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# Studies on ColE$_1$-plasmid DNA and its interactions with histones: sedimentation velocity studies of monodisperse complexes reconstituted with calf-thymus histones*

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ABSTRACT

Complexes between the four calf-thymus histones (H2A, H2B, H3 and H4) and ColE$_1$-plasmid DNA have been reconstituted using the procedure of Oudet et al. ((1975), Cell 4, 281-300). The sedimentation rates of the complexes formed were studied under a variety of conditions. In 0.4 M NaCl, 0.1 M Tris pH 7.50, 0.01 M EDTA and 0.02 M $NaHSO_3$, the final dialysis-solvent in the reconstitution procedure, the sedimentation coefficients $s_{23}$ were found to increase when the complexes were reconstituted at increasing histone to DNA ratios. True plateau regions were reached in the case of the relaxed circular and linear forms of the plasmid DNA. The sedimenting boundaries observed for the complexes at saturation are sharp, reflecting a narrow distribution of sedimentation coefficients and a homogeneity of the complex comparable to that of the uncomplexed DNA. Studies of the dependence of $s_{23}$ on the concentration of the complex at constant DNA to histones ratio have been undertaken at salt concentrations between 0.4 and 1.5 M NaCl in the above solvent. The formation of the complexes is reversible, at least at the higher ionic strengths. At salt concentrations below 0.36 M the complex precipitates from solution. Omission of histone H4 from the reconstitution mixture abolishes complex formation.

## INTRODUCTION

It is now accepted that the subunit structure derived from chromatin resembles a linear array of spherical subunits (beads, nucleosomes, ν-bodies), although some controversy still exists regarding the spacing of the beads in native chromatin.[1,2] In the nucleosome, two of each of the histones H2A, H2B, H3 and H4 are thought to interact with about 140 to 200 base pairs of DNA to give the spherical particles observed in the electron microscope.[3,4] Histone H1 and H5 as well as non-histone proteins are removed under these conditions. The DNA is approximately seven-fold compacted in the subunit, which has a diameter of about 80 Å as measured by electron microscopy,[5-8] although smaller values for the compaction and larger values for the diameter have been reported.[9,10] Evidence for the periodicity of the structure also comes from x-ray diffraction and

nuclease digestion studies[4,11,12] and for the globular shape of the subunit from hydrodynamic measurements.[1,13]

One of the more exciting aspects of the nucleosome structure from the biophysical chemist's point of view is the fact that it can apparently be reversibly dissociated and reconstituted. Oudet et al.[9] have shown by electron microscopy that the reconstituted structure also resembles a linear array of beads, which have the same diameter as those observed in native chromatin. These authors reconstituted the nucleosomes by dialyzing a mixture of DNA and the four histones H2A, H2B, H3 and H4 in 2 M NaCl, 0.1 M Tris pH 7.50 and 0.02 M $NaHSO_3$ against solutions of decreasing salt concentration (1.5 M, 1.0 M, 0.75 M, 0.5 M, 0.4 M) at constant concentrations of the other buffer salts. The reconstitution can be carried out at different histone to DNA ratios, leading to different degrees of saturation of the DNA. This can best be demonstrated by using monodisperse viral or bacterial plasmid DNA's, such as adenovirus-2 and λ-phage DNA[9] or SV-40 DNA.[10] A maximum number of beads (N) can be formed on a DNA with a given number of base pairs. Complexes reconstituted at various histone:DNA ratios show in the electron microscope a linear decrease in the total length of residual internucleosomal DNA with increasing average number of beads per chain ($\bar{\nu}$).[9] At intermediate degrees of saturation, the beads are found in random positions along the DNA.[9,10] They do not cluster together, indicating the absence of positively cooperative interactions among the beads. Recently, Voordouw,[14] in an analysis of the data of Germond et al.[10] on the average number ($\bar{\nu}$) of beads and the standard deviation of the bead count ($\sigma_\nu$) in SV-40 DNA, showed that the sites of the DNA on which the nucleosomes form are identical and independent. This conclusion is based on the fact that the experimental data ($\sigma_\nu$,$\bar{\nu}$) can be described by the relationship:

$$\sigma_\nu^2 = \bar{\nu}(N-\bar{\nu})/N \qquad (1)$$

which was derived for an identical, independent site model.[14] Equation (1) shows that $\sigma_\nu$=o for $\bar{\nu}$=o or $\bar{\nu}$=N. Hence the complex formed will be monodisperse only when either none or the maximal number of beads (N) is present on the chain. At intermediate values of $\bar{\nu}$ ($0 < \bar{\nu} < N$) the DNA molecules will show a distribution in the number of beads present on individual chains, the width of which is characterized by $\sigma_\nu$; $\sigma_\nu$ is maximal at $\bar{\nu} = N/2$, $\sigma_\nu = \sqrt{N}/2$. Hence for SV-40 DNA with N=25, maximal values $\sigma_\nu = 2.5$ are expected in agreement with the earlier data of Germond et al.[10]

Reconstitution of nucleosomes on λ-phage DNA (which does not occur in such a complex *in vivo*) shows that no special base-sequence is required for nucleosome-formation.[9] Also nucleosome formation, when monitored by electron microscopy, depends on the presence of all four histones H2A, H2B, H3 and H4.[9] Further evidence that the reconstituted structure closely resembles the native one comes from x-ray diffraction and nuclease digestion studies.[15,16] A random arrangement of nucleosomes along the DNA has also been deduced from digestion studies of the reconstituted structure with restriction endonucleases.[17] Recent nuclease digestion studies by Felsenfeld's group[18,19] indicate a central role for the histones H3 and H4 in creating and maintaining the structure of the DNA:histone complex.

It is clear from the above that the complexes reconstituted from monodisperse DNA and the four histones H2A, H2B, H3 and H4, represent a class of interesting macromolecules. Elucidation of their structure in solution, beyond what is provided by the electron micrographs, can be achieved by measuring typical physical chemical properties, such as the sedimentation and diffusion coefficients, the radius of gyration, intrinsic viscosity and molecular weight. We may expect these properties to change gradually from those characteristic of the worm-like coil of the free DNA[20] to the as yet unknown properties of the fully beaded chain. The properties obtained for DNA-molecules, which are intermediate between these two extremes, will be subject to the polydispersity condition, equation (1). Provided monodisperse DNA is used for the reconstitution, it follows that the fully beaded chain will also be monodisperse. Physical measurements done on the latter form thus have the advantage that they do not require polydispersity corrections in order to arrive at average properties of individual molecules.

The measurements referred to above are in progress in our laboratory, using complexes reconstituted from $ColE_1$-plasmid DNA and calf-thymus histones. Monodisperse $ColE_1$-plasmid DNA can be obtained in sufficiently large quantities and its physical chemical properties are now well known.[21] In this paper we report the results of sedimentation velocity experiments of DNA:histone complexes performed under a variety of conditions in order to characterize the system and evaluate the feasibility of other physical measurements. Using the reconstitution procedure of Oudet *et al.*[9], we find that monodisperse complexes with well-defined sedimentation coefficients are indeed obtained when $ColE_1$-plasmid DNA is saturated with histones.

MATERIALS AND METHODS

Materials. Whole calf-thymus histone, extracted by the method of Kobayashi and Iwai,[22] was a generous gift from Dr. H. Arfmann. Sterilized, double distilled water was used throughout. All chemicals were reagent grade.

Preparation of ColE$_1$-Plasmid DNA. Covalently closed supercoiled ColE$_1$-I, relaxed circular ColE$_1$-II and linear ColE$_1$-III plasmid DNA were prepared and purified as described in detail elsewhere.[21] All preparations were freed of proteins by phenol-extraction, whenever necessary. Preparations of ColE$_1$-III used in this study were very homogeneous as judged both by electron microscopy and analysis of sedimenting boundaries.[23] Preparations of ColE$_1$-I and ColE$_1$-II were less homogeneous due to slight cross-contamination and the presence of concatenated dimers. The principal monomer, e.g. I or II, represents the major portion (80-90% (w/w)) of all the forms present in the samples used.

Fractionation of Histones. Whole calf-thymus histone was fractionated according to the procedure of Böhm et al.[24] Whole histone (150 mg), dissolved in 1 ml of 8 M urea with 1% β-mercaptoethanol, was applied to a column of Biogel P-60 (2.6x90 cm) and equilibrated with 0.02 M HCl at room temperature. The sample was eluted at a flow rate of 0.2 ml/min, fractions of 2 ml being collected. The histones were separated into three well resolved peaks by this procedure, the first peak containing histone H1, the second histones H3, H2A and H2B and the third histone H4. The second and third peak were, either together or separately, precipitated by dialysis against 5 volumes of saturated ammonium sulphate. Gel electrophoresis, in 2.5 M urea-acetic acid, according to Panyim and Chalkley,[25] was done routinely to screen the various fractions obtained.

Reconstitution of Histone-DNA Complexes. Reconstitution was carried out following the procedure used by Oudet et al.[9] for their electron microscopy studies. Plasmid DNA in 2 M NaCl, 0.1 M Tris pH 7.5, 0.02 M $NaHSO_3$ and 0.01 M EDTA was mixed with various amounts of histones H2A, H2B, H3 and H4, dissolved in the same solvent. The mixture was transferred to a dialysis bag (Union-Carbide, dia 0.22 inch) and dialyzed for a few hours at 4°C against the above buffer. This was followed by dialysis against decreasing concentrations of NaCl, at constant concentrations of the other buffer salts according to the following scheme: 1.5 M (12 hr), 1.0 M (24 hr), 0.75 M (12 hr), 0.5 M (24 hr), 0.4 M (12 hr). We included 0.01 M EDTA in all of the solutions in order to prevent DNA degradation by mag-

nesium-dependent nucleases. DNA concentrations were in the range 50-150 μg/ml and were estimated using a determined E(1 mg/ml; 1 cm) = 20.4 at 260 nm. The histone:DNA ratios (w/w) of reconstitution mixtures were in the range 0 to 8. These were adjusted and calculated on the basis of an E(1 mg/ml;1 cm) = 0.4 at 270 nm[26] for the mixture of H2A, H2B, H3 and H4 as isolated by Biogel P-60 chromatography. The results presented in this paper do not depend on exact knowledge of extinction coefficients.

Sedimentation Velocity Analysis. Sedimentation velocities of the reconstituted complexes were measured directly in the final dialysis solvent (0.4 M NaCl, 0.1 M Tris pH 7.5, 0.01 M EDTA, 0.02 M $NaHSO_3$) using a Beckman Model E Analytical Ultracentrifuge, equipped with electronic speed control, a photoelectric scanner system and multiplex accessory unit. A 12 mm aluminum filled epon double sector centerpiece and quartz or sapphire windows were used. The two sectors contained dialyzed histone:DNA complex ($0.1 < OD_{260} < 1.0$) and dialysis buffer. The cells were loaded in either of a two-hole An-H or four-hole An-F titanium rotor. Centrifugation was performed at rotor speeds from 15,000 to 44,000 rpm at temperatures close to room temperature (23°C). The RTIC temperature regulating unit was not used during the run. The cells and rotor were instead kept at the required temperature by adjusting the temperature of the Freon cooling fluid in the range 11-16°C. The rotor temperature (t) did not, in general, deviate more than one degree from 23°C, the actual value being constant to within 0.1°C during a run. Cells were scanned at 265 nm at 4 minute intervals. Sedimentation coefficients ($s_t$) were obtained from the measured change in radial position of the point of maximum concentration gradient in the sedimenting boundary with time. Boundary analysis[23] of some of the scans was performed in order to quantitatively evaluate the distribution of sedimentation coefficients. The sedimentation coefficient, $s_t$, was corrected to its value at 23°C in the same solvent, $s_{23}$, by means of the equation:

$$s_{23} = s_t \, \eta_t / \eta_{23} \qquad (2)$$

where $\eta_{23}$ and $\eta_t$ are the viscosities of the solvent (0.4 M NaCl, 0.1 M Tris pH 7.50, 0.02 M $NaHSO_3$, 0.01 M EDTA in most cases). Corrections in the buoyancy factor $(1-\phi'\rho_{23})/(1-\phi'\rho_t)$ were considered unnecessary as t was always close to 23°C making the densities $\rho_t$ and $\rho_{23}$ equal within 0.03%. As the apparent specific volume, $\phi'$, of the histone:DNA complex has not been determined, corrections to standard conditions (the viscosity and

density of pure water at 20°C) cannot be performed reliably.

RESULTS

The apparent sedimentation coefficient $s_{23}$ of the complexes formed between $ColE_1$-plasmid DNA and the four calf-thymus histones was measured as a function of several variables in order to determine whether specific complexes, with possible similarities to those observed with the electron microscope,[9,10] are indeed formed. When the ratio (r) of histones to DNA in the reconstitution mixture was increased, significant increases in $s_{23}$ were observed for all three conformational forms of the $ColE_1$-plasmid. This increase does not continue indefinitely, however, but levels off and finally reaches a plateau at histone to DNA ratios between 2 and 3. Further increase in the concentration of histones relative to that of the plasmid DNA does not result in a continued increase in $s_{23}$ in the case of relaxed circular $ColE_1$-II and linear $ColE_1$-III, whereas a relatively small increase only is observed in the case of covalently closed supercoiled $ColE_1$-I (Fig.1). These results show that the interaction of histones with plasmid DNA leads to specific complexes. The sedimentation coefficients of the free and the histone-saturated DNA are presented for the three conformational forms of the plasmid in Table 1. It is seen that saturation with histones leads to a 5-6 fold increase in $s_{23}$ under the given experimental conditions. The data shown in Table 1 have been obtained for a DNA-concentration of approximately 30 µg/ml; $s_{23}$ is almost independent of concentration under the given experimental conditions (Fig.4) and does thus closely approximate the value extrapolated to zero concentration $s^o_{23}$.

Apart from observing the significant increase in $s_{23}$ as the ratio of histones to DNA in the reconstitution mixture is increased, it is interesting to examine the sedimenting boundaries at different degrees of saturation. These are shown in Fig.2 for a $ColE_1$-I sample, which apart from the $ColE_1$-I monomer (90% w/w) contains approximately 10% (w/w) of concatenated $ColE_1$-I-dimer. In the absence of histones (Fig.2, r=0.00) two boundaries are observed for this sample: one for the monomer sedimenting with an apparent $s_{23}$ = 22.2 S and one for the dimer with $s_{23}$ = 31.7 S. The boundaries are sharp, reflecting a narrow distribution of sedimentation coefficients and, therefore, homogeneity of the DNA. Boundary sharpening effects are ruled out in view of the small concentration dependence of $s_{23}$ (see Fig.4, 0.4 M NaCl). With increasing histone to DNA ratios (Fig.2: r = 0.48; r = 0.96, r = 1.44) the boundaries are seen to broaden

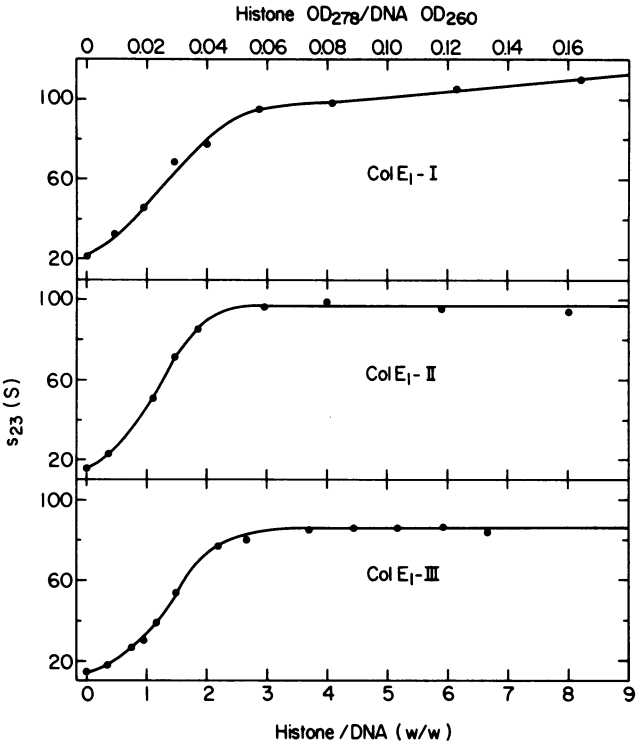


Figure 1. Uncorrected sedimentation coefficients ($s_{23}$) for the reconstituted complexes of the three conformational forms of $ColE_1$-plasmid DNA with calf-thymus histones H2A, H2B, H3 and H4 as a function of the ratio(r) of total histone to DNA (w/w). The concentration of the plasmid DNA is constant, approximately 30 µg/ml in 0.4 M NaCl, 0.1 M Tris pH 7.50, 0.01 M EDTA, 0.02 M $NaHSO_3$.

TABLE 1. Sedimentation coefficients of the various conformational forms of the $ColE_1$-plasmid, free or complexed with saturating amounts of histones, at 23°C in 0.4 M NaCl, 0.1 M Tris pH 7.50, 0.02 M $NaHSO_3$, 0.01 M EDTA.

| Form | $s^*_{23}$ (free) | $s^*_{23}$ (saturated) |
|---|---|---|
| $ColE_1$-I | 22.4 | 100-110 |
| $ColE_1$-II | 17.0 | 96 |
| $ColE_1$-III | 14.8 | 86 |

* In Svedberg units (S), the data are for a concentration of approximately 30 µg of DNA/ml.

significantly, causing overlap of the monomer and dimer boundaries. Values for $s_{23}$ obtained by midpoint analysis are indicated with the boundaries (Fig.2: 32.4 S, 45.6 S and 68.9 S) and are likely to correspond to the average $s_{23}$ of the monomer fraction, as this is by far the major species in the sample. When approaching saturation of the complex (Fig.1), the boundaries sharpen again and at r = 2.88 (Fig.2) two clearly resolved boundaries are once more observed for the histone complexed monomer and the histone complexed dimer, sedimenting with apparent sedimentation coefficients ($s_{23}$) of 95.6 S and 145.3 S, respectively. The sharp boundaries observed for the complex at saturation indicate a narrow sedimentation coefficient distribution and consequent homogeneity of the complex, similar to that of the free, uncomplexed DNA. In addition, these results show unambiguously that the complex comprises only a single DNA molecule. A complex involving several DNA molecules "crosslinked" by the positively charged histones (open association) is not likely to display the saturation phenomenon shown in Fig.1, or the separate boundaries for monomeric and dimeric molecules shown in Fig.2.

The dependence of $s_{23}$ on the concentration of NaCl in the solution has also been investigated and the results are presented in Fig.3. At NaCl concentrations in excess of 1.5 M, free DNA in the absence of added histones (Fig.3, open circles) sediments at the same rate as the DNA in the presence of histones indicating absence of interaction. With decreasing concentration of NaCl a continuous increase in $s_{23}$ is observed ($s_{23}$ = 86 A at 0.4 M NaCl), indicating increasing interaction. Single sedimenting boundaries were observed at all salt concentrations investigated, with no evidence of the presence of either faster or slower sedimenting material. However, at NaCl concentrations below 0.36 M, strong intermolecular association of the histone-complexed DNA molecules was observed leading to the formation of large, polydisperse aggregates ($\langle s_{23} \rangle \simeq 10^5$ S) and, finally, to quantitative precipitation of the histone-complexed DNA. At NaCl concentrations in excess of 0.36 M, where the reconstituted complex does not aggregate intermolecularly, complete reversibility of the changes in $s_{23}$ with salt concentration was observed (Fig.3). Hence the data obtained by decreasing the NaCl concentration by dialysis (Fig.3, open triangles) fall on the same smooth curve as those obtained after stepwise dilution of complexes first reconstituted to 0.4 M NaCl, to a given NaCl concentration (Fig.3, filled triangles).

In order to characterize further the association between histones

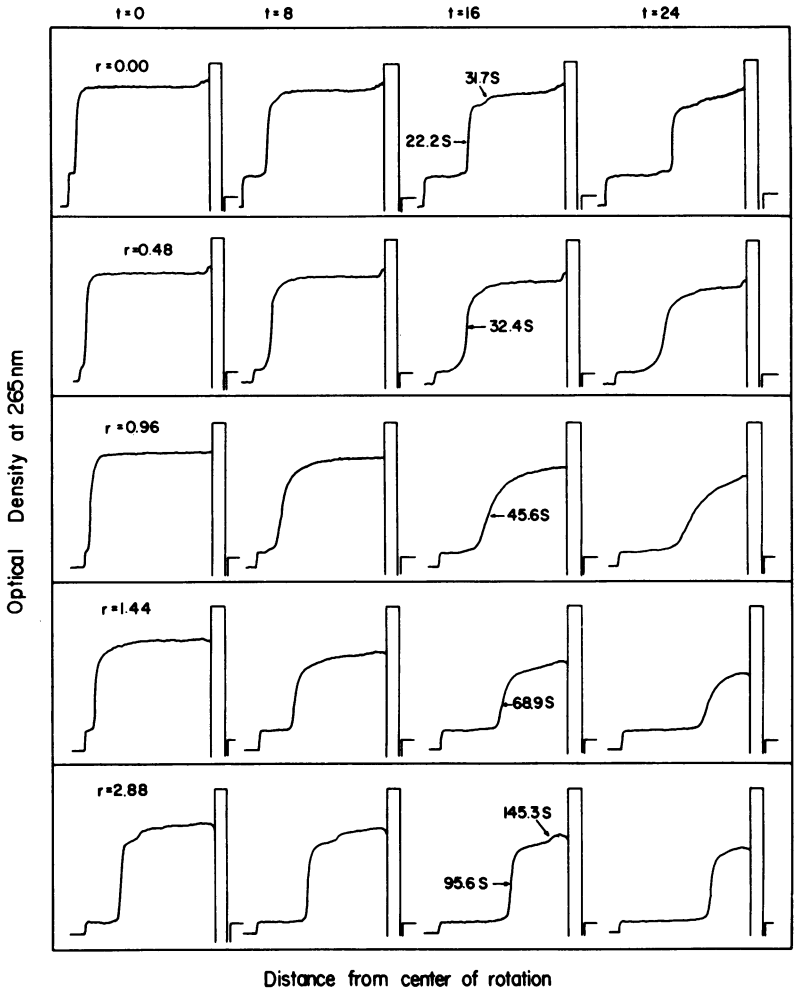


Figure 2. Scanner tracings for covalently closed, supercoiled $ColE_1$-I plasmid DNA and its complexes with the histones H2A, H2B, H3 and H4. Scans are shown at eight minute intervals, with time increasing from left to right. Histone to DNA ratios (r) are indicated for each row of four scans. The sedimentation velocity experiment for free DNA (r=0.00) was performed at 44,000 rpm, all others (r=0.48, 0.96, 1.44, 2.88) at 30,000 rpm. Values for the sedimentation coefficients of the boundaries observed ($s_{23}$) are indicated with the third scan (t=16). Solution conditions as in Fig.1.

and $ColE_1$-plasmid DNA studies on the concentration dependence of $s_{23}$ were done at the five concentrations of NaCl used during dialysis in the reconstitution procedure (1.5 M, 1.0 M, 0.75 M, 0.5 M, 0.4 M). In these experiments a stock solution of histones and DNA ($OD_{260} \simeq 1.6$), dialyzed

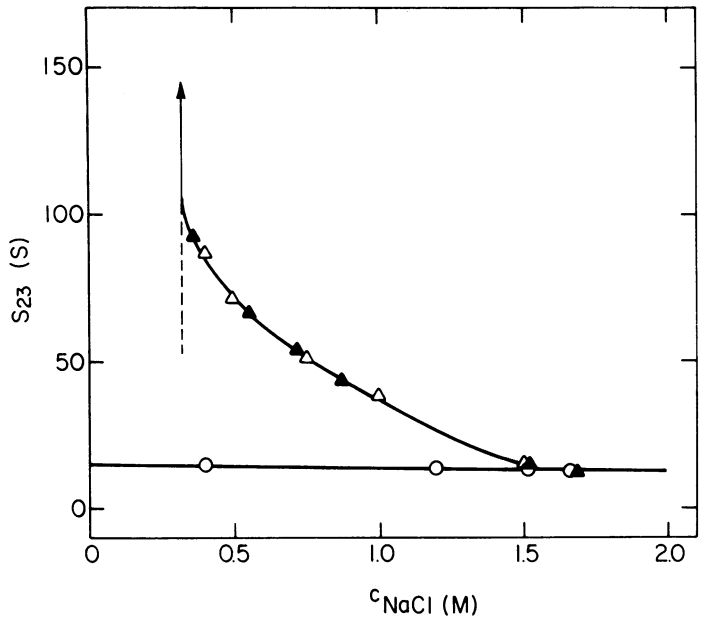


Figure 3. Sedimentation coefficients ($s_{23}$) for linear ColE$_1$-III DNA (o) and the ColE$_1$-III DNA:histone complex (Δ,▲) as function of NaCl concentration. The ratio (w/w) of total histones to DNA is approximately 6 and the DNA concentration is 30 µg/ml. The data for the complex were obtained by decreasing the concentration of NaCl slowly by dialysis starting from 2 M NaCl (Δ) or by stepwise dilution of the complex in 0.4 M NaCl, 0.1 M Tris pH 7.5, 0.01 M EDTA, 0.02 M $NaHSO_3$, to the indicated NaCl concentration (▲). The arrow at 0.32 M NaCl indicates the formation of large intermolecular aggregates.

to the given concentration of NaCl, was diluted up to ten-fold with dialysis buffer and $s_{23}$ was immediately determined. The ratio of histones to DNA (r=6) was thus constant for every dilution. It is seen (Fig.4) that even at 1.5 M NaCl there is a slight residual interaction between the ColE$_1$-III plasmid DNA and the histones, as $s_{23}$ increases slightly with concentration (expressed as $OD_{260}$), whereas the sedimentation coefficient of the plasmid DNA in the absence of added histones is known[21] to decrease slightly with increasing concentration in this same range of $OD_{260}$. At 0.5 M, 0.75 M and 1.0 M NaCl a continuous increase in $s_{23}$ with increasing concentration is observed, indicative of a reversible association-dissociation equilibrium. In contrast, at 0.4 M NaCl $s_{23}$ is seen to decrease slightly with increasing concentration in the range $0.2 < OD_{260} < 1.0$, in accordance with the usual behavior of macromolecules in the absence of intermolecular association. The observed decrease

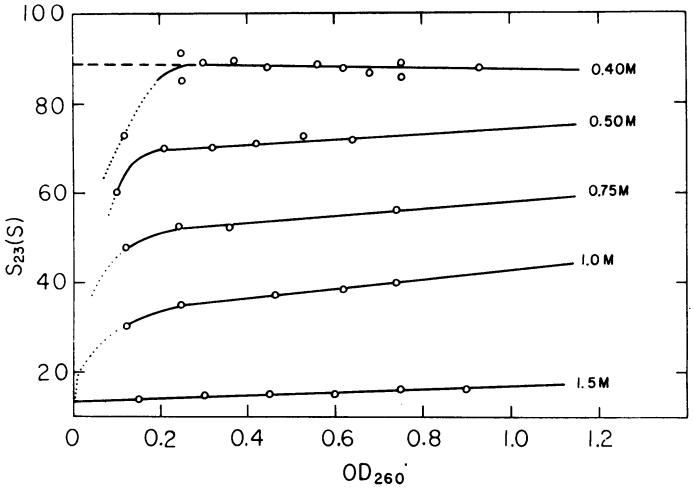


Figure 4. Sedimentation coefficient ($s_{23}$) of the linear $ColE_1$-III plasmid Histone:DNA complex as a function of concentration ($OD_{260}$) at various concentrations of NaCl. A stock solution of complex ($OD_{260}$ = 1.6) reconstituted at a ratio of total histone to DNA, r=6, was diluted with the dialysis buffer (0.1 M Tris pH 7.5, 0.01 M EDTA, 0.02 M $NaHSO_3$ and a given concentration of NaCl) and $s_{23}$ measured immediately.

indicates therefore that the association-dissociation equilibrium is not rapidly reversible, or, alternatively, that the affinity of the DNA for histones is very high under these experimental conditions: the saturated histone:DNA complex does not dissociate over a five-fold range in concentration. At still lower concentrations ($OD_{260}$ < 0.2) association may nevertheless occur (Fig.4, dotted line) although concentrations of this magnitude are studied only with difficulty with the 12 mm-centerpieces used here. A slow association-dissociation equilibrium between the histones and the DNA at 0.4 M NaCl is also reflected by the boundary-broadening observed at lower histone to DNA ratios (Fig.2). This effect is likely to be absent, and the boundaries sharp at any degree of saturation of the DNA with histones, if there is a rapid association-dissociation equilibrium.

Results obtained by reconstituting the $ColE_1$-III DNA-histone complex in the absence of histone H4 are shown in Fig.5. The latter can be conveniently eliminated from the histone mixture, since it is eluted as a distinct peak on the Biogel P-60 columns (see "Methods"), usually well-resolved from the preceding peak containing the other three histones.[24] The fractions containing H2A, H2B, and H3 were pooled, concentrated and rerun on the Biogel column. The resulting preparation shows a negligible

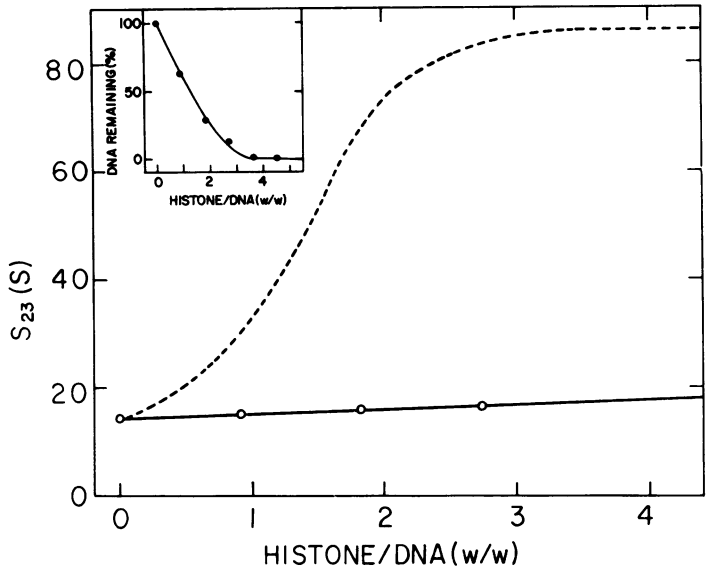


Figure 5. Sedimentation coefficients ($s_{23}$) of linear $ColE_1$-III plasmid DNA reconstituted with histones H2A, H2B and H3 as function of the ratio of total histones to DNA (w/w). The dotted line shows the change in $s_{23}$ when H4 is also included (see Fig.1). Solution conditions as in Fig.1. Inset: fraction of soluble DNA remaining as function of the ratio of histones (H2A, H2B, H3) to DNA.

increase in $s_{23}$ with increasing histone to DNA (w/w) ratio compared to the increase in $s_{23}$ when histone H4 is included in the reconstitution mixture. The presence of H4 is thus an obligatory constituent for the formation of the histone:DNA complexes with the specific sedimentation properties shown in Fig.1. From the foregoing, it must not be concluded that the histones H2A, H2B and H3, either in combination or separately, do not interact with DNA. Addition of a four-fold excess of H4-depleted histone leads to quantitative precipitation of the plasmid DNA, indicating extensive crosslinking of DNA duplexes by histones (Fig.5, inset). Adding less histone leads to formation of smaller amounts of precipitate. The remaining soluble DNA has a sedimentation coefficient essentially equal to that of free DNA (Fig.5). Hence we conclude that the H4-depleted histone fraction is capable of forming non-specific precipitates by cooperative interaction of histones and DNA. The formation of specific complexes, which may have a structure resembling the linear array of beads observed by electron microscopy, is dependent on the presence of histone H4.

DISCUSSION

In the present study we have attempted to give some further insights into the assembly system comprising DNA and the histones H2A, H2B, H3 and H4. Other workers[9,10,18,19] have already shown that the complexes that can be reconstituted from these components have a subunit structure resembling that of native chromatin. Physical studies on native chromatin generally require that the molecule is degraded either by shearing, sonication or enzymatic digestion in order to reduce its size to a level at which meaningful data can be obtained. Such procedures lead to polydisperse solutions; the width of the distribution can be ascertained or reduced by application of fractionation techniques. The reconstitution method, on the other hand, offers the advantage that using monodisperse DNA as starting material, homogeneous DNA:histone complexes can be obtained, resembling a comparable segment of the native structure. It has been our primary aim here to show that homogeneous complexes are indeed obtained in solution. The elucidation of the solution structure of these complexes can only be achieved by the combined use of a variety of structure-probing techniques. Several such studies are presently under way in our laboratory. By electron microscopy in the presaturation range (histone:DNA about 1.5 w/w) we observe that the structure is similar to the linear array of beads found by other workers.[9,10] About 15 beads per complex were counted under these conditions. Several points require additional discussion.

Monodispersity of the Complexes. The sedimentation coefficients of the complexes at saturation are quite reproducible and lie for all preparations of $ColE_1$-DNA and histones within 5% of the reported mean values (Table 1). At saturation, the complexes sediment with narrow boundaries, comparable to those of the free DNA. The boundary broadening effects observed when the DNA is only partially saturated can be readily explained on the basis of multiple equilibria considerations (equation (1)). Calculations for linear $ColE_1$-III by boundary analysis show a standard deviation of the weight-distribution of sedimentation coefficients, $\sigma_s$ = 1.6 S for the free DNA, and $\sigma_s$ = 4.0 S for the saturated complex. This standard deviation does not reflect the experimental error in the mean, which is generally only about 1%, but is a defined parameter describing the distribution of sedimentation coefficient values about the mean. As probably both diffusion and convection contribute more to the boundary broadening of the saturated complex than that of the free DNA,

we cannot state whether the complex is less homogeneous than the free DNA on the basis of this result. On a relative basis, the distribution for the complex is actually narrower than that for the free DNA. However, at intermediate degrees of saturation (40 S < $s_{23}$ < 80 S) $\sigma_s$ was found to be distinctly higher: $\sigma_s$ = 6-7 S. The width of the distribution of sedimentation coefficients thus passes through a maximum on going from the naked to the fully saturated chain. A similar effect has been described for the width of the distribution of nucleosomes on SV-40 DNA at different degrees of saturation (equation (1).

Non-Specific Aggregation of DNA by Histones. Intermolecular association leading to the formation of large aggregates was observed for the DNA-histone complex below 0.36 M NaCl and in the case of the free DNA in the presence of H4-depleted histones at 0.4 M NaCl (Figs. 3 and 5). The latter observation indicates that the H4-depleted histones can certainly bind to DNA, but most likely in a non-specific manner. Formation of specific complexes (Table 1) depends on the presence of H4 and, presumably, on that of all four of the histones H3, H4, H2A and H2B,although this still remains to be investigated. The non-specific binding of histones to DNA, usually giving rise to huge aggregates or precipitates, is not unexpected.[27,28] It finds its origin in the fact that the histones and the dimer, tetramer and higher order complexes derived therefrom are ligands with "multipoint attachment". Binding of the first ligand thus brings otherwise distant inter- or intramolecular DNA-segments together, thereby increasing the binding constants for attachment of successive ligands. The complexes that are formed during this open association will clearly be huge and precipitation of the DNA with all or part of the histones will usually result. Recognizing that histones and their quaternary aggregates have the ability to enter into non-specific open-association reactions with DNA, it follows that association leading to specific complexes will be observed only when the sites of multiple attachment between the DNA and the core-histone complex are located close together along the DNA helix. The latter requirement implies precisely defined winding of small sections of the DNA with respect to the central histone complex core: none of the multiple attachement sites can be allowed to interact with a remote, statistically independent segment of the DNA molecule, as this would greatly increase the chance of formation of an irregular and thus biologically irrelevant structure. It follows that all of the multiple attachment sites have to be well within the length

of DNA beyond which different segments of the chain become statistically independent.

The intermolecular aggregation of the saturated complex (Fig.3) below 0.36 M NaCl is not without precedent and similar aggregation properties have been reported for chromatin fragments[29] with maximal intermolecular association at 0.2 M NaCl. At very low ionic strengths (1 mM NaCl), complexes reconstituted by related procedures have also been shown to be soluble,[18,19] but strong long range electrostatic interactions makes physical studies more complicated under these solution conditions.

Relationship Between the Complexes and Chromatin. As seen from Fig.1, a two-to-three-fold excess of histones over DNA was required for saturation of the complex. The question thus naturally arises whether this represents stoichiometric binding of the histones to DNA under the conditions of this reconstitution procedure or whether the ratio is actually lower with part of the histones not being bound to the DNA. In chromatin the ratio of histones to DNA is approximately 1:1, but many other components (e.g. the histones H1 or H5 and the non-histone proteins) can still bind to this structure. In some recent experiments[21] uncomplexed histones were separated from a histone:ColE$_1$-III DNA complex (reconstituted at an input ratio of 6:1) by sucrose gradient centrifugation. The histone content of the resulting purified complex was determined by the method of Lowry and by UV spectroscopy. Both methods yielded a ratio (w/w) of 2.4:1. The good agreement of this value with the location of the saturation point (Fig.1) indicates stoichiometric binding of the histones in the presaturation range. The high histone content of the complex at saturation is somewhat puzzling, yet does not appear unreasonable if we consider that native chromatin indeed contains about 2.5:1 (w/w) total (including non-histone) protein to DNA.[30] It at all events appears that, in the absence of non-histone proteins, and under the saturation conditions used in this work, monodisperse DNA yields well-defined monodisperse histone:DNA complexes by binding histones far in excess of what has been reported for chromatin. The question of how closely the reconstituted complexes resemble the native chromatin structure remains open. Light scattering studies now in progress[21] indicate a considerable compaction of the DNA in the purified DNA:histone complexes as manifested in a reduced radius of gyration and

an increased diffusion coefficient of the complexes compared to those of the free DNA.

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* This paper is dedicated to the memory of Jerome Vinograd, pioneer, scientist and friend.

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