might have been expected from the gel filtration and sucrose gradient profiles obtained. However, some rapidly equilibrating systems exhibit single approximately symmetrical boundaries even though the individual species involved have different sedimentation coefficients (34). Also, no fast sedimenting, aggregated material was observed as the centrifuge approached the set speed in any of the samples. Thus, the sample appears to be homogeneous by boundary sedimentation, although not by band sedimentation, gel filtration, sedimentation equilibrium, or from the value of $\bar{M}_n$ by osmotic pressure.

Analysis of the Data According to a Dimer + Hexamer ⇌ Octamer Equilibrium—The value of $\bar{M}_n$ (75,000) obtained from the osmotic pressure measurements at 25°C, the small virial coefficient (see "Discussion"), the fractionation of a low molecular weight (H2A H2B) complex from the sample, the only slightly increased value of $\bar{M}'_n$ (80,000) for the column-fractionated sample, and the increase in $\bar{M}_n^{app}$ with concentration at 35°C all indicate that an association equilibrium occurs. The existence of about 10% of the mass of the sample as low molecular weight species consisting of H2A + H2B (Fig. 3*A*) precludes the existence of an appreciable amount of tetramer of any kind in the sample at concentrations greater than 1 or 2 mg/ml. This follows because the remaining material must then have a number average molecular weight of at least 92,500 according to Equation 5, and a still higher weight average molecular weight. Therefore the data are not consistent with a heterotypic tetramer ⇌ octamer equilibrium (19). However, the data seem consistent with an equilibrium whereby the octamer dissociates by losing (H2A H2B) dimers (35). If the dissociation constant for the second (H2A H2B) dimer (that leading to a $(H3\ H4)_2$ tetramer) is roughly the same as or less than that for the first, the dissociation of only the first dimer needs to be considered at concentrations greater than about 1 mg/ml, where only about 20% of the H2A + H2B are present as dimers. Therefore, we next examined whether the measured concentration dependence of the osmotic pressure at 25°C (Fig. 2) is consistent with this association equilibrium.

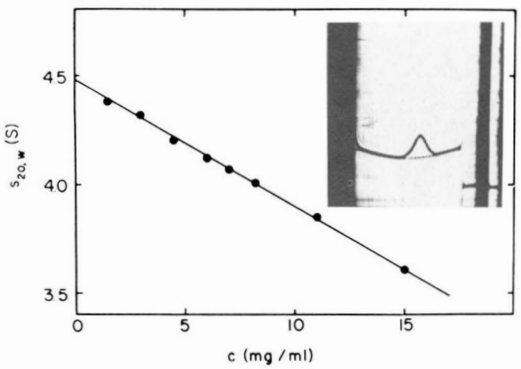

FIG. 6. **Concentration dependence of the sedimentation coefficient for core histones.** *Inset*, a Schlieren pattern taken after 121 min of sedimentation at 60,000 rpm, 20°C for a 4.5 mg/ml sample; the boundary was sedimenting from right to left.

The concentrations (milligrams per ml) of octamer, hexamer, and dimer, denoted by Subscripts 1, 2, and 3, respectively, are written as:

$$c_1 = (1 - \theta)c \tag{6}$$

$$c_2 = \theta c \frac{M_2}{M_1} \tag{7}$$

$$c_3 = \theta c M_3 / M_1 \tag{8}$$

where θ is the fraction of octamers dissociated, c is the total protein concentration (milligrams per ml), and the M_i values are the molecular weights of the three complexes, 108,768, 80,990, and 27,778, respectively (29). The association constant, K, is then given by

$$K = \frac{1 - \theta}{\theta^2 \frac{c}{M_1}} \tag{9}$$

And therefore, θ is given by

$$\theta = \frac{-1 + \sqrt{1 + 4K\frac{c}{M_1}}}{2K\frac{c}{M_1}} \tag{10}$$

Using Equations 6 through 8 and Equation 3, the number average molecular weight $\bar{M}_n$ is given simply by

$$\bar{M}_n = \frac{M_1}{1 + \theta} \tag{11}$$

Thus π/c as a function of K, c, and B, the second virial coefficient, is obtained by using Equations 10 and 11 in Equation 1, and is given by

$$\frac{1}{RT}\frac{\pi}{c} = \frac{1}{M_1} + \frac{1}{2Kc}\left(-1 + \sqrt{1 + 4K\frac{c}{M_1}}\right) + Bc \tag{12}$$

The curve through the points in Fig. 2 is given by Equation 12 with $K = (1.5 \pm 0.5) \times 10^5$ M^{-1} and $B = (1.77 \pm 0.17) \times 10^{-4}$ cm^3 mol g^{-2}. The fit and the values of the parameters K and B (see "Discussion") are quite reasonable. For values of the parameters K and B outside of the indicated error limits, the fit is significantly poorer. Therefore, we conclude that the concentration dependence of the osmotic pressure at 25°C is consistent with the dimer + hexamer ⇌ octamer equilibrium.

We then checked whether the osmotic pressures measured

for the column fractionated sample (Fig. 4*A*) were consistent with the values of K and B obtained from the fit in Fig. 2. Using $K = 1.5 \times 10^5$ M^{-1}, we calculated the concentrations of octamer, hexamer, and dimer, at equilibrium, which would result from a given concentration of core histones deficient in 10% by weight H2A + H2B. The value of $\bar{M}'_n$ at each concentration was then obtained from Equation 3 and π/c from Equation 1 using $B = 1.77 \times 10^{-4}$ cm^3 mol g^{-2}. The theoretical curve is in good agreement with the experimental points. The point at 17.5 mg/ml may be low because there was insufficient sample available here to rinse the osmometer sample compartment as thoroughly as desired. Thus, the π/c values did not decrease to the dashed line upon fractionation of the sample because more octamer dissociated after removal of the H2A + H2B, to the extent consistent with $K = 1.5 \times 10^5$ M^{-1}.

Using this value of K, the weight fraction octamer, hexamer, and dimer as a function of concentration (for a sample containing equimolar amounts of the core histones) is given by c_i/c using Equations 6 through 8 along with Equation 10. The curves are shown in Fig. 7*A*. Thus, at concentrations greater than 1 mg/ml, the octamer is the predominant species present at 25°C; at lower temperatures, the octamer may be more stable. These weight fractions of (H2A H2B) provide an explanation for the separation of 10% of the histone mass as H2A + H2B at rather high concentrations (Fig. 3*A*). With these weight fractions, the "true" number average and weight average molecular weights as a function of concentration are plotted in Fig. 7*B*. Any measured molecular weights must have lower values due to the positive virial coefficient. For example, $\bar{M}_w^{app}$ should be approximately 60,000 at 20 mg/ml, a factor of $1/(1 + 2B\bar{M}_w c)$ lower than $\bar{M}_w$. At lower concentrations, where there is a mixture of species present, $\bar{M}_w^{app}$ cannot be calculated in this way for comparison with sedimentation equilibrium data because B is then a rather complex average (36).

In order to fit the 35°C data (Fig. 4*B*), the dissociation of the second (H2A H2B) dimer would also have to be considered for meaningful parameters. Moreover, very high concentrations are required for an accurate estimation of the virial coefficient. Therefore we present these data only qualitatively.

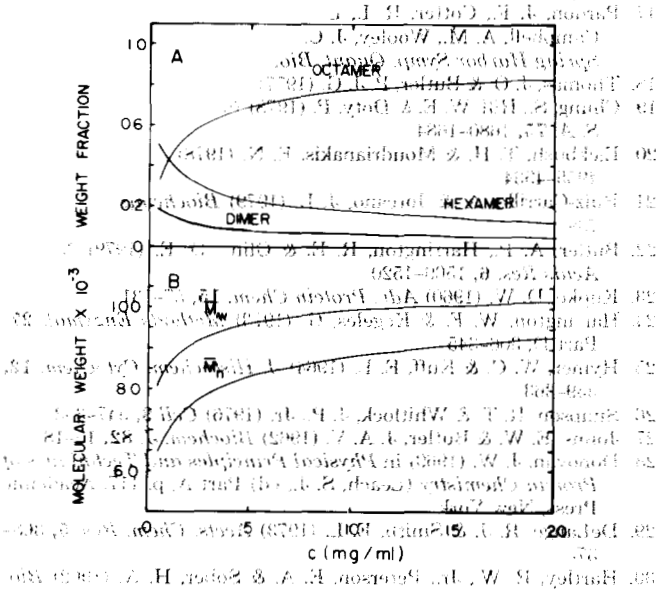

FIG. 7. **Calculated weight fractions (*A*) and molecular weights (*B*) for the equilibrium mixture of octamers, hexamers, and dimers as functions of the total protein concentration.** The curves were calculated as described in the text for $K = 1.5 \times 10^5$ M^{-1}.

DISCUSSION

We have measured the concentration dependence of the osmotic pressure of core histones in 2.0 M NaCl, pH 8.0, at 25°C. Three independent runs on different preparations were in close agreement, with $\bar{M}_n^{app}$ nearly constant at 75,000 from 1 to 20 mg/ml (Fig. 2). The samples appeared homogeneous by visual inspection of the sedimentation boundary (Fig. 6), but were found to be heterogeneous by gel filtration (Fig. 3) at a concentration of about 4 mg/ml; 10% of the total histone by weight, consisting of only H2A + H2B, eluted at a volume consistent with a dimer molecular weight, whereas 90% eluted at a volume close to that of a cross-linked octamer marker. The existence of approximately 10% by weight of the sample as dimers must necessarily have a substantial influence on $\bar{M}_n$, although not on $\bar{M}_w$. We calculated that the number average molecular weight of the sample components larger than dimer was at least 92,500, and therefore not much tetramer of any kind could be present. The measured value of $\bar{M}'_n$ for a fractionated sample (dimer removed) was only about 80,000 (Fig. 4*A*), indicating that the dimer was involved in an association equilibrium. At 35°C, the concentration dependence of the osmotic pressure clearly indicated that an association reaction occurs (Fig. 4*B*).

A dimer + hexamer $\rightleftharpoons$ octamer association equilibrium is completely consistent with the data. First, the concentration dependence of the osmotic pressure at 25°C (Fig. 2) fits at least as well to Equation 12 with $K = 1.5 \times 10^5$ M^{-1} and $B = 1.77 \times 10^{-4}$ cm^3 mol g^{-2} as it does to an empirical straight line. Additionally, this value of B is more reasonable than the value estimated from the slope of the straight line. Both the excluded volume effect and the Donnan equilibrium contribute to the second osmotic virial coefficient; see, for example, Tanford (31). The minimal contribution of the excluded volume term, that for a sphere, is approximately 4 v_2/M_2, where v_2 is the solute specific volume (approximately 1.0 g/cm^3) and M_2 is the solute molecular weight (75,000). Thus, excluded volume makes a contribution of at least 5×10^{-5} cm^3 mol g^{-2}, which alone exceeds the value of B obtained if the concentration dependence of $\bar{M}_n$ is ignored (2×10^{-5}). The Donnan term generally exceeds the excluded volume term for charged proteins (31), and is given by $1000\ Z^2 v_1/4m_3M_2^2$, where Z is the average net charge on the protein, v_1 the solvent specific volume, and m_3 the concentration of the third component (NaCl). Here v_1 is approximately 1.0 g/cm^3, $m_3 = 2.0$ M, and $M_2 = 75{,}000$. Since there are 142 basic amino acid residues/histone octamer (lysine, arginine) that are not approximately neutralized by the acidic residues (aspartic acid, glutamic acid) (29), we estimate Z is roughly 100 (in units of protonic charges) at pH 8. Thus, the Donnan term is approximately 1.25×10^{-4} cm^3 mol g^{-2}, and the sum of the two terms is 1.75×10^{-4} cm^3 mol g^{-2}, consistent with the value obtained from the fit of the data to Equation 12 (1.77×10^{-4} cm^3 mol g^{-2}). In contrast, if the concentration dependence of $\bar{M}_n$ is ignored, the virial coefficient is smaller than that expected for an uncharged globular protein.

We feel that the apparent discrepancies among various laboratories are best explained on the basis of experimental difficulties, rather than on details of sample preparation leading to different modes of histone-histone interactions. One major factor leading to disagreement clearly is the value of the density increment, or the analogous quantities for scattering methods; knowledge of the density increment was not required here. We suggest that another factor which contributed to discrepancies was the presence of varying amounts and types of DNA contamination in samples that were analyzed using the photoelectric scanner at 280 nm. The ratio A_{230}/A_{260} should be a measure of the extent of DNA contam-

ination; higher values indicate less contamination. In our samples A_{230}/A_{260} was 18. Even with this high value, comparison of equilibrium sedimentation measurements at 280 and 265 nm (Fig. 5) indicated that DNA contributed to the absorbance signal. Chung *et al.* (19) reported absorbance ratios of 17 to 18, about the same as ours, whereas, Butler *et al.* (22) reported a value of 22.3, and Thomas and Butler (18) reported a value of 12.9. Thus it is likely that in some of the studies, DNA contributed to the absorbance at 280 nm. Moreover, it seems unlikely that the contaminating DNA was of sufficiently high molecular weight to not interfere with the measurement because the samples were, in all cases (except here) subjected to extensive high speed centrifugation during preparation. It is also unlikely that the contaminating DNA was sufficiently small so as not to redistribute in the gravitational field at sedimentation equilibrium because samples were dialyzed extensively, and this amount of nonsedimenting material would have been readily observed in the baseline or in sedimentation velocity studies. Thus, different extents and size distributions of small amounts (less than 1% by weight) of contaminating DNA could cause differences in observed $\bar{M}_w^{app}$ values. In contrast, levels of DNA contamination on the order of 1% are negligible when interference optics are used. Consistent with this, the data of Eickbush and Moudrianakis (20) at 24°C using interference optics are in good agreement with our osmotic pressure measurements (compare with $\bar{M}_w$ *versus* c, Fig. 7*B*).

In addition to these two problems, there are additional factors which can lead to apparent discrepancies. We have found that although the octamer is the major species present (by weight) at concentrations greater than 1 mg/ml, it appears that the equilibrium can be shifted by temperature (this work, 20) and pH (20, 21). Clearly, these variables will have a greater effect at lower sample concentrations. This can therefore lead to the observation of different molecular weights if measurements are performed only at low concentrations at slightly different conditions (21, 22). Another factor that can obviously lead to erroneous molecular weights is the presence of low molecular weight material in the sample. A substantial amount of slow sedimenting material in the sample, apparently histones, was revealed by boundary sedimentation in the study by Lilley *et al.* (37), whereas the samples used here and by some others appeared homogeneous. Apparently both the light- and neutron-scattering studies (16, 17) were made on such samples as used by Lilley *et al.* (37), consistent with the low molecular weights reported. Still more possible sources of error have been discussed by others (18-22). Additionally, after submission of this manuscript, Philip *et al.* (38) reported that histone associations in 2 M NaCl are pressure-dependent and, consequently, that ultracentrifugation studies on this system should be interpreted with caution. In the study reported here, the histones were prepared and the osmotic pressure measurements made at atmospheric pressure. Thus, it seems that the discrepancies can, to a large extent, be accounted for without postulating different modes of histone association behavior.

The tendency of histones to associate into octamers in high salt solutions is consistent with our recent observations that histones assemble into octamers upon interaction with negatively charged macromolecules such as chromatin (39) and acidic proteins (40), as well as DNA. In all cases, the screening of the histone positive charges apparently promotes assembly (around pH 8). Also, it seems likely that the $(H3\ H4)_2$ tetramer is a very stable species, even in the presence of histones H2A and H2B. Two (H2A H2B) dimers can interact with the $(H3\ H4)_2$ tetramer when the histone charges are sufficiently screened, as in high salt solutions. This was initially suggested by Thomas and Kornberg (35) from cross-linking studies, and shown rather convincingly in the recent studies at lower protein concentrations by Eickbush and Moudrianakis (20) and Ruiz-Carrillo and Jorcano (21). Our measurements here indicate that at 25°C, pH 8.0, 2.0 M NaCl, the association to octamers is not complete even at very high sample concentrations, and that the hexamer, $(H3\ H4)_2$ (H2A H2B) is also a rather stable species. Thus, whereas the possible existence of heterotypic histone interactions *in vivo* cannot be excluded, their possible functional significance should perhaps be reconsidered.

Acknowlegments—We thank Doctors Robert T. Simpson and Peter Künzler for helpful discussions and for critically reading the manuscript. We also thank Dr. Marc Lewis for use of the microcomparator, Ms. Linda Propst for preparation of nuclei and for photography, and Mrs. Bonnie Richards for preparation of the manuscript.

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