Reconstitution of Chromatin Core Particles†

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ABSTRACT: Chromatin core particles, containing 140 base pairs (bp) of DNA plus the inner histones, can be nearly quantitatively formed either by reassociation from 2 M NaCl or by reconstitution from salt extracted histones and DNA. The reassociated or reconstituted particles appear to be identical with the native particles in all physical properties examined (sedimentation velocity, histone content, circular dichroism, and melting) as well as in their patterns of digestion by micrococcal nuclease, DNase I, and trypsin. In the presence of excess DNA, no "half-particles" are formed. In the presence of excess histone, aggregated structures are formed in addition to 11S core particles.

That the nucleosome is a fundamental unit of chromatin structure seems now to be well established. Furthermore, it has recently become clear that while the size of the DNA fragments observed as structural repeats by nuclease digestion studies may vary from one cell type to another, the nucleosomal *core particle,* containing 140 base pairs (bp)[1] of DNA and 8 histone molecules (Van Holde et al., 1975; Sollner-Webb and Felsenfeld, 1975; Axel, 1975; Shaw et al., 1976; Noll, 1976a; Morris, 1976a; Compton et al., 1976; Lohr et al., 1977), is truly ubiquitous in eukaryotes. The general structure appears to involve identical or nearly identical core particles, containing two copies each of H2A, H2B, H3, and H4, connected by spacer DNA segments which carry H1 (and H5, in avian erythrocytes) (Shaw et al., 1976). The average spacer length varies from one organism and even one tissue or cell type to another (Compton et al., 1976; Lohr et al., 1977; Spadafora et al., 1976; Morris, 1976b; Thomas and Thompson, 1977). Further, there is considerable evidence that these particles are not confined to nonexpressed regions of the DNA, but may be present in active genes as well (Axel et al., 1975; Reeves, 1976). There is some evidence that their structure or stability may differ in genetically active regions, for core particles in such regions appear to be selectively susceptible to the action of DNase I (Weintraub and Groudine, 1976; Garel and Axel, 1976). A model has recently been proposed which attempts to explain how transcription might occur in the presence of such structures (Weintraub et al., 1976). Clearly, any evidence about the stability and/or possible structural transformations of core particles will be of the greatest interest.

Reconstitution of chromatin has been attempted in many laboratories, but there have been until recently few detailed investigations of the structures of the reconstituted material (Richards and Pardon, 1970; Axel et al., 1974; Kornberg and Thomas, 1974; Oudet et al., 1975; Boseley et al., 1976; Woodcock, 1977). Felsenfeld and co-workers (Camerini-Otero et al., 1976; Sollner-Webb et al., 1976) have shown that chromatin reconstituted with selected portions of the histones exhibits some of the limit digestion pattern shown by whole chromatin. Germond et al. (1976) have recently shown that the SV-40 minichromosome can be reconstituted by a variety of procedures, so as to yield structures very similar to the "native" minichromosomes, as judged by gel electrophoresis and electron microscopy. Laskety et al. (1977), using a cell-free extract from *Xenopus laevis,* have reconstituted nucleosomes onto SV-40 supercoiled DNA without the use of salt dialysis.

It seemed to us that the ubiquity of the core particle and the importance of reconstitution studies pointed to the necessity for a physical investigation of the simplest possible reconstitution experiment—the reconstitution of core particles from short DNA segments plus the "inner" histones (H2A, H2B, H3, and H4). We have been especially concerned with two questions: (1) Can quantitative reconstitution be obtained? (2) How do the properties of the reconstituted core particles compare with those of particles obtained by micrococcal nuclease digestion of intact nuclei? We felt that a wide variety of physical and chemical tests should be applied to examine fidelity of reconstitution.

This report describes our first experiments along these lines. Part of this work was reported at the Biophysical Society Meeting in February, 1976, and in discussions at the Dahlem conference on Organization and Expression of Chromosomes in May, 1976.

Materials and Methods

(A) *Isolation of Erythrocyte Nuclei.* Blood was obtained from White Leghorn chickens by exsanguination in the presence of 6% sodium citrate. Nuclei were prepared according to Shaw et al. (1976) with the following modifications: (1) 0.15 M NaCl–0.015 M cacodylic acid (pH 7.2) was substituted for the standard saline citrate; (2) 0.1 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) was present at all times during preparation and digestion of the nuclei to inhibit serine protease action.

(B) *Preparation and Digestion of Histone H1 and H5 Depleted Chromatin.* Freshly prepared nuclei from 6 mL of packed RBC were lysed in 250 mL of 10 mM Tris-cacodylic acid–0.7 mM EDTA–0.1 mM $PhCH_2SO_2F$ (pH 7.2) (lysis buffer), then made 0.6 M NaCl with solid NaCl, and allowed to swell overnight at 4 °C. The chromatin gel was transferred to 250 mL of lysis buffer containing 0.65 M NaCl and allowed to stand 24 h again with gentle stirring. The chromatin was

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[1] Abbreviations used are: bp, base pairs of DNA; *Hae*III, endonuclease R *Hae*III obtained from *Hemophilus aegyptius*; PM2, *Pseudomonas* bacteriophage 2; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Tos-LysCH_2Cl, N^{α}-*p*-tosyl-l-lysine chloromethyl ketone; bis, *N,N'*-methylenebis(acrylamide); RBC, red blood cells; CD, circular dichroism; UV-vis, ultraviolet-visible.

centrifuged at 17 000g for 15 min and the pelleted chromatin was brought up in 250 mL of lysis buffer and stirred for 4–8 h. The chromatin, now in low ionic strength buffer, was centrifuged at 17 000g for 15 min, brought up in 10–15 mL of lysis buffer, and digested at 37 °C with 125 units/mL micrococcal nuclease (Worthington). The reaction was terminated after 1 h at 27% acid solubility by making the solution 10 mM EDTA and cooling on ice.

(C) *Preparation of Core Particles from Digested Chromatin.* Core particles were isolated from chromatin digests by the method of Shaw et al. (1976), using gel chromatography on Bio-Gel A-5m. As in previous studies, the core particles were obtained in a well-resolved peak in the included volume. Peak fractions were chosen for the experiments which follow. Unless otherwise indicated, all experiments were carried out in the same buffer used for column elution: 10 mM Tris (pH 7.2), containing 0.7 mM Na_2EDTA.

(D) *Isolation of Core Particle DNA.* DNA from isolated core particles was extracted by a modified Marmur procedure involving Pronase digestion of the core particles, followed by phenol–chloroform extraction of the DNA (Britten et al., 1974). The DNA was precipitated with ethanol, and then redissolved in 2 M NaCl–10 mM Tris-cacodylic acid, containing 0.7 mM EDTA (pH 7.2), and dialyzed extensively against this buffer.

(E) *Preparation of Histones for Reconstitution.* H1 and H5 depleted chromatin prepared as described above was dissolved in lysis buffer containing 2 M NaCl by stirring overnight at 4 °C. The solution which contained about 10 mg/mL DNA was centrifuged at 170 000g for 24 h to pellet the DNA away from the inner histones. The nonviscous supernatant was chromatographed through a G-100 Sephadex column equilibrated with lysis buffer containing 2 M NaCl and the peak fraction corresponding to the histone complex was used for the reconstitution.

(F) *Reconstitution of Core Particles.* Two types of reconstitution experiments were carried out. In the first, which may better be described as "reversible dissociation" or "reassociation", a solution of core particles (in lysis buffer) was first made 2 M in NaCl. The salt was then removed by stepwise dialysis (as below).

In the second technique, salt-extracted histones and purified DNA were mixed together in the desired weight ratio and dialyzed against dialysates of successively lower ionic strengths. The total reconstitution time was approximately 24 h at 4 °C with 4-h steps of 1.5 M NaCl, 1.0 M NaCl, 0.75 M NaCl, and 0.5 M NaCl, and, finally, 2 changes of 10 mM Tris–0.7 mM EDTA (pH 7.2). The concentration of DNA in the reconstitution mixtures was 50 μg/mL except as otherwise specified. When we wanted to separate the reconstituted monomer from any DNA or histone that had not reconstituted, the material was first concentrated using an Amicon ultrafiltration cell (Model 12 with a PM10 membrane); at least 90% of the material absorbing at 260 nm was recovered. The concentrate was then centrifuged through 5–25% isokinetic sucrose gradients according to McCarty et al. (1974) in an SW40 rotor. Gradients were dripped from the bottom and fractions were dialyzed to remove the sucrose.

(G) *Sedimentation Experiments.* All sedimentation experiments were performed with a Beckman Model E analytical ultracentrifuge equipped with scanner optics. A wavelength of 265 nm was used in most experiments. The linearity and accuracy of the scanner had been checked with nucleotide solutions of known absorbance. The accuracy of temperature recording and control had been recently checked by diphenyl ether melting, using the technique of Gropper and Boyd (1965).

Sedimentation velocities were determined from the midpoints of the sedimenting boundaries, using a calculation program that automatically compensates for the change in scanner transit time to the boundary. All data were recorded at or near 4 °C, and corrected for temperatures and buffer effects to yield $s_{20,w}$ values.

In most cases, the integral distribution of sedimentation coefficients was calculated, using a modification of the method of Gralén and Lagermaln (1952). Details of this method will be described in a subsequent publication (Van Holde and Weischet, 1977).

(H) *Circular Dichroism.* The CD spectra of core particles were recorded with a Jasco J-SP CD apparatus calibrated against camphorsulfonic acid. All spectra were recorded at 10–20 °C.

(I) *Thermal Denaturation of Native and Reconstituted Core Particles.* Core particles for thermal denaturation studies were dialyzed extensively against 0.25 mM EDTA (pH 8.0). Samples were placed in 1-cm path length quartz cuvettes, bubbled with helium to degas the solution, and then overlayered with Dow Corning 200 silicone fluid and fitted with ground glass stoppers. Absorbances and temperatures were recorded directly on punch tape from a Beckman Acta III spectrophotometer. The rate of temperature increase was 0.25 °C/min and one set of data (absorbance, reference absorbance, and temperature) was collected each minute. The data were processed through a Hewlett Packard 9521 minicomputer, correcting for thermal expansion, to give percent hyperchromicity (h) vs. temperature (T) or the first derivative of h with respect to T. The percent hyperchromicity at temperature T is defined as:

$$h = \frac{A_{260} - A_{260^\circ}}{A_{260^\circ}} \times 100$$

where A_{260} is the absorbance at T and $A_{260}°$ is the absorbance at the base temperature (20 °C). A 20-point linear least-squares fit was used to obtain the derivative plot. A quadratic least-squares fit was tried but it was unsatisfactory.

(J) *Redigestion of Native and Reconstituted Core Particles by Pancreatic DNase I, Micrococcal Nuclease, and Trypsin.* Core particles, at a concentration of 50 μg/mL DNA, were made 4 mM in Mg^{2+} and digested with 100 units/mL pancreatic DNase I (Worthington) at 37 °C. Digestion was terminated by making the solution 10 mM in EDTA and cooling on ice.

Core particles were digested similarly with 100 units/mL micrococcal nuclease, except that 3 mM Ca^{2+} replaced the Mg^{2+}. The high levels of Mg^{2+} and Ca^{2+} used in these experiments were in part to compensate for the presence of EDTA in the core particle solutions. Repetition of the experiments with lower Mg^{2+} or Ca^{2+} concentrations gave identical results.

Core particles in 10 mM Tris-HCl (pH 8.0)–0.7 mM EDTA were incubated with various concentrations of trypsin (Sigma) at 4 °C for 16 h. The reaction was stopped by making the reaction mixture 10 μg/mL N^{α}-p-tosyl-l-lysine chloromethyl ketone (Tos-LysCH_2Cl); the mixture was immediately lyophilized to dryness.

(K) *DNA Extraction and Electrophoresis.* DNA was extracted using a modified Marmur technique (Britten et al., 1974), precipitated with 3 vol of ethanol, and redissolved in 0.1 strength electrophoresis buffer. The DNA was characterized by gel electrophoresis. Six percent polyacrylamide slab gels were prepared and run following the procedure of Loening (1967). Eight percent polyacrylamide–urea gels for examining

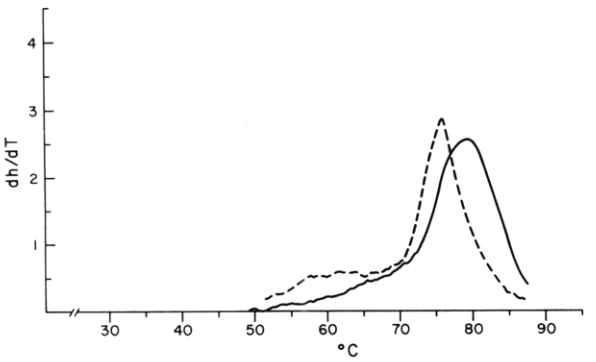

FIGURE 1: Derivative thermal denaturation profiles. Core particles soluble in 0.12 M NaCl (dashed line) and insoluble in 0.12 M NaCl (solid line) were thermally denatured as described under Materials and Methods. All samples melted to 35% hyperchromicity.

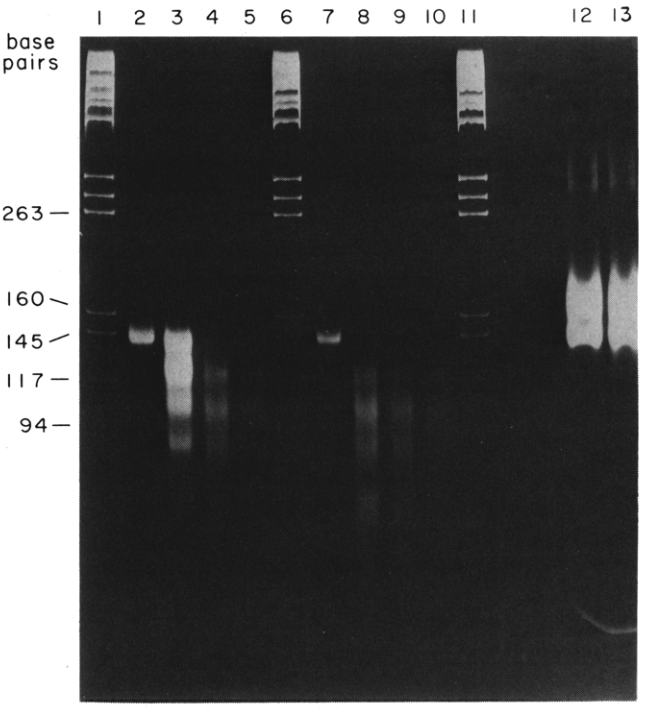

FIGURE 2: DNA from native and reconstituted core particles and their digestion with micrococcal nuclease. DNA was extracted from particles and electrophoresed on 6% polyacrylamide gels. The origin is at the top. The sizes indicated for the PM2–*Hae*III fragments (channels 1, 6, and 11) are taken from Kovacic and Van Holde (1977). Channel 2 shows the DNA from core particles isolated from H1 depleted chromatin by the Noll (1976b) procedure. Channel 7 shows the DNA from the reconstituted particles. Channels 3, 4, and 5 illustrate the products of digestion of the native core particles by 125 units/mL of micrococcal nuclease for 5, 15, and 60 min, respectively. Channels 8, 9, and 10 show comparable digestions of the reconstituted particles. Channels 12 and 13 illustrate the DNA from monomer particles prepared by digestions of whole chromatin in nuclei. Both 140 bp and larger DNA are present.

denatured DNA were prepared according to Maniatis et al. (1975). PM2–*Hae*III fragments were used to calibrate the DNA sizes (Kovacic and Van Holde, 1977). Gels were either photographed after staining with ethidium bromide or stained with toluidine blue or Stains All (Eastman) (Shaw et al., 1976).

The negatives from the ethidium bromide photographs or toluidine blue stained gels were scanned on an Ortec UV–vis densitometer.

(L) *Protein Characterization.* Proteins were electrophoresed on sodium dodecyl sulfate–15% polyacrylamide slab gels according to the procedure of Laemmli (1971). Fifteen percent polyacrylamide gels (1 mm × 200 mm × 200 mm) with 6% polyacrylamide stacking gel were run for 22 h at 95 V. When the protein composition of core particles was to be studied, the particles were lyophilized, dissolved in sample buffer containing 2.0% sodium dodecyl sulfate, 5% β-mercaptoethanol, 0.001% bromophenol blue, and 10% glycerol, and then heated at 100 °C for 1 min prior to electrophoresis. Gels were stained in 0.05% Coomassie blue R-250 (Sigma), 25% 2-propanol, and 10% acetic acid, and destained in 10% acetic acid. The destained gels were either photographed or scanned on an Ortec UV–vis densitometer. Lowry protein analyses according to Hartree (1972) were done using calf-thymus H4 as the standard. This protein was chosen since it was available in a highly purified form, of known extinction coefficient, and is representative of the inner histones.

Results

When nucleosome core particles are prepared by the standard procedure (digestion of chromatin in nuclei by micrococcal nuclease) DNA fragments of 160 and 130 bp, together with some smaller contaminating fragments, are obtained along with the 140-bp core DNA. The relative amounts of these various DNA fragments depend upon the time of digestion and the concentration of micrococcal nuclease, with longer digestions producing less of the 160-bp class and relatively more of the 130-bp and smaller fragments. Along with heterogeneity in the DNA size, small amounts of H1 and H5 are also found, specifically associated with particles containing 160 bp of DNA or more (Noll, 1976b; Noll and Kornberg, 1977; Varshavsky et al., 1976).

Such heterogeneity in DNA and protein content could seriously cloud observations concerning the physical structure of core particles. Approximately one-third of a standard core particle preparation will precipitate in 0.1–0.15 M salt (Olins et al., 1976). The fraction that precipitates is enriched in the 160-bp DNA and histones H1 and H5. The soluble fraction is enriched in the 140-bp core DNA and contains no H1 and H5. The differences in the thermal denaturation profiles are most striking, as shown in Figure 1. The fraction containing longer DNA and H5 and H1 has a considerably higher T_m and less premelting than the fraction soluble in 0.12 M salt. A more detailed account of the differences observed in different particle preparations will be published elsewhere (Weischet et al., manuscript in preparation).

The reconstitution and physical studies in this work were done with core particles very homogeneous in DNA size and histone content. To prepare such particles we used the method of Noll (1976b) in which most of the H1 and H5 are removed from a chromatin preparation with a 0.6 M NaCl wash before digestion of the chromatin with micrococcal nuclease. As shown in Figure 2 this procedure produces from chicken erythrocyte chromatin a very homogeneous core particle containing DNA with a size centered at 138 bp. The half-width of the band is only ±5 bp. There is only insignificant contamination by lower and higher molecular weight DNA size classes. That there is no significant "nicking" of the DNA can be seen by examining the zero-time columns in Figure 10; the sample remains entirely homogeneous on a single-strand gel. Histones H1 and H5 along with nonhistone proteins are present in only very low percentages. All the physical studies and reconstitution were done with this same core particle preparation.

A large number of reconstitution experiments have been carried out, using the salt-extracted core histones and core DNA. The results are summarized below.

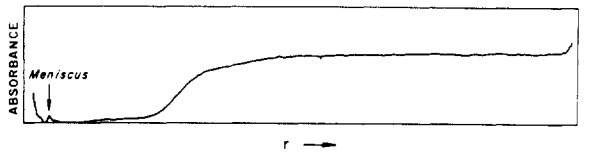

FIGURE 3: Analytical ultracentrifuge scanner trace of reconstituted core particles. This scanner trace shows the boundary obtained after reconstitution at a 2:1 histone tetramer/DNA ratio. The slower and faster sedimenting components comprise about 5 and 15% of the 11S boundary.

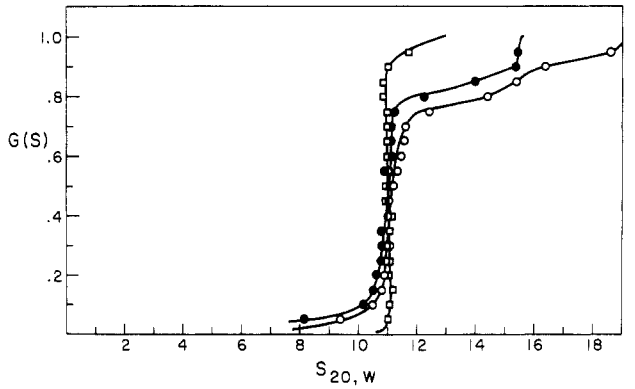

FIGURE 4: Integral distribution of s. The integral distribution of s (G (S)) for: (□) native core particles, (●) reconstituted core particle at 2:1 histone tetramer/DNA ratio, and (○) reassociation of core particles as described under Materials and Methods.

Nearly Complete Reconstitution Can Be Attained. Figure 3 shows an ultracentrifuge scanner trace from a typical reconstitution using nearly equivalent amounts of histones and DNA (2 mol of each histone per mol of core DNA). Nearly identical results were obtained with DNA concentrations of 300 μg/mL. The prominent leading boundary corresponds to reconstituted core particles. In this case, about 80% of the OD_{260} is present in this boundary. The small trailing boundary (<5%) which has a sedimentation coefficient of about 4.5 S (an expected value for 140-bp DNA at this low ionic strength) corresponds to DNA that has not reassociated. The faster boundary (~15%) corresponds to a particle with a sedimentation coefficient of ~16 S. Since UV absorption at 265 nm will not be very sensitive to histones at the concentration used here, we cannot tell whether there is a small amount of unassociated histone, or if we have not used exactly equivalent quantities. Concentrations were estimated assuming an extinction coefficient of 6600 cm^{-1} (mole base pair)$^{-1}$ for the DNA at 260 nm and 2.02 × 10^4 cm^{-1} M^{-1} for a histone heterotypic tetramer at 275.5 nm. In particular, we do not have evidence that the latter value is exact for the histone tetramers in 2 M NaCl since the histone extinction coefficient was a simple extrapolation from individual histones in distilled water (D'Anna and Isenberg, 1974).

In other similar experiments native core particles were dissociated by making them 2 M NaCl and reassociating them in the same manner as the reconstituted cores. In such "reassociation" experiments the stoichiometry should be exact. The scanner trace from the reassociated core particles is very similar to that of the reconstituted cores. In sedimentation experiments, the scanner profile is somewhat complicated by the fact that some of the boundary spreading is due to diffusion. Using the boundary analysis method of Van Holde and Weischet (1977), an integral distribution of sedimentation coefficients can be obtained. This effectively removes the diffusion spreading and gives a picture of the true heterogeneity

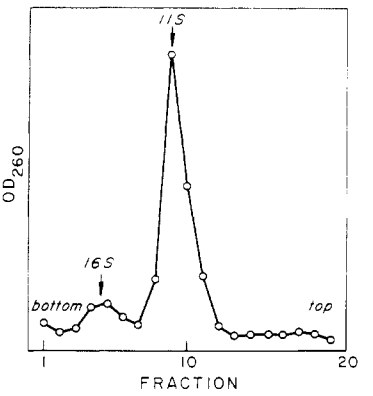

FIGURE 5: Sucrose gradient fractionation of reconstituted core particles. A reconstituted core particle preparation was run on 5–25% isokinetic sucrose gradients and gave the fractionation pattern shown. This procedure was used to preparatively separate 5, 11 and 16S, and larger components after a reconstitution.

of the sample. Figure 4 compares the heterogeneity in reconstituted core particles, reassociated core particles, and native core particles. Notice that the distribution for the native core particle used in this work is nearly a step function with the step at 11 S. Both the reconstituted and the reassociated core particles have small amounts of faster and slower sedimenting components. It is of interest that the reassociated core, showing this somewhat heterogeneous distribution, was very homogeneous prior to disassociation and reassociation. The similarity between the reconstituted and reassociated cores is evidence that the failure to attain 100% reconstitution is not a result of the wrong stoichiometry of DNA and histone.

The reconstituted and reassociated mixtures were run on preparative isokinetic sucrose gradients in order to separate the 5S, 11S, and 16S components (Figure 5). The fractions corresponding to these 5S, 11S, and 16S components were isolated, and the DNA run on 6% polyacrylamide slab gels and the proteins run on 15% sodium dodecyl sulfate slabs. Figure 6 shows that the reassociated and reconstituted 11S components contained just the 138 ± 5-bp DNA size class. The 16S components in each case contain 90% 138-bp DNA and 10% 265-bp DNA while the 5S component contained sizable quantities of small DNAs (50 and 90 bp). The protein contents of the 11S and 16S fractions are nearly identical. There is almost a total lack of nonhistone proteins. Two very faint, slowly migrating bands are occasionally observed in the protein gels. We do not know the nature of these bands. It is of importance that only a very small amount of the histones H1 and H5 are associated with the 16S component (less than 1%). This indicates that H1 and H5 are *not* responsible for the binding of two 11S cores to form a 16S dimer. This phenomenon will be discussed in more detail below.

Comparative Properties of Native, Reconstituted, and Reassociated Core Particles. We have examined the physical properties of native, reconstituted, and reassociated core particles. In each case the purified 11S component, isolated by the sucrose gradient sedimentation, was used in the comparative studies.

Histone Composition. Figure 7 compares scans of sodium dodecyl sulfate gels of proteins from native and reconstituted particles. In each case, the only proteins present in significant quantities are the "inner" histones H2A, H2B, H3, and H4, and the proportions of each appear to be the same in each case. Results with reassociated particles and 16S particles (not shown) appear identical. The histone stoichiometry cannot be determined directly due to the possibility of differential dye

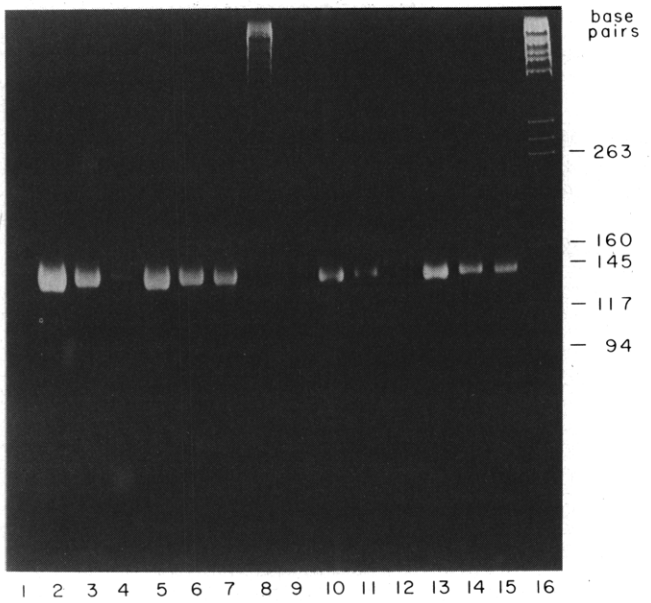

FIGURE 6: DNA content of reconstituted core particle fractions. DNA was extracted from 5, 11, and 16S sucrose gradient fractions of reconstituted and reassociated core particle preparations and run on 6% polyacrylamide slab gels. Channels 8 and 16 are PM2–*Hae*III fragments. Channels 1 and 9 are free or unreconstituted DNA (5 S) from reconstituted preparations. Channels 4 and 12 are free DNA from reassociated preparations. Channels 2,10 and 5,13 are DNA from the reconstituted and reassociated 11S fractions, respectively. Channels 3,11 and 6,14 are DNA from the reconstituted and reassociated 16S fractions, respectively. Channels 7 and 15 are the entire DNA before fractionation on sucrose gradients. The higher DNA loading of the channels on the left allows better observation of minor DNA size classes, specifically the 265-bp DNA in the 16S components and the low molecular weight DNA in the free DNA components.

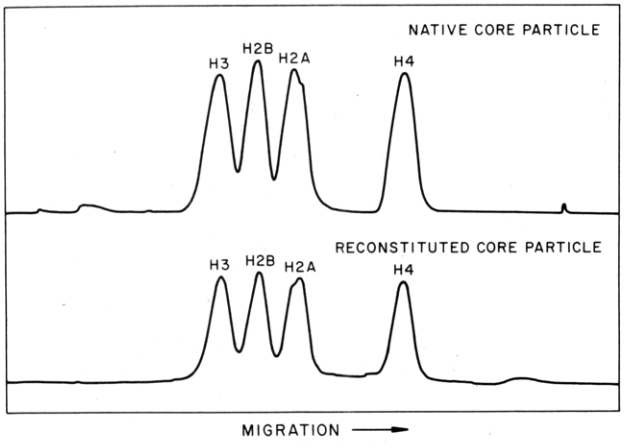

FIGURE 7: Densitometer tracings of sodium dodecyl sulfate–polyacrylamide gel. Sodium dodecyl sulfate (15%) slab gels were stained with Coomassie blue and destained and a densitometer tracing made of each sample. Scans were integrated and the area ratios of the four inner histones were determined for each fraction. The area ratios H3:H2B:H2A:H4 were: native core particle, 1.3:1.1:1.1:1.0; reassociated 16S fraction, 1.3:1.3:1.2:1.0 (not shown); reconstituted 16S fractions, 1.2:1.3:1.3:1.0 (not shown); reassociated 11S core particle, 1.3:1.2:1.3:1.0 (not shown); reconstituted 11S core particle, 1.3:1.3:1.2:1.0.

binding (McMaster-Kaye and Kaye, 1974); nevertheless, the stoichiometry of the inner histones in native and reconstituted core particles appears to be the same, and very likely corresponds to equimolar quantities of each.

CD Spectra. Figure 8 shows the CD spectra, in the range between 310 and 250 nm, of native particles, reconstituted particles, and free "core" DNA, under identical conditions.

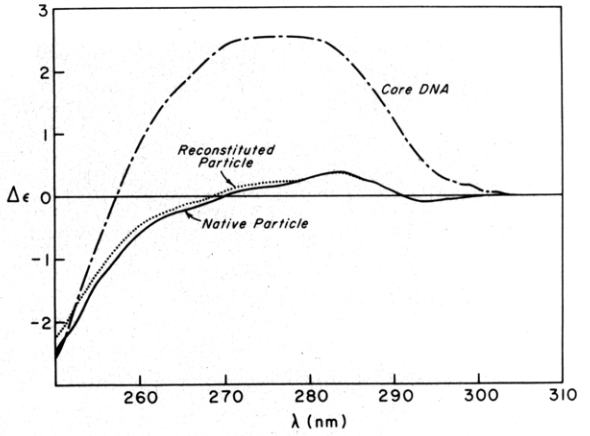

FIGURE 8: Circular dichroism spectra of native and reconstituted core particles. The CD spectra of native core particles, reconstituted core particles, and core particle DNA were normalized to the DNA concentration assuming the same extinction coefficient for free DNA and core particles (6600 cm^{-1} mol base $pair^{-1}$).

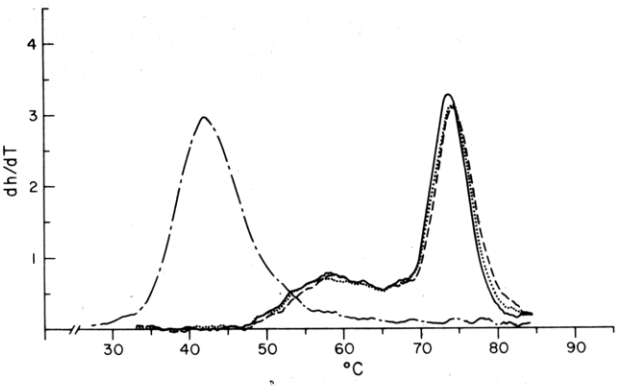

FIGURE 9: Thermal denaturation of native and reconstituted core particles. Core particle DNA (-·-), reconstituted particles (—), reassociated particles (···), and native particles (---) were thermally denatured in 0.25 mM EDTA (pH 8.0) and the hyperchromicity was followed at 260 nm. We have plotted the derivative of percent hyperchromicity as a function of temperature. The total percent hyperchromicities for the core particle DNA, reconstituted particle, reassociated particle, and native particle were 36.0, 35.0, 35.0, and 34.6, respectively.

Within the experimental uncertainties of our measurements, the native and reconstituted particles yield identical CD spectra. While we do not understand the reason for the unusual CD spectrum of the DNA in core particles, Figure 8 may be taken as strong evidence that at least the gross conformation of the DNA is identical in native and reconstituted particles. Preliminary results indicate that the CD spectra of native, reassociated, and reconstituted particles are also identical between 210 and 250 nm.

Thermal Denaturation. Native, reassociated, and reconstituted core particles were thermally denatured in 0.25 mM EDTA (pH 8.0). Free DNA and core particle samples gave ~35% hyperchromicity upon denaturation. Figure 9 shows the derivative plots of the thermal denaturations. It is quite evident that both native and reconstituted cores melt nearly identically, with a wide premelt centered at 59 °C and a main transition at ~74 °C. The thermal denaturation of nucleosomes is sensitive to the overall physical structure of the particles from various preparations (see Figure 1). Although we cannot explain the significant premelt, it is evident that the reconstituted and native core particles behave nearly identically.

Nuclease Digestion. The digestion of nuclei or chromatin by pancreatic deoxyribonuclease I (DNase I) produces a series

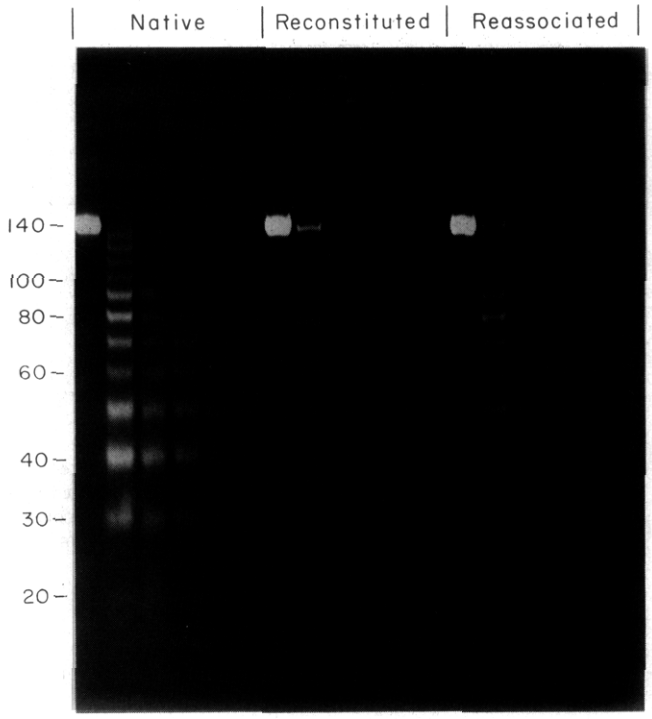

FIGURE 10: Pancreatic DNase I digest of native and reconstituted core particles. DNA was extracted from a DNase I digestion of core particles and run on an 8% polyacrylamide–urea slab gel. The times of digestion for each component are, from left to right: 0, 30 s, 2 min, 5 min, 10 min, and 20 min. The numbers to the left give the DNA sizes in bases.

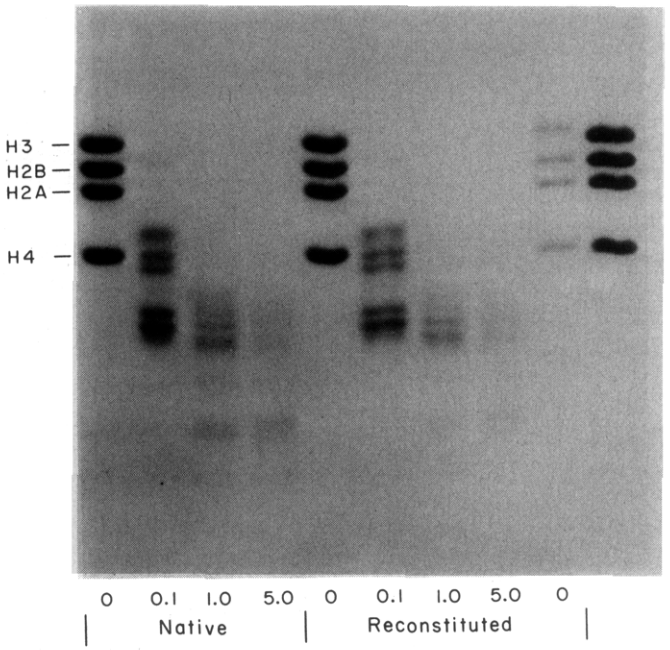

FIGURE 11: Trypsin digest of native and reconstituted core particles. Core particle preparations were digested for 16 h with various concentrations of trypsin (in μg/mL) at 4 °C. The reaction was stopped with the addition of Tos-LysCH_2Cl; samples were then lyophilized and run on 15% sodium dodecyl sulfate slab gels. The numbers under each channel refer to concentration of trypsin in μg/mL.

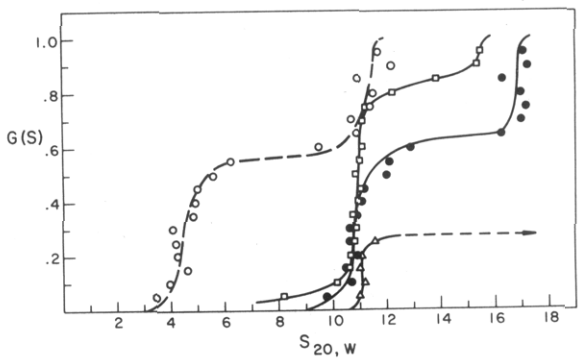

FIGURE 12: Integral distribution of s (G (S)). Salt-extracted histones and DNA were reconstituted at various histone tetramer/140-bp DNA ratios and the resultant products analyzed by analytical sedimentation. The integral distribution of s (G (S)) is plotted here: 1:1 histone tetramer/DNA (○), 2:1 (□), 2.5:1 (●), and 3.2:1 (△). The dashed line and arrow in the 3.2:1 sample are used to indicate aggregation, in this case 70%. A histone tetramer/DNA ratio of 4:1 resulted in 100% aggregation.

of DNA fragments at 10-b intervals when electrophoresed on denaturing gels (Noll, 1974). This pattern is also produced upon digestion of native and reconstituted core particles with DNase I (Figure 10). The reconstituted core particles not only give the entire series of fragments from 140 to 20 b but the relative rates of cleavage seem nearly identical with those obtained with native particles in each case.

Evidence that the reconstituted core particle is nearly identical with the native particle also comes from the digestion of cores with micrococcal nuclease. Digestion with this enzyme also produces specific submonomer digestion patterns reflecting in some manner the structure of the core particle. Figure 2 indicates that the native and reconstituted cores have a similar structure with very similar micrococcal nuclease digestion patterns.

Trypsin Digestion. Further evidence for the similarity between native and reconstituted cores comes from trypsin digestion studies. Sahasrabuddhe and Van Holde (1973) have shown that mild trypsin digestion of nucleosome particles leads to a more extended structure with a lower s value. Weintraub and Van Lente (1974) have shown that trypsin cleaves the N-terminal ends of the inner histones leaving a more resistant core protein particle made up of the C-terminal portions of the histones. Electrophoresis of these particles on sodium dodecyl sulfate–polyacrylamide gels reveals a specific pattern of specific histone cleavage products. Weintraub et al. (1975) have shown that these cleavage products are not produced with histones alone at low ionic strength while Sollner-Webb et al. (1976) have indicated that reconstitution with all four inner histones plus DNA gives the specific cleavage products. We have trypsin treated both native and reconstituted core particles and find an identical pattern of protein cleavage (Figure 11). This provides further evidence for the specificity of reconstitution.

In summary we have provided evidence that a core particle nearly if not wholly identical with the native core can be reconstituted from its protein and DNA components, with at least 80% yield.

Reconstitutions at Different Histone/DNA Ratios. We investigated the occurrence of faster and slower sedimenting components in the ultracentrifuge scan of reconstituted or reassociated core particles by varying the histone/DNA stoichiometry in reconstitutions. Salt-extracted histone and 140-bp DNA were mixed in various proportions ranging from 1 mol of histone tetramer per mol of DNA to 4 mol of histone tetramers per mol of DNA; these were reconstituted in the same manner as above and the resultant product analyzed with the analytical ultracentrifuge. Figure 12 illustrates the results of the experiment. The 1:1 (histone tetramer/140-bp DNA) reconstitution appears just as would be expected if two tetramers combine with one DNA molecule to form a core particle. The 4.5S and 11S boundaries correspond to free DNA and core

TABLE I: Histone/DNA Ratios.[a]

Sample	Histone/DNA (g/g)	Av
Native 11S particle	1.20, 1.16, 1.02	1.13
Reconst. 11S particle	1.19, 1.02, 1.06	1.09
Reconst. 16S particle	1.14, 1.21	1.18
Aggregated material	1.86, 1.86	1.86

[a] Protein concentrations were determined by Lowry analysis (Hartree, 1972), calibrated against calf-thymus histone H4. DNA concentrations were determined from absorbance at 260 nm.

particles, respectively; roughly half of the DNA remains free. Also of importance is the fact that no DNA sediments between the 4.5S and the 11S boundaries, indicating that a "half-core" particle made up of a core DNA fragment and only one histone tetramer is not a stable product in the reconstitution process. When the histone/DNA ratio is raised to 2:1 (histone tetramer/140-bp DNA) almost all the DNA sediments as an 11S boundary with little of the DNA left free in solution. Again it is important to mention that the faster and slower sedimenting components in the 2:1 reconstitution are not the result of the wrong stoichiometry because a simple reassociation of native core particles produces the same components to the same extent.

The 16S component in the 2:1 reconstitution was analyzed further after purification on sucrose gradients. It sedimented in the ultracentrifuge as a homogeneous boundary at 15.5 S, the same sedimentation coefficient observed for dimer particles, although it contained mostly core particle length DNA (see Figure 6). The protein/DNA ratio of the 16S component was identical with the 11S reconstituted component (see Table I).

Reconstitution at a 2.5:1 ratio leads to a further increase in the amount of material sedimenting at $s_{20,w} > 11$ S (see Figure 12). This complex has a sedimentation coefficient significantly greater than 15.5 S (~17 S). Raising the histone tetramer/140-bp DNA ratio to 3.2:1 leads to considerable aggregation. The solution is visibly turbid with 70% of the DNA pelleting out of solution before the sedimentation run is up to speed. The nonaggregating material sediments as a homogeneous boundary at 11 S. A further increase in the histone tetramer/140-bp DNA ratio leads to greater aggregation; at a ratio of 4:1 all the DNA aggregates and no 11S material is left in solution. The histone/DNA ratio (w/w) of this aggregated material is 1.86 (see Table I). Possible mechanisms will be discussed below.

Discussion

The nearly quantitative reconstitution that can be obtained from either dissociated core particles or mixtures of core DNA and histone tetramers argues that formation of the core particles is a spontaneous process in low salt media. The virtual identity of the physical properties and DNase sensitivity of the native and reconstituted core particles strongly supports this contention.

It would be of importance to know whether we are dealing with histone octamers or heterotypic tetramers in the reconstitution from 2 M NaCl. On the basis of cross-linking studies, Thomas and Kornberg (1975) have suggested that the structure is an octamer. Other workers, using physical techniques (Weintraub et al., 1975; Wooley et al., 1977) have argued that the heterotypic tetramer is the dominant species. Judgment is complicated on the one hand by the possibility that the cross-linking studies may be selecting for a small population of octamers in dynamic equilibrium with tetramers, and on the other by the fact that physical studies in high salt, where preferential hydration effects may be severe, may easily be misinterpreted. We do not believe that the answer is *critical* to our studies. As will be argued below, the evidence is that at the point of association with DNA, or immediately thereafter, the octamer is the stable species.

It is significant, however, to note that the presence of DNA appears to be essential for the integrity of octamers, or even tetramers, in low salt. It has been demonstrated that the complex present in high salt will disproportionate into H3–H4 and H2A–H2B complexes similar to those studied at low ionic strength by others (D'Anna and Isenberg, 1974; Kornberg and Thomas, 1974; Roark et al., 1974; Sperling and Bustin, 1975; and Lilley et al., 1976) when the salt concentration is lowered below 1 M (Weintraub et al., 1975). In the presence of DNA, however, a very different result is found; the tetramers (if present) dimerize to form the octamer core of the core particle as salt is removed.

This is dramatically demonstrated by the experiments in which an excess of DNA was used. The DNA concentration was such as to provide one DNA molecule per tetramer. A conceivable result would have been the association of *each* tetramer with one DNA molecule, to form a structure with $5 \text{ S} < s_{20,w} < 11$ S. This was not observed. Instead, about half of the DNA remains free; half associates with histones to form 11S particles. There are two possible explanations for this: (1) association is initially between one tetramer and one DNA molecule, with an obligatory subsequent addition of a second tetramer, or (2) at some point in the gradient dialysis, a significant fraction of octamers is formed, which possesses a very strong binding tendency for DNA. There is, in fact, some evidence for the latter possibility in the sense that histone octamers and higher aggregates (Thomas and Kornberg, 1975) can be obtained in cross-linking studies. On the other hand, the first possibility cannot be excluded, for formation of a transient DNA–tetramer complex might lead to a very strong tendency to incorporate another tetramer.

A fundamental question concerns the fidelity with which the DNA is placed upon the set of binding sites on the protein core. Are all of the DNA molecules wholly bound, or are there projecting "tails"? Of course, if the "re-entrant path" model proposed by Cantor (1976) is correct, one would expect that the DNA, wherever it first attached, would lie completely in the path. However, this model has not been demonstrated, to our knowledge. If the path is not re-entrant, it would seem possible that DNA molecules could irreversibly attach at an "incorrect" point, and leave a projecting region. The evidence from our experiments is that this either does not happen frequently, or represents an unstable intermediate at least in reconstitution or reassociation experiments where the DNA/histone ratio is close to that of the native particles. The fact that the sedimentation coefficient and CD of such particles are very close to those of the native particle is evidence of a sort. More convincing is the evidence from DNase I digestion. The reconstituted or reassociated particles show the same pattern, including the higher (>100 b) bands, and do not exhibit significant "background" of randomly cleaved fragments, suggesting that in most cases there must be very little "projecting" DNA. Finally, we note that the melting curves for the reconstituted and reassociated particles are nearly identical with those for native particles.

However, especially at higher histone/DNA ratios, there is evidence for poorer fidelity in DNA placement. This comes from the existence of higher aggregates, which increase in both size and amount as the histone/DNA ratio is increased. It is difficult to explain the formation of such structures other than

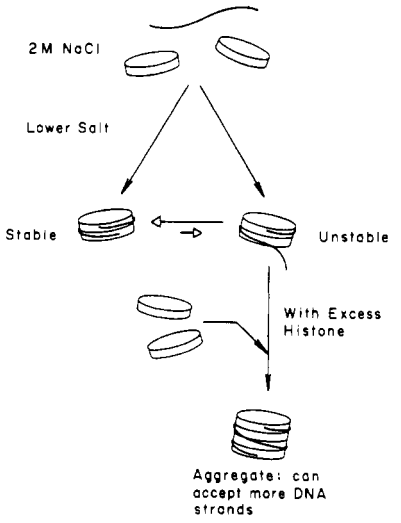

FIGURE 13: A schematic diagram of our model for the reconstitution process. In 2 M NaCl, the components are postulated to be free DNA and histones, mainly present as heterotypic tetramers. As the salt concentration is reduced, association of DNA and tetramer takes place to form core particles. Both "correct" (left) and "incorrect" (right) alignment of the DNA can initially occur; however, the latter structures are relatively unstable, and tend to rearrange to form "correct" particles. In the presence of excess histone, however, the incorrectly aligned DNA will tend to serve as a "cross-link" between adjacent octamers. These structures can in turn add more DNA to form large aggregates.

by the formation of DNA cross-links between octamers. Our tentative model for the reassociation process is shown in Figure 13. It presumes that at some intermediate stage in the gradient dialysis, the salt concentration will be such that DNA-histone complexes are only marginally stable. At low histone/DNA ratios, incorrect placement of a DNA on an octamer will be corrected by exchange, for a partially attached DNA will be relatively unstable, compared to a "fully" attached DNA. However, at a high histone/DNA ratio, a poorly attached DNA may be stabilized if another protein core is immediately available to "stack" on the first. This generates an "out of phase" structure which can grow to a larger aggregate by successive attachment of DNA fragments and protein cores.

As might be expected, this process can occur to some degree even when the stoichiometry is exact, as in the case of reassociation experiments (Figure 4). It is noteworthy that the small amount of dimer found when a 2:1 tetramer/DNA ratio is used *cannot* be accounted for by dimer-size DNA (there is only 10% dimer DNA in the *isolated* dimer peak (Figure 6)), nor is sufficient H1 or H5 present to account for the dimer found. Only in cases where the histone tetramer/140-bp DNA ratio is less than 2:1 do we see a complete absence of structures larger than the core particles.

The techniques described here provide a way for making large quantities of core particles, using specifically modified histones or mixtures of histones. Chromatin reconstitution experiments with individually purified histones (Camerini-Otero et al., 1976; Sollner-Webb et al., 1976) and even acid purified histones (Germond et al., 1976) indicate that reconstitution need not be done with histone complexes as a starting material. We have carried out reassociation experiments from 6 M urea-2 M NaCl in which the urea was dialyzed away prior to salt dialysis. These experiments give the same result as salt reassociations. Along with evidence that urea will denature histone complexes (Weintraub et al., 1975) this gives further evidence that reconstitution experiments need not start with the histone complexes, but can begin with unfolded isolated histone molecules. Even if a small fraction of dimers or larger structures is formed, these can easily be separated. The 11S particles so produced appear to be, in every way tested so far, identical with the native particles.

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Isoelectric Focus Analysis of Rat Anti-phosphocholine Antibodies†

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ABSTRACT: Anti-phosphocholine (PC) antibodies in sera from four strains of rats were examined before and after immunization with either *Streptococcus pneumoniae* R36A, which contains PC as a cell wall component, or with PC-coupled keyhole limpet hemocyanin (PC-KLH). PC-specific protein was purified from pooled immune sera and shown by a combination of isoelectric focus (IEF) in acrylamide and crossed immunoelectrophoresis, as well as by molecular weight determination in $NaDodSO_4$-acrylamide, to be immunoglobulin. An additional, small molecular weight, nonimmunoglobulin protein (pI = 7.1-7.3) was present in sera from normal and germ-free rats which had the ability to bind the C-carbohydrate of *S. pneumoniae* R36A, but without specificity for PC. The IEF profile of normal and immune sera showed marked sharing of bands of anti-PC antibody between individual rats as well as between strains. In addition, other anti-PC antibodies which focused between pH 8.5 and 9.5 were less regularly shared. The uniformity of IEF profile of the bulk of anti-PC antibodies in rats is most consistent with their being the products of germ line genes.

Detailed studies of antibodies with similar antigen binding specificities have revealed striking structural similarities. In particular, the analysis of mouse anti-phosphocholine (PC)[1] immunoglobulins by antigenic (Claflin and Davie, 1974a), functional (Claflin and Davie, 1974b), and structural means (Claflin and Rudikoff, 1977) has shown that all mice regardless of genetic background produce similar antibodies. This conservation of variable region structure is consistent with the existence of a germ line gene for anti-PC antibodies in mice.

In addition, preliminary examination of the fine specificity of anti-PC antibodies raised in different species of rodents showed that each species developed distinctive binding patterns for PC and its analogues and that within a species, indistinguishable patterns were found in all individuals examined (Claflin and Davie, 1974b). These studies were based on the ability of soluble haptens to inhibit plaque formation by antibody secreting cells. The present paper extends these studies in the rat by examining the isoelectric focusing pattern of 7S anti-PC antibodies in normal and immune rat serum. While minor differences in these spectra exist, extensive sharing of antibody patterns is seen regardless of the genetic background of the animal.

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[1] Abbreviations used are: PC, phosphocholine; KLH, keyhole limpet hemocyanin; IEF, isoelectric focusing; PBS, phosphate-buffered saline; BGG, bovine γ-globulin; BSA, bovine serum albumin; CFA and IFA, complete and incomplete Freund's adjuvant, respectively; PnC, C polysaccharide.