Vârô, G., & Lanyi, J. K. (1991) *Biochemistry* (preceding paper in this issue).
Vârô, G., Duschl, A., & Lanyi, J. K. (1990) *Biochemistry 29*, 3798-3804.
Walmsley, A. R., & Lowe, A. G. (1987) *Biochim. Biophys. Acta 901*, 2229-2238.
Zimányi, L., Keszthelyi, L., & Lanyi, J. K. (1989) *Biochemistry 28*, 5165-5172.

Different Mechanisms for in Vitro Formation of Nucleosome Core Particles†

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ABSTRACT: The interaction of different histone oligomers with nucleosomes has been investigated by using nondenaturing gel electrophoresis. In the presence of 0.2 M NaCl, the addition of the pairs H2A,H2B or H3,H4 or the four core histones to nucleosome core particles produces a decrease in the intensity of the core particle band and the appearance of aggregated material at the top of the gel, indicating that all these histone oligomers are able to associate with nucleosomes. Equivalent results were obtained by using oligonucleosome core particles. Additional electrophoretic results, together with second-dimension analysis of histone composition and fluorescence and solubility studies, indicate that H2A,H2B, H3,H4, and the four core histones can migrate spontaneously from the aggregated nucleosomes containing excess histones to free core DNA. In all cases the estimated yield of histone transfer is very high. Furthermore, the results obtained from electron microscopy, solubility, and supercoiling assays demonstrate the transfer of excess histones from oligonucleosomes to free circular DNA. However, the extent of solubilization obtained in this case is lower than that observed with core DNA as histone acceptor. Our results demonstrate that nucleosome core particles can be formed in 0.2 M NaCl by the following mechanisms: (1) transfer of excess core histones from oligonucleosomes to free DNA, (2) transfer to excess H2A,H2B and H3,H4 associated separately with oligonucleosomes to free DNA, (3) transfer to excess H2A,H2B initially associated with oligonucleosomes to DNA, followed by the reaction of the resulting DNA–(H2A,H2B) complex with oligonucleosomes containing excess H3,H4, and (4) a two-step transfer reaction similar to that indicated in (3), in which excess histones H3,H4 are transferred to DNA before the reaction with oligonucleosomes containing excess H2A,H2B. The possible biological implications of these spontaneous reactions are discussed in the context of the present knowledge of the nucleosome function.

In the nucleosome, DNA interacts with the core histone octamer and forms a stable folded structure (Richmond et al., 1984). However, under appropriate solution conditions, the nucleosome core particle can undergo various conformational transitions without modification of the normal histone composition (Uberbacher et al., 1983; Yager & van Holde, 1984; Oohara & Wada, 1987b; Daban & Cantor, 1989). Moreover, the results obtained in several laboratories indicate that histones and DNA can form various complexes different from the typical nucleosome core particle. It has been found that core particle DNA can associate with different oligomers of histones H3,H4 (Simon et al., 1978; Read et al., 1985) and H2A,H2B (Oohara & Wada, 1987a; Aragay et al., 1988). It has also been found that nucleosome core particles are capable of binding more than 1 equiv of core histones in addition to the inner histone octamer (Voordouw & Eisenberg, 1978; Stein, 1979; Stein et al., 1985). The sedimentation analysis of these complexes has indicated that the additional histones are bound to the exterior of the nucleosome (Eisenberg & Felsenfeld, 1981). The histone pairs H3,H4 and H2A,H2B can also interact separately with nucleosome core particles (Eisenberg & Felsenfeld, 1981).

The altered nucleosome structures and the different DNA–histone complexes considered in the preceding paragraph could be related to the cellular function of the nucleosome. In particular, they could be involved in the mechanism of chromatin assembly. In this regard, previous physicochemical studies have demonstrated the existence of conformational transitions in the final part of the nucleosome self-assembly reaction (Daban & Cantor, 1982a,b). The involvement of DNA–(H3,H4) and DNA–(H2A,H2B) complexes in the mechanism of nucleosome self-assembly has also been investigated. It has been demonstrated that, in the presence of 0.2 M NaCl, a reaction of exchange of histone pairs allows the formation of complete nucleosome core particles from incomplete structures containing only histones H3,H4 or H2A,H2B (Aragay et al., 1988).

As in the case of DNA–(H3,H4) and DNA–(H2A,H2B) complexes, the association of histones with nucleosome core particles must also be considered in the context of the reaction of nucleosome assembly. In fact, in an early study, Stein (1979) showed that during nucleosome reassociation in 0.6 M NaCl a fraction of the core particles initially formed are complexed with an excess histone octamer, which finally is

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transferred to free core DNA. Taking into account that, even during chromatin replication, most of the DNA in the cell nucleus is packaged into nucleosomal structures (DePamphilis & Wassarman, 1980), the association of excess histones with nucleosomes could also occur in vivo. Obviously, it is likely that in the cell nucleus, acidic proteins such as nucleoplasmin and N1/N2 (Earnshaw et al., 1980; Kleinschmidt et al., 1985, 1990; Sealy et al., 1986; Dilworth et al., 1987; Cotten & Chalkley, 1987) and HMG1 (Bonne-Andrea et al., 1984), and polyanions such as RNA (Nelson et al., 1981), all of them factors that facilitate core particle assembly, compete with nucleosomes for binding histones. However, in a more recent study, Stein et al. (1985) have shown that, even in the presence of polyglutamic acid, which also facilitates nucleosomes assembly (Stein et al., 1979), core particles are able to bind excess core histones.

The studies on the interaction of histones with nucleosomes have been carried out at high salt concentrations [0.5 M NaCl (Voordouw & Eisenberg, 1978; Eisenberg & Felsenfeld, 1981) or 0.6 M NaCl (Stein, 1979)], or at lower salt concentration (0.1 M NaCl) but in the presence of polyglutamic acid (Stein et al., 1985). In the present paper, in order to continue our physicochemical studies on the basic mechanisms of nucleosome self-assembly (in the absence of additional factors) at physiological ionic strength, we have carried out a detailed investigation of the stability and reactivity under these conditions of mononucleosome and oligonucleosome core particles containing excess core histones. In this study we have used polyacrylamide gel electrophoresis under nondenaturing conditions (Garner & Revzin, 1981, 1986; Fried & Crothers, 1981; Aragay et al., 1988; Fried, 1989). Although the initial ionic strength of the samples is not maintained during electrophoresis on the gels containing 1× TBE used in this work, our results show that this electrophoretic system allows the direct analysis of DNA–histone complexes under a wide variety of initial conditions.

Moreover, taking into account that at physiological salt concentration histones H2A,H2B and H3,H4 free in solution do not interact with each other (Kornberg & Thomas, 1974; Eickbush & Moudrianakis, 1978; Ruiz-Carrillo & Jorcano, 1979; Sperling & Wachtel, 1981), we have also investigated the reactivity in 0.2 M NaCl of nucleosome core particles and oligonucleosomes containing an excess of either H2A,H2B or H3,H4. Our findings, together with previous results, allow us to present a general model for the self-assembly of nucleosome core particles.

Materials and Methods

Preparation of Histones, Nucleosome Cores, Oligonucleosome Core Particles, and DNA. Core histones, nucleosome core particles, and core DNA (146 base pairs) were obtained from chicken erythrocyte nuclei as described (Diaz & Daban, 1986; Aragay et al., 1988). To prepare histones H2A,H2B and H3,H4, core histones (180 mg) in 9 mL of a buffer containing 0.2 M NaCl, 0.4 mM phenylmethanesulfonyl fluoride, 0.01% 2-mercaptoethanol, and 10 mM Tris-HCl, pH 7.4, were chromatographed at 4 °C on a Sephadex G-100 column (150 × 3 cm) eluted with the same buffer (Ruiz-Carrillo & Jorcano, 1979; Daban & Cantor, 1982b). Oligonucleosome core particles were prepared by micrococcal nuclease digestion of soluble chromatin that had been previously depleted of histones H1 and H5 following the method of Ruiz-Carrillo et al. (1980). The digested chromatin was fractionated by sedimentation on a 5–20% linear sucrose gradient, and the DNA of the different fractions was analyzed on polyacrylamide and/or agarose gels. Generally, in the experiments described below, we used chromatin fragments containing 1–3 or 3–8 nucleosome core particles. Circular double-stranded DNA from the replicative form of bacteriophage M13 and pBR322 plasmid DNA were isolated by conventional procedures (Maniatis et al., 1982; Messing, 1983).

Histone Transfer Reactions. Histones were dissolved in water before the addition of the required buffer solution. A fresh solution of H2A,H2B, H3,H4, or core histones in a buffer containing 0.2 M NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl, pH 7.4, was added to a mononucleosome or oligonucleosome core particle solution prepared in the same buffer. The resulting samples were incubated for 15–30 min at room temperature and then treated with free core DNA. After the addition or core DNA, the samples were incubated for 15–30 min at room temperature and, unless otherwise indicated, centrifuged for 5 min at 10000*g*. The supernatants were analyzed on nondenaturing gels. In the experiments involving M13 and pBR322 DNA as histone acceptors, the samples were incubated for 2–8 h in the presence of circular DNA and, finally, analyzed by the different methods described below. The detailed reaction sequences corresponding to the different experiments are indicated in the figure legends. The concentration of histone solutions was determined spectrophotometrically, taking A_{230nm} = 4.2 for 1 mg of histone/mL (Thomas & Oudet, 1979). The concentration of DNA (free or complexed with histones) was calculated by using A_{260nm} = 20.0 for 1 mg of DNA/mL and by assuming that the absorbance of histones at 260 nm is negligible. The DNA concentration and the histone to DNA weight ratio corresponding to each sample are indicated in the figure legends.

Electrophoretic Analysis of DNA–Histone Complexes. The different reaction products were analyzed on nondenaturing 6% polyacrylamide gels containing 1× TBE buffer as described previously (Aragay et al., 1988). These gels were also used to analyze reconstituted nucleosome core particles prepared by direct mixing of the four core histones and core DNA in 0.2 M NaCl. Samples in 0.2 M NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl, pH 7.4, were mixed with 0.1 volume of 10× TBE buffer containing Ficoll, loaded onto the gel (15 cm long), and electrophoresed at 200 V for 2 h. The DNA of the nucleoprotein bands was stained with ethidium bromide and the gel was photographed over an ultraviolet light. Photographic negatives were scanned with a Shimadzu CS-9000 densitometer. For the analysis of histones of the nucleoprotein bands, second-dimension 15% polyacrylamide gels containing sodium dodecyl sulfate were prepared as described (Aragay et al., 1988).

To study the effect of trypsin on the electrophoretic mobility of nucleosome cores, reconstituted core particles (100 μg of DNA/mL), prepared by direct mixing of core histones and core DNA in 0.2 M NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl, pH 7.4, were digested with a fixed concentration of trypsin (Sigma) for the indicated times (see legend to Figure S1b). The digestion was stopped by the addition of bovine lung trypsin inhibitor (Bayer, 2 μg/μg of trypsin), and the resulting samples were analyzed on nondenaturing gels containing 1× TBE and on 15% polyacrylamide–sodium dodecyl sulfate gels as described elsewhere (Diaz & Daban, 1986).

To study the effect of the stoichiometry of core histones on the electrophoretic properties of the reconstituted DNA–histone complexes, different volumes of solutions of histones H2A,H2B and H3,H4 in 0.2 M NaCl, 0.2 mM EDTA, and 10 mM Tris–HCl, pH 7.4 (see legend to Figure S1d), were added to a fixed volume of a solution of core DNA in the same buffer. The samples corresponding to the studies of the effect

of salt and particle concentration on the dissociation of nucleosome cores were diluted to the indicated final NaCl and DNA concentrations (see Figures 1 and S2), incubated for 15 min at room temperature, and electrophoresed on 6% polyacrylamide–1× TBE gels as indicated above. The relative intensities of the bands corresponding to free core DNA were determined from the densitometric analysis of the photographic negatives of the gels stained with ethidium bromide.

Fluorescence and Solubility Studies. Fluorescent labeling of the single thiol group of chicken erythrocyte histone H3 with *N*-(1-pyrenyl)maleimide was carried out as described elsewhere (Daban & Cantor, 1982a, 1989). The transfer reactions with samples containing pyrene-labeled histone H3 were performed as indicated above but in the presence of 0.45 or 0.14 M NaCl (additional experimental details are given in Table I). The yield of histone transfer was estimated from the increase in the fluorescence intensity at 460 nm, corresponding to the formation of pyrene excimers (Daban & Cantor, 1982a, 1989). Fluorescence was measured on a Perkin-Elmer 650-40 spectrofluorometer interfaced to a Perkin-Elmer 3600 data station.

In the solubility studies, the samples containing mononucleosome or oligonucleosome core particles plus excess histones, in both the absence and presence of additional free DNA (see Figures 2d and S4), were centrifuged for 15 min at 10000*g* in an Eppendorf microfuge. The amount of soluble histone remaining in the supernatant was determined by the method of Bradford (1976).

Supercoiling Assay. The supercoiling assay for nucleosome formation was carried out essentially following the method of Sealy et al. (1986). Generally, M13 DNA or pBR322 DNA (forms I and II) in a buffer containing 0.2 M NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl, pH 7.4, was treated with topoisomerase I (GIBCO-BRL, 2 units/μg of DNA) for 2–3 h at 30 °C. The resulting relaxed DNA was mixed with different volumes of a solution of oligonucleosomes complexed with excess core histones prepared in the same buffer and further digested with an additional amount of topoisomerase I (2 units/μg of circular DNA) for 2–3 h at 30 °C. In some experiments the initial topoisomerase digestion was omitted; in other cases, the second digestion was allowed to proceed for 6 h at 30 °C. The digestions were terminated by the addition of sodium dodecyl sulfate to a final concentration of 0.2–1% and treatment with proteinase K (Merck, 18 μg/μg of circular DNA) or phenol (equilibrated with 0.2 M NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl, pH 7.4). Finally, the DNA was precipitated with ethanol and analyzed on 1% agarose gels (25 cm long) containing 1 mM EDTA and 50 mM Tris–phosphate buffer (pH 7.2) at 1–3 V/cm for 18–23 h (Shure et al., 1977).

Electron Microscopy. For analysis by electron microscopy, spread preparations of the samples corresponding to histone transfer reactions were made essentially according to Thoma et al. (1979). Most of the samples were prepared directly in a buffer containing 0.2 M NaCl, 0.2 mM EDTA, and 5 mM triethanolamine hydrochloride, pH 7.4, and fixed with 0.1% glutaraldehyde (Merck) for about 15 h at 4 °C in the presence of the same buffer. In some experiments, samples were prepared initially in buffers containing Tris as indicated above, dialyzed against 0.2 M NaCl, 0.2 mM EDTA, and 5 mM triethanolamine hydrochloride, pH 7.4, for 3 h at room temperature, and finally fixed at 4 °C for 15 h by adding 0.1% glutaraldehyde to the dialysis buffer.

The fixed samples were diluted with the fixation buffer containing 0.2 M or 10 mM NaCl (see legend to Figure 6) to a final concentration of 2–0.8 μg of DNA/mL. A 10-μL drop of each sample was applied to a carbon-coated grid (pretreated with Alcian blue; see below) placed on a Parafilm sheet. After a 5-min adsorption, the excess liquid was removed with the edge of a filter paper and the grids were washed by immersion in water for 5 min, dehydrated in ethanol for 2–3 s, and allowed to dry on a filter paper. Finally, the grids were rotary-shadowed with platinum at an angle of about 6°. Carbon-coated 400-mesh copper grids, prepared as described by Coggins (1987), were made hydrophilic by treatment with 0.002% Alcian blue 8GX (Serva) for 5 min according to Sogo et al. (1987) and used within 30 min. Micrographs were taken at a magnification of 30000× with a Hitachi H7000 electron microscope operated at 75 kV.

Results

Gel Electrophoresis of Native and Reconstituted Nucleosome Core Particles. In our previous studies using gels containing 1× TBE, we observed that reconstituted nucleosome cores with the normal histone stoichiometry produce a band with a mobility slightly lower than that corresponding to native core particles (Aragay et al., 1988). The results shown in Figure S1a–c indicate that even a very low extent of proteolytic digestion of core histones produces significant changes in the mobility of the core particle band. At 4 °C endogenous proteolysis starts immediately after the preparation of native core particles. This degradation is responsible for the observed mobility differences between native and reconstituted core particles. Unfortunately, we cannot store nucleosomes at lower temperatures to prevent proteolysis, because we have observed (not shown) that storage at −20 °C produces aggregated structures (dinucleosomes and higher aggregates) that make difficult the interpretation of the results obtained with the different samples analyzed on these gels. Moreover, the results shown in Figure S1d indicate that departures from the normal stoichiometry of core histones produce bands with mobilities different from that found for core particles.

The appropriate conditions for the studies described below were established from experiments about the stability of the nucleosome core particle. We have used 1× TBE-containing gels to analyze the dissociation of nucleosome cores as a function of salt (Figure 1) and particle concentration (Figure S2). Under the conditions used in most of the experiments of this work (0.2 M NaCl and core particles at a concentration of 45 μg of DNA/mL), the extent of dissociation is lower than 9%.

Binding of Additional Histones to Nucleosomes Core Particles in 0.2 M NaCl. Increasing amounts of histones H2A,H2B were added to nucleosome core particles in 0.2 M NaCl, and the resulting mixtures were analyzed on gels containing 1× TBE. As shown in Figure 2a (see lanes with symbol −), for a fixed concentration of nucleosome cores (45 μg of DNA/mL), increasing the concentration of histones H2A,H2B produces a progressive decrease in the intensity of the core particle band and the appearance of excluded material at the top of the gel. This indicates that histone H2A,H2B are able to interact with core particles and induce the formation of high molecular weight aggregates that cannot be resolved by the gel. Equivalent results were obtained in experiments in which H3,H4 (Figure S3a) or core histones (Figure S3b) were mixed with core particles in 0.2 M NaCl. In agreement with these observations, the fraction of soluble histone remaining after centrifugation of core particle samples with an excess of H2A,H2B, H3,H4, or core histones decreases with increasing the weight ratio of excess histones to core DNA (see Figure 2b). Core particles are completely precipitated at values of the excess histones to core DNA weight ratio

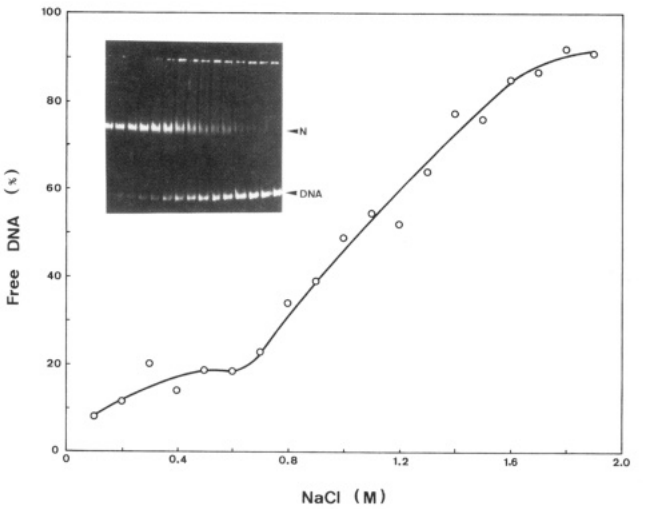


FIGURE 1: Electrophoretic analysis of the stability of nucleosome core particles as a function of NaCl concentration. Core particles (40 μg of DNA/mL) in the presence of the indicated NaCl concentrations were electrophoresed on a nondenaturing gel containing 1× TBE. The NaCl concentrations of the samples of the inset gel were (from left to right) 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, and 1.6 M. The positions of core particles (N) and core DNA are indicated.

Table I: Transfer of Excess Histones from Nucleosome Core Particles to Free Core DNA[a]

excess histones	[NaCl] (M)	transfer yield[b] excimer fluorescence[c]	soluble histone[d]	gel densitometry[e]
core histones	0.45	0.9		
	0.14	1		
	0.2		0.8	0.7
H3,H4	0.2		0.8	0.8
H2A,H2B	0.2		1	1

[a] The values shown correspond to the samples that produce the maximum yield of histone transfer. Unless otherwise indicated, the input weight ratio of excess histones to core particle DNA (45 μg/mL) was 0.8, and the weight ratio of the additional free core DNA to core particle DNA was 1. [b] Transfer yields are expressed relative to the values expected for samples with a 100% transfer efficiency. [c] The transfer yield was estimated from the increase of excimer fluorescence (460 nm) observed after the addition of free core DNA to nucleosome–core histone samples labeled with *N*-(1-pyrenyl)maleimide (see Materials and Methods). In this case the ratio of excess histones to core particle DNA (3 μg/mL) was 0.6, and the weight ratio of the additional core DNA to core particle DNA was 0.5. [d] Values calculated from the increase in the amount of soluble histone observed after the addition of free core DNA (see Figure 2b). [e] Values calculated from the intensity of the bands corresponding to the different DNA–histone complexes resolved by nondenaturing gels (see Figures 2a and S3).

between 0.6 and 0.8. At higher values of this ratio, the samples containing an excess of H2A,H2B are partially soluble, but in the case of samples in which H3,H4 or core histones are in excess, the amount of histones remaining in the supernatant is negligible.

Transfer of Excess H2A,H2B, H3,H4, and Core Histones in 0.2 M NaCl. (1) Transfer from Nucleosome Core Particles to Core DNA. In early studies we used fluorescence spectroscopy to investigate whether excess histones bound to nucleosome core particles can be transferred to free core DNA under different conditions. Although these studies were carried out at a particle concentration in which nucleosome cores are partially dissociated (see above), our estimates (Table I) suggested that this transfer is possible both in the presence of relatively high concentrations of salt (0.45 M NaCl) and at ionic strength close to physiological.

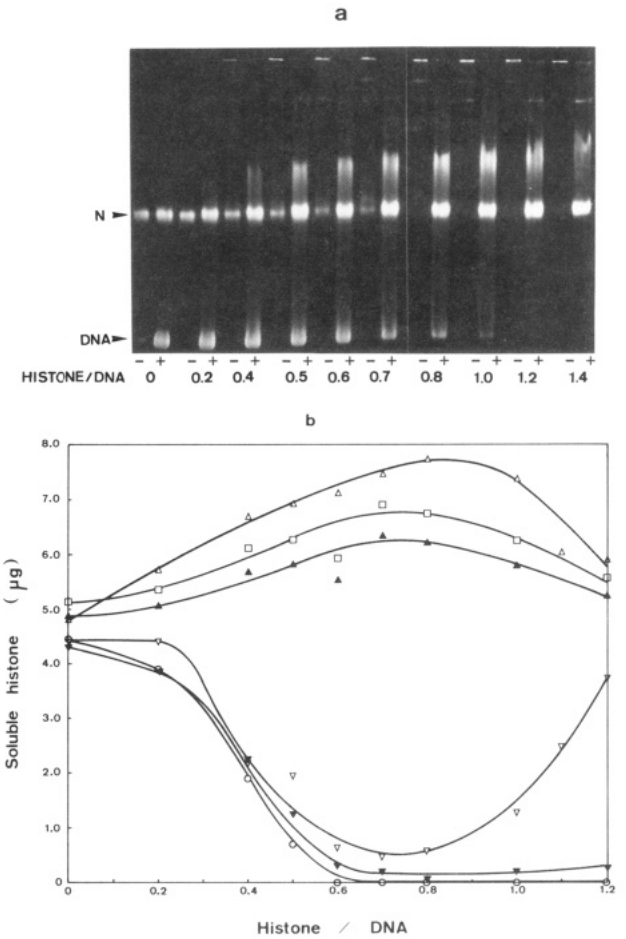


FIGURE 2: Association of H2A,H2B, H3,H4, and core histones with nucleosome core particles and further transfer of these histones to free core DNA in 0.2 M NaCl. A fixed amount of core DNA was added to different core particle–excess histone complexes. The resulting samples were analyzed on a nondenaturing gel (a) or centrifuged for 15 min at 10000*g* for the determination of the amount of soluble protein (b). The weight ratio of excess histones to core DNA before the addition of core DNA is indicated. (a) Core particle samples containing excess H2A,H2B before (−) and after (+) the addition of core DNA. (b) Core particle samples plus excess H2A,H2B (▽), H3,H4 (▼), or core histones (○) before the addition of free core DNA; core particle samples plus excess H2A,H2B (△), H3,H4 (▲), or core histones (□) after the addition of free core DNA. In all samples the final concentration of DNA corresponding to core particles was 45 μg/mL, and in the samples that contain additional core DNA the total DNA concentration was 90 μg/mL.

The above findings stimulated us to carry out a detailed study of the reactivity of nucleosome core particles complexed with excess H2A,H2B, H3,H4, or core histones in the presence of 0.2 M NaCl. As can be seen in Figure 2a (lanes with symbol +), the addition of free core DNA to core particle–(H2A,H2B) complexes results in (1) the disappearance of the aggregated material observed at the top of the gel in the case of samples without additional DNA (lanes with −), (2) the reappearance of the core particle band, and (3) the formation of an ill-defined band corresponding to core DNA–(H2A,H2B) complexes (Aragay et al., 1988). Similar results are obtained when free core DNA is added to samples containing nucleosome core particles associated with an excess of H3,H4 (Figure S3a) or core histones (Figure S3b). These observations indicate that core DNA is able to dissociate the aggregated structures produced by excess histones bound to nucleosome core particles in the presence of 0.2 M NaCl (see the preceding section). This is also observed in the solubility studies presented in Figure 2b. Taken together, these findings

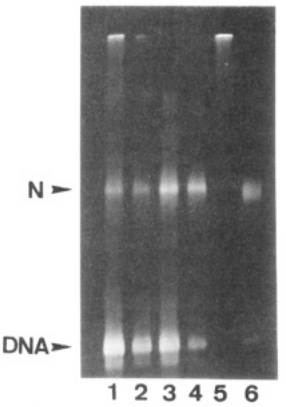


FIGURE 3: Transfer of excess core histones from oligonucleosomes to free core particle DNA in 0.2 M NaCl. Aliquots of a solution containing oligonucleosome–core histone complexes were mixed with different amounts of free core DNA. The supernatants of the resulting samples were analyzed on a 1× TBE gel. The input weight ratio of excess histones to oligonucleosome DNA was 1.2, and the weight ratios of additional core DNA to oligonucleosome DNA were 1 (lane 2) and 2 (lane 1). Lanes 3 and 4 correspond, respectively, to samples similar to those shown in lanes 1 and 2 but prepared (without oligonucleosomes) by direct mixing of the four core histones and core DNA. (Lane 5) oligonucleosomes; (lane 6) native core particles. The positions of core particles (N) and free core DNA are indicated. The final concentration of DNA corresponding to the oligonucleosomes was 45 μg/mL.

allow us to conclude that core particle–histone complexes can transfer histones to free core DNA. In all cases the maximum efficiency of transfer is observed when the excess histones to core DNA weight ratio is around 0.8. The yield of histone transfer has been estimated from the results considered above as indicated in Table I. All the samples show a high yield of histone transfer, but the highest yield of transfer is observed with core particles containing excess H2A,H2B.

(*2*) *Transfer from Oligonucleosome Core Particles to Core DNA.* As shown in Figure 3, in the presence of 0.2 M NaCl, the addition of free core DNA to oligonucleosome core particles (lane 4) previously mixed with core histones gives rise to the typical nucleosome core particle band (lanes 1 and 2). Moreover, second-dimension gels (Figure 4d, lane 2) indicate that this band contains the four core histones with the same stoichiometry as that of the normal nucleosome core particle. The intensity of this band increases with increasing the concentration of the free core DNA added to oligonucleosome–core histone samples (compare lanes 1 and 2 in Figure 3), but the nucleosome bands obtained in these reactions show lower intensities than those corresponding to the nucleosome bands obtained by direct mixing of core DNA and the four core histones (lanes 3 and 4). Thus, the possibility that the new core particle bands of lanes 1 and 2 are due to the direct association of free core histones with core DNA must be considered. However, solubility studies indicate that under the conditions used in these experiments (see legend to Figure 3) the amount of free histones in oligonucleosome–core histone samples is negligible. This demonstrates that the new core particle bands result from the reaction of core DNA with the excess core histones associated with oligonucleosomes and allows us to estimate the yield of histone transfer corresponding to these reactions (see Table II).

We next examined whether oligonucleosome–(H2A,H2B) and oligonucleosome–(H3,H4) complexes can transfer histones to core DNA to form nucleosomes. As can be seen in Figure 4b, in the presence of 0.2 M NaCl, the addition of histones H2A,H2B or H3,H4 to oligonucleosome core particles results in the formation of high molecular weight aggregates (lanes

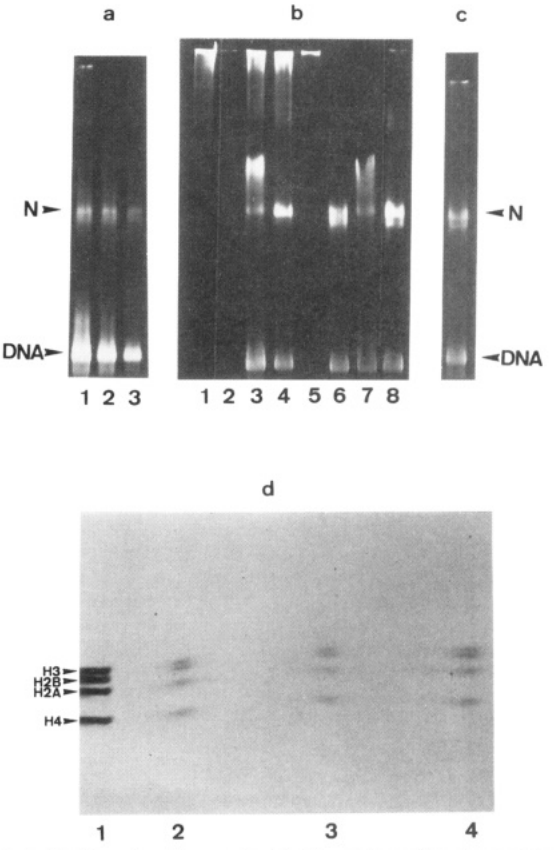


FIGURE 4: Different pathways for the formation of nucleosome core particles by transfer of excess H2A,H2B and H3,H4 from oligonucleosomes to free core particle DNA in 0.2 M NaCl. (a) Different amounts of core DNA were added to a sample containing oligonucleosome–(H2A,H2B) complexes premixed with oligonucleosome–(H3,H4) complexes. The weight ratios of the additional core DNA to oligonucleosome DNA were 4 (lane 1), 2 (lane 2), and 1 (lane 3). (b) Oligonucleosome–(H3,H4) complexes were added to a sample containing oligonucleosome–(H2A,H2B) complexes premixed with core DNA (lane 4). A portion of this sample before the addition of oligonucleosome–(H3,H4) complexes is shown in lane 3. The oligonucleosome preparation used in this gel (lane 1), oligonucleosome–(H2A,H2B) (lane 2), oligonucleosome–(H3,H4) (lane 5), core DNA–(H3,H4) (lane 6), core DNA–(H2A,H2B) (lane 7), and core particles prepared by mixing core DNA and the four core histones (lane 8) are shown as references. (c) Oligonucleosome–(H2A,H2B) complexes were added to a sample containing oligonucleosome–(H3,H4) complexes premixed with core DNA. In all samples the input weight ratio of excess histones (H2A,H2B or H3,H4) to DNA in oligonucleosomes was 1.2, and the final weight ratio of H3,H4 to H2A,H2B was 1. The final concentration of the oligonucleosome DNA was 45 μg/mL, and unless otherwise indicated, the final concentration of the additional core DNA was 45 μg/mL. (d) Lanes 2–4 of the second-dimension gel (stained for protein) correspond, respectively, to the nucleoprotein bands of samples similar to those shown in Figure 3 (lane 2) and in panels a (lane 3) and c of this figure. In lane 1, the four core histones are shown as reference.

2 and 5). However, when free core DNA is added to oligonucleosome–(H2A,H2B) complexes premixed with oligonucleosome–(H3,H4) complexes, the typical band corresponding to nucleosome core particles is observed in the nondenaturing gel (see Figure 4a). In addition, a faster minor band, originated by the departure from the normal histone stoichiometry found in nucleosome cores (see Figure S1d), is also produced in these reactions. On the other hand, as shown in Figure 4b (lane 3), the addition of free core DNA to oligonucleosome–(H2A,H2B) complexes produces the appearance of the ill-defined band corresponding to core DNA–(H2A,H2B) complexes [lane 7; see also Aragay et al. (1988)]. Furthermore, these DNA–(H2A,H2B) complexes can react with oligonucleosome–(H3,H4) complexes and give rise to

Table II: Transfer of Excess Histones from Oligonucleosome Core Particles to Free Core DNA

reaction sequence[a]	ratio of excess histones to oligonucleosome DNA	ratio of free DNA to oligonucleosome DNA	transfer yield[b]
mechanism 1	0.6	1	0.5
		2	0.6
	1.2	1	0.3
		2	0.5
mechanism 2	1.2	1	0.4
		2	0.6
mechanism 3	1.2	1	0.6[c]

[a] The different reaction sequences are indicated in the text (see Results, mechanisms 1–3). The experimental details are given in Figures 3 and 4. [b] Transfer yields are expressed relative to the values expected for samples with a 100% transfer efficiency. The values shown were estimated from the intensity of the core particle bands obtained in the different reactions. The existence of a small fraction of free histones in some samples has not been taken into account in these calculations (see text). [c] The reaction described by mechanism 3 shows the same yield when histones H3,H4 are the first pair transferred from oligonucleosomes to core DNA.

nucleosome core particles (Figure 4b, lane 4). A similar two-step reaction sequence, in which histones H3,H4 are transferred from oligonucleosome–(H3,H4) complexes to core DNA before the addition of oligonucleosome–(H2A,H2B) complexes, has also been investigated. The analysis of the products of this reaction (Figure 4c) also indicates the formation of nucleosome core particles. In all cases, second-dimension gels show that the nucleosome bands obtained in these reactions have the same histone composition as that of the normal nucleosome core particle (see Figure 4d). The estimates of the histone transfer yield corresponding to the different reactions considered above are shown in Table II. Solubility studies indicate that 6–10% histone remains in the supernatant after centrifugation of oligonucleosome–(H2A, H2B) and oligonucleosome–(H3,H4) samples. This suggest that the actual values of the transfer yields corresponding to the reactions involving these samples are probably lower than those shown in Table II. However, direct association of these soluble histones with core DNA can only be responsible for the formation of a small fraction of core particles (6–10%). Thus, the high nucleosome formation yields (40–60%) observed with these samples demonstrate that nucleosome core particles can be formed by excess histone transfer according to the different reaction pathways studied in this paper.

Transfer of Excess Histones from Oligonucleosome Core Particles to Circular DNA in 0.2 M NaCl. (1) Solubility Studies. In order to study the transfer reactions further, we carried out experiments in which the acceptor core DNA was replaced with circular DNA of high molecular weight. The results presented in Figure S4 indicate that in 0.2 M NaCl when the input weight ratio of excess core histones to oligonucleosome DNA is 1, even at low concentrations of oligonucleosome core particles (1.5–4.5 μg of DNA/mL), the fraction of histones remaining in the supernatant is lower than 10%. If M13 DNA is added to these aggregated oligonucleosome–core histone samples, the fraction of soluble protein increases to about 50%. This suggests that excess histones are transferred from oligonucleosomes to circular DNA. The extent of solubilization produced by the addition of M13 DNA decreases with increasing the concentration of the oligonucleosome–core histone sample (Figure S4). At relatively high concentrations of oligonucleosome–core histone complexes (34 μg of DNA/mL), the amount of free histones observed before the addition of DNA is negligible, and the extent of solubilization observed after the addition of circular DNA is very small (about 7%).

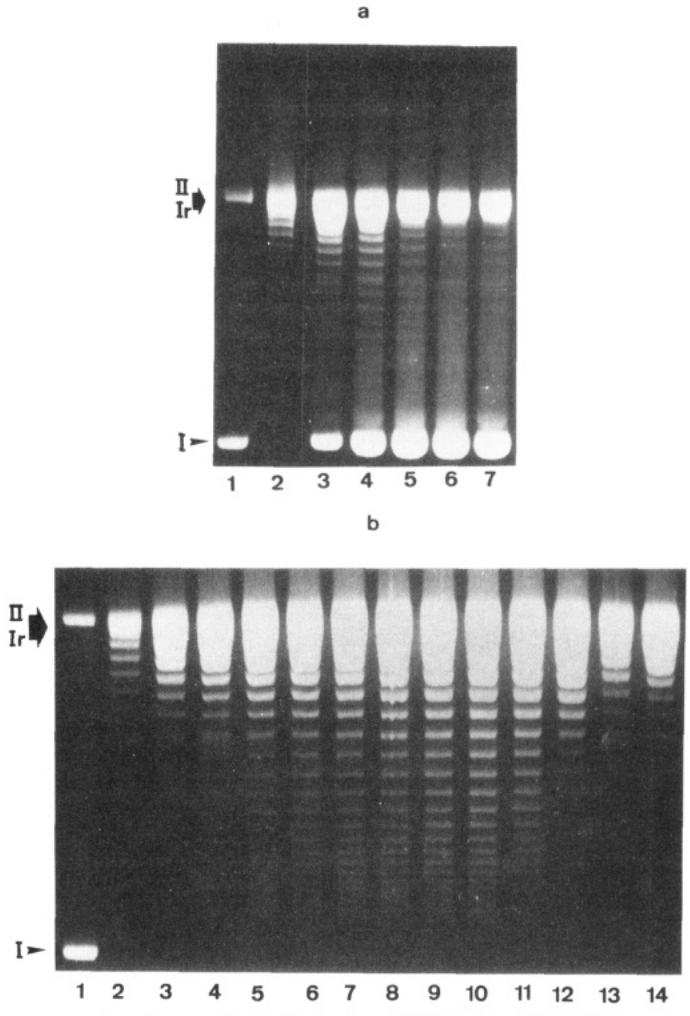


FIGURE 5: Supercoiling of circular DNA induced by histones transferred from oligonucleosome core particles complexed with excess core histones. All the reactions were carried out in the presence of 0.2 M NaCl, and the DNA of the resulting samples was analyzed on 1% agarose gels. (a) M13 DNA (forms I and II, lane 1) was mixed with increasing amounts of oligonucleosomes premixed with core histones and then treated with topoisomerase I. The weight ratios of oligonucleosome DNA to M13 DNA (38 μg/mL) were (lanes 2–7) 0, 1, 1.5, 2, 3, and 4, respectively. (b) M13 DNA (lane 1) was digested with topoisomerase I (lane 2), treated with oligonucleosomes containing excess core histones, and finally digested with an additional amount of topoisomerase I. Samples were prepared at an oligonucleosome DNA to M13 DNA (23 μg/mL) weight ratio of (lanes 3–13) 0.5, 1, 1.3, 1.5, 1.8, 2, 2.3, 2.5, 3, 4, and 0, respectively. A sample prepared as in lane 8 but containing oligonucleosomes without excess histones is shown in lane 14. In all samples the weight ratio of excess core histones to DNA in oligonucleosomes was 1. The positions of DNA forms I (native supercoiled), Ir (relaxed covalently closed circular), and II (nicked circles) are indicated.

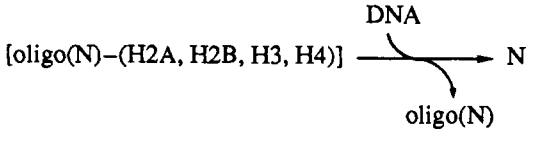
(2) Induction of Supercoiling in Circular DNA. In early experiments, carried out in the presence of 0.2 M NaCl, M13 DNA was mixed with increasing amounts of oligonucleosome–core histone complexes, and then the resulting samples were treated with topoisomerase I. Apparently, as shown in Figure 5a, the increase in the concentration of oligonucleosome–core histone complexes produces a progressive increase in the intensity of the band corresponding to the fully supercoiled DNA. Nevertheless, when circular DNA was relaxed with topoisomerase I before the addition of the oligonucleosome–core histone complexes and then the resulting sample was treated with an additional amount of topoisomerase I, the deproteinized circular DNA was only partially super-

coiled (see Figure 5b). Equivalent results were obtained by using circular DNA of different molecular weights (M13 and pBR322) and oligonucleosome core particles with different lengths. The apparent contradiction between these two sets of results could be related to previous observations indicating that the topoisomerase supercoiling assay for nucleosome formation is highly dependent on the solubility of the reconstituted material (Cotten & Chalkley, 1985). The results presented in Figure 5 indicate that excess core histones are able to migrate from oligonucleosome core particles to circular DNA. However, when the acceptor DNA is initially supercoiled and the amount of transferred histones is relatively high, it is likely that the circular DNA becomes involved in the formation of aggregated structures (see the preceding section) and cannot be relaxed with topoisomerase I (Figure 5a, lanes 4–7). On the other hand, if DNA is relaxed before the histone transfer reaction (Figure 5b), presumably only the circular DNA molecules with a relatively low content of transferred core histones are soluble and can be digested with topoisomerase I. After deproteinization, these circular DNA–core histone complexes give rise to the topoisomer bands seen in agarose gels (e.g., Figure 5b, lanes 8–10); the DNA molecules with a high histone content become aggregated and remain relaxed after deproteinization.

(*3*) *Electron Microscopy.* Experiments similar to those described in the preceding sections were performed in order to analyze by electron microscopy the different reaction products obtained in the presence of 0.2 M NaCl. The results of these analyses are shown in Figure 6. The addition of M13 DNA to oligonucleosome–core histone complexes results in the formation of structures with different degrees of compactness (panels d–g). Particles with the typical morphology of the nucleosome cores can be clearly seen in the less compact regions. Similar results are obtained when M13 DNA is added to a solution containing oligonucleosome–(H2A,H2B) complexes premixed with oligonucleosome–(H3,H4) complexes (panel h) and when a solution of oligonucleosome–(H3,H4) [or oligonucleosome–(H2A,H2B)] complexes premixed with M13 DNA is added to a solution of oligonucleosome–(H2A,H2B) [or oligonucleosome–(H3,H4)] complexes (panels i–l). In these studies we have not seen any difference using oligonucleosome core particle preparations with different lengths. Although the dilution carried out to obtain spread preparations of the samples could cause solubilization and even partial dissociation of the analyzed products (e.g., see the reference chromatin shown in panel c), the results obtained in this electron microscopic study strongly support the data obtained by the other techniques used in this work.

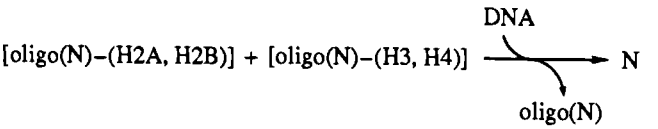
Mechanisms Consistent with the Experimental Results. The results presented in the preceding sections demonstrate the existence of several spontaneous reactions involving excess histones by which nucleosome core particles can be self-assembled in vitro at physiological ionic strength. First, oligonucleosome core particles [oligo(N)] complexed with excess core histones are able to react with DNA and give rise to nucleosome core particles (N):

mechanism 1



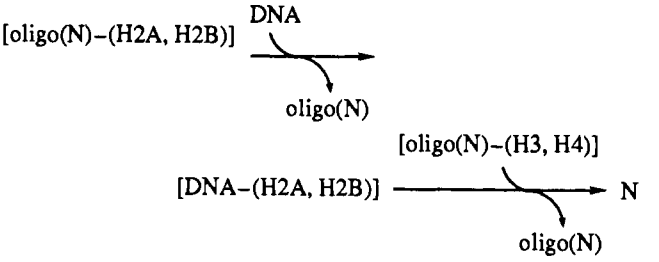
Second, a more complex mechanism for the formation of nucleosome cores, involving the histone pairs H2A,H2B and H3,H4 bound separately to oligonucleosomes, is also consistent with our experimental results:

mechanism 2



Finally, we have shown that the two-step transfer mechanism summarized below also allows the assembly of nucleosome core particles:

mechanism 3



In this case, a similar mechanism, in which histones H3,H4 are transferred to DNA before the reaction with oligo(N)–(H2A,H2B) complexes, is also consistent with our results.

Discussion

Study of DNA–Histone Complexes in Nondenaturing Gels. The ionic strength of 1× TBE gels is relatively low. Thus the ionic strength of the samples is not maintained during electrophoresis. Moreover, core DNA–(H2A,H2B) complexes dissociate during electrophoresis in these gels (Aragay et al., 1988). However, this electrophoretic system allows the analysis of samples containing initially high salt concentrations (up to 2 M NaCl). In fact, the results obtained with this method in our study of nucleosome stability are in agreement with those found in studies carried out by using other techniques (Jorcano & Ruiz-Carrillo, 1979; Cotton & Hamkalo, 1981; Oohara & Wada, 1987b). In keeping with previous suggestions (Garner & Revzin, 1981; Aragay et al., 1988), these observations confirm that layering of samples onto the gel surface does not produce changes in the ionic composition of the sample and, consequently, allows the determination of the initial amount of free DNA.

Transfer of Excess Histones at Physiological Ionic Strength. The binding of additional histones to nucleosome core particles in the presence of 0.5 M NaCl (Voordouw & Eisenberg, 1978; Eisenberg & Felsenfeld, 1981) or in solutions containing 0.1 M NaCl and polyglutamic acid (Stein et al., 1985) produces soluble complexes. In contrast, our electrophoretic and solubility studies performed in 0.2 M NaCl, in the absence of additional factors, indicate that the association of core histones, H2A,H2B or H3,H4 with mononucleosome or oligonucleosome core particles originates aggregated structures. Nevertheless, the high yield of formation of core particles, DNA–(H2A,H2B) and DNA–(H3,H4) complexes observed after the addition of free core DNA, reveals that these aggregated complexes are extremely reactive at ionic strength close to physiological. Furthermore, our results obtained by using different methods indicate that the aggregated complexes containing excess histones are also able to react with circular DNA to produce nucleosome core particles. In this case, however, the extent of solubilization is lower than that observed by using core DNA as histone acceptor.

We have shown, using nondenaturing gels, supercoiling assays, and electron microscopy, that core particles can be

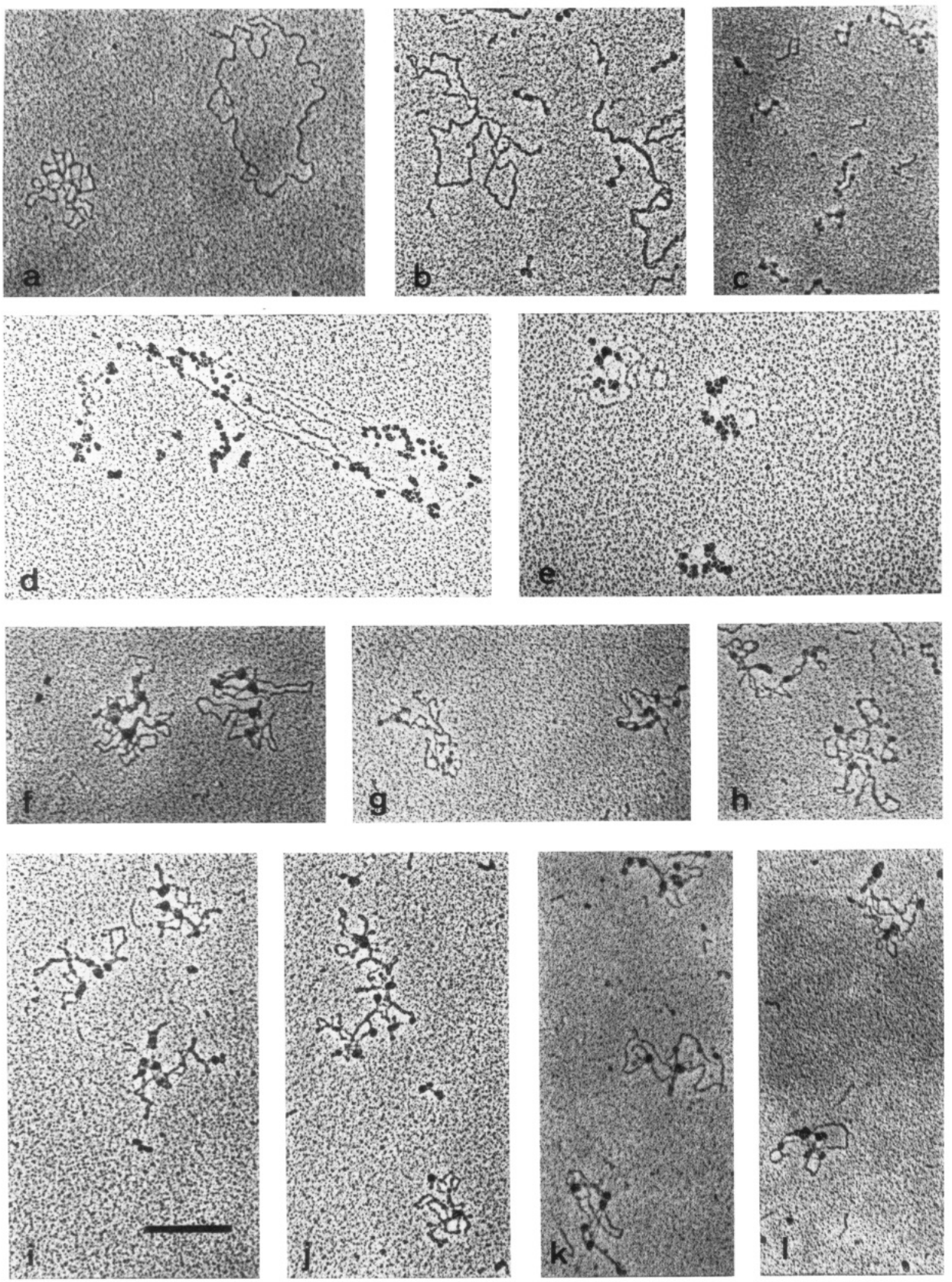


FIGURE 6: Analysis by electron microscopy of the complexes obtained by transfer of histones from oligonucleosomes containing excess histones to free M13 DNA. All the reactions and the fixation were performed in the presence of 0.2 M NaCl. The final NaCl concentration was 0.2 M (b, d–f) or 10 mM (a, c, g–l). (Panels d–g) oligonucleosome–core histone complexes were mixed with M13 DNA. (Panel h) M13 DNA was added to a sample containing oligonucleosome–(H2A,H2B) complexes premixed with oligonucleosome–(H3,H4) complexes. (Panels i and j) oligonucleosome–(H2A,H2B) complexes were added to a sample containing oligonucleosome–(H3,H4) complexes premixed with M13 DNA. (Panels k and l) oligonucleosome–(H3,H4) complexes were added to a sample containing oligonucleosome–(H2A,H2B) complexes premixed with M13 DNA. Oligonucleosome core particles (c), M13 DNA (a), and oligonucleosomes plus M13 DNA (b) are shown as references. In all samples the input weight ratio of excess histones to DNA in oligonucleosomes was 1, and the final weight ratio of oligonucleosome DNA to M13 DNA was 1.5. The concentration of M13 DNA (before the dilution for the spreading) was 15 (b, d, e) or 40 (a, c, f–l) μg/mL. The bar represents 250 nm.

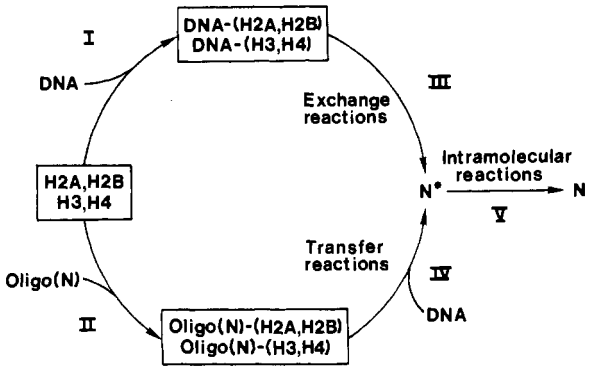


FIGURE 7: Model for the self-assembly of nucleosome core particles at physiological ionic strength. This scheme summarizes the different spontaneous reactions that are probably involved in the in vitro formation of nucleosomes. Additional information and references are given in the text (see Discussion). Oligo(N) represents oligonucleosome core particles; N and N* represent, respectively, the nucleosome core particle and the intermediate nucleosomal structure produced after the initial binding of the four core histones to DNA.

formed at physiological ionic strength following the reaction indicated in mechanism 1 (see Results). This reaction has also been demonstrated in studies performed at higher ionic strength (0.6 M NaCl; Stein, 1979) or at ionic strength close to physiological (0.1 M NaCl) but in the presence of polyglutamic acid (Stein et al., 1985). Our results are also consistent with more complex mechanisms of nucleosome formation involving the pairs H2A,H2B and H3,H4 bound separately as excess histones to different oligonucleosome fragments in the presence of 0.2 M NaCl [see mechanisms 2 and 3 in Results]. Moreover, our results do not exclude the possibility that the excess core histones indicated in mechanism 1 dissociate transiently, forming H2A,H2B dimers and H3,H4 tetramers, during the transfer reaction. Our studies on nucleosome stability in the presence of 0.2 M NaCl (this work) indicate that the bulk of histones involved in the different nucleosome formation reactions considered in this paper are the excess histones bound initially to oligonucleosome cores. However, the observations of Louters and Chalkley (1984), indicating that exogenous histones H2A,H2B (but not H3,H4) can exchange with inner nucleosome histones under physiological conditions, suggest that, before the transfer reactions considered above, a relatively small fraction of excess H2A,H2B can be replaced with histones of the oligonucleosome core particles.

Relationship with Previous in Vitro Studies. A General Model for Nucleosome Self-Assembly. In the absence of DNA, at ionic strength close to physiological, there is no association between H2A,H2B and H3,H4 oligomers (Kornberg & Thomas, 1974; Eickbush & Moudrianakis, 1978; Ruiz-Carrillo & Jorcano, 1979; Sperling & Wachtel, 1981). This behavior is observed even at high histone concentrations. In fact, histones H2A,H2B and H3,H4 in 0.2 M NaCl produce two separate peaks (corresponding to H2A,H2B dimers and H3,H4 tetramers) when a mixture of the four core histones at a concentration of 20 mg/mL is chromatographed on a Sephadex G-100 column (see Materials and Methods). Taking these findings into account, we present in Figure 7 a dynamic model, which includes the different observed spontaneous reactions that are probably involved in the in vitro self-assembly of core particles in the presence of 0.2 M NaCl. Since both H2A,H2B (Oohara & Wada, 1987a; Aragay et al., 1988) and H3,H4 (Simon et al., 1978; Read et al., 1985) can form complexes with DNA, it is likely that during the assembly process these histone pairs associate separately with different fragments of DNA or with different regions of the same DNA molecule (reaction I). Moreover, as demonstrated in this work, histones H2A,H2B and H3,H4 can also associate separately with preformed mononucleosome or oligonucleosome core particles (reaction II). Thus, it is very likely that part of the histones H2A,H2B and H3,H4 interact with core particles formed during the assembly reaction and produce intermediate structures containing excess histones. The histone exchange reactions (III) that take place spontaneously between DNA–(H2A,H2B) and DNA–(H3,H4) complexes (Daban & Cantor, 1982b; Aragay et al., 1988) and the transfer reactions (IV) studied in this work allow the formation of core particles from the different intermediates indicated in Figure 7.

Previous kinetic studies have indicated that the binding of all four core histones to DNA is a very fast process (Diaz & Daban, 1986). This suggests that during self-assembly the exchange and transfer reactions considered above take place very rapidly. In fact, our results indicate that exchange reactions (III) are very fast (Aragay et al., 1988) and that the transfer of excess histones to core DNA is completed in less than 15 min (this work). In addition to these complex reactions that must involve second-order mechanisms, after the binding of the four core histones to DNA, a slower intramolecular first-order reaction (Figure 7, reaction V) that allows the complete folding of the assembled core particle has been detected by using the fluorescent label *N*-(1-pyrenyl)maleimide (Daban & Cantor, 1982a). This intramolecular change that occurs during the self-assembly reaction in the presence of 0.2 M NaCl is equivalent to the conformational transition that is found between 0.6 and 0.2 M NaCl with pyrene-labeled nucleosome cores (Daban & Cantor, 1982a) and, probably, is also equivalent to the transition that can be clearly detected in this ionic strength region by using circular dichroism (Ausio & van Holde, 1986; Oohara & Wada, 1987b), various fluorescent probes (Daban & Cantor, 1989), and other techniques (Wilhelm & Wilhelm, 1980; Harrington, 1982). Although these studies have indicated that the core particles are more compact in 0.2 M NaCl than at higher salt concentrations, the change in the sedimentation coefficient of the nucleosome cores observed in this ionic strength region is relatively small (Eisenberg & Felsenfeld, 1981; Yager & van Holde, 1984; Ausio & van Holde, 1986). The N-terminal regions of the histones are not involved in this conformational transition (Diaz & Daban, 1986; Ausio et al., 1989). From the comparison of different experimental results (Daban & Cantor, 1989), it has been suggested that this transition is related to the flexibility of the core particle structure in the regions containing the DNA tails. Furthermore, the results obtained in kinetic studies (Daban & Cantor, 1982b) have suggested that although the four core histones participate in the final part of the nucleosome self-assembly process, histones H3,H4 are the driving force of this intramolecular reaction. In this regard, these studies have also indicated that, in the absence of H2A,H2B, the core DNA–(H3,H4) complexes evolve slowly to an altered structure. This structure can bind histones H2A,H2B and produce the normal electrophoretic band corresponding to nucleosomes containing the four core histones (Aragay et al., 1988). However, the addition of histones H2A,H2B to these altered DNA–(H3,H4) complexes does not produce the high excimer fluorescence typically obtained with pyrene-labeled core particles (Daban & Cantor, 1982b). This suggests that histones H3,H4 in the absence of H2A,H2B induce local structural changes that do not preclude the subsequent binding of H2A,H2B but modify the final positioning of these histones.

Biological Implications. The spontaneous tendency of DNA–histone mixtures to form the folded nucleosome core particle indicates that this final structure is thermodynamically stable. Moreover, the different reaction pathways considered in the preceding section are kinetically competent to form core particles at physiological ionic strength. However, in the nucleus, if changes in local conditions (i.e., interaction with polymerases and other proteins, chemical modifications, ionic strength changes) perturb the thermodynamic equilibrium of this system, the reactions shown in Figure 7 may be reversed and, consequently, the transient intermediate structures considered above could be involved in different biological functions. In fact, the physicochemical model presented in Figure 7 is compatible with the dynamic model of DNA–histone interactions suggested by Jackson (1990) from in vivo studies on chromatin replication and transcription.

According to Ellis and Hemmingsen (1989), nucleosome formation in vivo can be considered as a case of assisted self-assembly (i.e., the primary sequences contain all the steric information necessary for the assembly, but additional factors are required to prevent the formation of incorrect structures). This suggests that the basic dynamic properties of the nucleosome observed in physicochemical studies are probably modulated by additional factors in the cell nucleus. Nevertheless, taking into account the in vivo results suggesting that newly synthesized histones are weakly associated with chromatin as excess H3,H4 tetramers and H2A,H2B dimers (Seale, 1981; Jackson, 1990), it is reasonable to suggest that the reactions of transfer of excess H2A,H2B and H3,H4 demonstrated in this work could participate in the mechanism of chromatin replication. On the other hand, the findings of several laboratories indicating that nucleosomes are dissociated during transcription (Baer & Rhodes, 1983; Karpov et al., 1984; González et al., 1987; Lorch et al., 1988) suggest that the displaced histones could associate with the nucleosomes of chromatin regions located near the transcribed DNA. Normal nucleosome structures could be reassembled by spontaneous migration of excess histones (probably H2A,H2B) from these storage intermediates to DNA previously involved in transcription. The results of Riehm and Harrington (1989), indicating that diffusion-limiting matrices of polyacrylamide stabilize chromatin against precipitation at high temperature under physiological salt concentrations, suggest the possibility that the transient association of excess histones with nucleosomes could take place within the nuclear matrix (Nelson et al., 1986) in localized chromatin regions without formation of the high molecular weight aggregates observed in our in vitro studies.

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SUPPLEMENTARY MATERIAL AVAILABLE

Figures S1–S4, showing the effect of proteolysis and histone stoichiometry on the electrophoretic mobility of nucleosome core particles, the electrophoretic analysis of the stability of nucleosome cores as a function of particle concentration, the electrophoretic study of the transfer of excess H3,H4 and core histones from core particles to core DNA, and the solubility study of the transfer of excess core histones from oligonucleosome core particles to M13 DNA (5 pages). Ordering information is given on any current masthead page.

REFERENCES

Aragay, A. M., Diaz, P., & Daban, J. R. (1988) *J. Mol. Biol. 204*, 141–154.
Ausio, J., & van Holde, K. E. (1986) *Biochemistry 25*, 1421–1428.
Ausio, J., Dong, F., & van Holde, K. E. (1989) *J. Mol. Biol. 206*, 451–463.
Baer, B. W., & Rhodes, D. (1983) *Nature, 301*, 482–488.
Bonne-Andrea, C., Harper, F., Sobczak, J., & De Recondo, A. M. (1984) *EMBO J. 3*, 1193–1199.
Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.
Coggins, L. M. (1987) in *Electron Microscopy in Molecular Biology: A Practical Approach* (Sommerville, J., & Scheer, U., Eds.) pp 1–29, IRL Press, Oxford, England.
Cotten, M., & Chalkley, R. (1985) *Nucleic Acids Res. 13*, 401–414.
Cotten, M., & Chalkley, R. (1987) *EMBO J. 6*, 3945–3954.
Cotton, R. W., & Hamkalo, B. A. (1981) *Nucleic Acids Res. 9*, 445–457.
Daban, J. R., & Cantor, C. R. (1982a) *J. Mol. Biol. 156*, 749–769.
Daban, J. R., & Cantor, C. R. (1982b) *J. Mol. Biol. 156*, 771–789.
Daban, J. R., & Cantor, C. R. (1989) *Methods Enzymol. 170*, 192–214.
DePamphilis, M. L., & Wassarman, P. M. (1980) *Annu. Rev. Biochem. 49*, 627–666.
Diaz, P., & Daban, J. R. (1986) *Biochemistry 25*, 7736–7744.
Dilworth, S. M., Black, S. J., & Laskey, R. A. (1987) *Cell 51*, 1009–1018.
Earnshaw, W. C., Honda, B. M., Laskey, R. A., & Thomas, J. O. (1980) *Cell 21*, 373–383.
Eickbush, T. H., & Moudrianakis, E. N. (1978) *Biochemistry 17*, 4955–4964.
Eisenberg, H., & Felsenfeld, G. (1981) *J. Mol. Biol. 150*, 537–555.
Ellis, R. J., & Hemmingsen, S. M. (1989) *Trends Biochem. Sci. 14*, 339–342.
Fried, M. G. (1989) *Electrophoresis 10*, 366–376.
Fried, M. G., & Crothers, D. M. (1981) *Nucleic Acids Res. 9*, 6505–6525.
Garner, M. M., & Revzin, A. (1981) *Nucleic Acids Res. 9*, 3047–3060.
Garner, M. M., & Revzin, A. (1986) *Trends Biochem. Sci. 11*, 395–396.
González, P. J., Martinez, C., & Palacián, E. (1987) *J. Biol. Chem. 262*, 11280–11283.
Harrington, R. E. (1982) *Biochemistry 21*, 1177–1186.
Jackson, V. (1990) *Biochemistry 29*, 719–731.
Jorcano, J. L., & Ruiz-Carrillo, A. (1979) *Biochemistry 18*, 768–774.
Karpov, V. L., Preobrazhenskaya, O. V., & Mirzabekov, A. D. (1984) *Cell 36*, 423–431.
Kleinschmidt, J. A., Fortkamp, E., Krohne, G., Zentgraf, H., & Franke, W. W. (1985) *J. Biol. Chem. 260*, 1166–1176.
Kleinschmidt, J. A., Seiter, A., & Zentgraf, H. (1990) *EMBO J. 9*, 1309–1318.
Kornberg, R. D., & Thomas, J. O. (1974) *Science 184*, 865–868.
Louters, L., & Chalkley, R. (1984) *Biochemistry 23*, 547–552.
Lorch, Y., LaPointe, J. W., & Kornberg, R. D. (1988) *Cell 55*, 743–744.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp 86-96, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Messing, J. (1983) *Methods Enzymol. 101*, 20-89.
Nelson, T., Wiegand, R., & Brutlag, D. (1981) *Biochemistry 20*, 2594-2601.
Nelson, W. G. Pienta, K. J., Barrack, E. R., & Coffey, D. S. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 457-475.
Oohara, I., & Wada, A. (1987a) *J. Mol. Biol. 196*, 389-397.
Oohara, I., & Wada, A. (1987b) *J. Mol. Biol. 196*, 399-411.
Read, C. M., Baldwin, J. P., & Crane-Robinson, C. (1985) *Biochemistry 24*, 4435-4450.
Richmond, T. J., Finch, J. T., Rushton, B., Rhodes, D., & Klug, A. (1984) *Nature 311*, 532-537.
Riehm, M. R., & Harrington, R. E. (1989) *Biochemistry 28*, 5787-5793.
Ruiz-Carrillo, A., & Jorcano, J. L. (1979) *Biochemistry 18*, 760-768.
Ruiz-Carrillo, A., Puigdomenech, P., Eder, G., & Lurz, R. (1980) *Biochemistry 19*, 2544-2554.
Seale, R. L. (1981) *Biochemistry 20*, 6432-6437.
Sealy, L., Cotten, M., & Chalkley, R. (1986) *Biochemistry 25*, 3064-3072.
Shure, M., Pulleyblank, D. E., & Vinograd, J. (1977) *Nucleic Acids Res. 4*, 1183-1205.
Simon, R. H., Camerini-Otero, R. D., & Felsenfeld, G. (1978) *Nucleic Acids Res. 5*, 4805-4818.
Sogo, J., Stasiak, A., De Bernardin, W., Losa, R., & Koller, Th. (1987) in *Electron Microscopy in Molecular Biology: A Practical Approach* (Sommerville, J., & Scheer, U., Eds.) pp 61-79, IRL Press, Oxford, England.
Sperling, R., & Wachtel, E. J. (1981) *Adv. Protein Chem. 34*, 1-60.
Stein, A. (1979) *J. Mol. Biol. 130*, 103-134.
Stein, A., Whitlock, J. P., & Bina, M (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5000-5004.
Stein, A., Holley, K., Zeliff, J., & Townsend, T. (1985) *Biochemistry 24*, 1783-1790.
Thoma, F., Koller, Th., & Klug, A. (1979) *J. Cell Biol. 83*, 403-427.
Thomas, J. O., & Oudet, P. (1979) *Nucleic Acids Res. 7*, 611-623.
Uberbacher, E. C., Ramakrishnan, V., Olins, D. E., & Bunick, G. J. (1983) *Biochemistry 22*, 4916-4923.
Voordouw, G., & Eisenberg, H. (1978) *Nature 273*, 446-448.
Wilhelm, M. L., & Wilhelm, F. X. (1980) *Biochemistry 19*, 4327-4331.
Yager, T. D., & van Holde, K. E. (1984) *J. Biol. Chem. 259*, 4212-4222.

Structure of the O-Specific, Sialic Acid Containing Polysaccharide Chain and Its Linkage to the Core Region in Lipopolysaccharide from *Hafnia alvei* Strain 2 As Elucidated by Chemical Methods, Gas-Liquid Chromatography/Mass Spectrometry, and ^{1}H NMR Spectroscopy†

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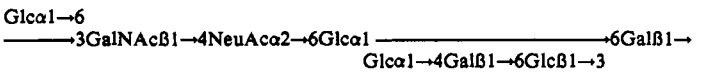
ABSTRACT: Mild acid hydrolysis of *Hafnia alvei* strain 2 lipopolysaccharide released no O-specific polysaccharide but instead gave a monomeric octasaccharide repeating unit with *N*-acetylneuraminic acid as the reducing terminus. In addition, a dimer of the octasaccharide repeating unit, and also a decasaccharide composed of a fragment of the O-specific polysaccharide chain and the core region, were obtained in minute amounts. On the basis of the sugar and methylation analyses, periodate oxidation, and ^{1}H NMR spectroscopy of the lipopolysaccharide hydrolytic products, the biological repeating unit of the O-specific polysaccharide was shown to be a branched octasaccharide:



The linkage between the O-specific polysaccharide chain and core region has also been determined and has yielded strong evidence that *N*-acetylneuraminic acid is an inherent lipopolysaccharide component. The lipopolysaccharide of *H. alvei* strain 2 is the first lipopolysaccharide reported to contain 4-substituted neuraminic acid in its O-specific polysaccharide region.

Hafnia alvei microorganisms form one of the lesser known enterobacterial genera, as far as structural and serological aspects of their O antigens are concerned. Preliminary chemical characterization of the lipopolysaccharides isolated from 33 strains of this genus showed that the lipopolysaccharide of strain 2 contains sialic acid as a component (Romanowska et al., 1988). The presence of sialic acid in lipopolysaccharides of some strains of *Salmonella*, *Escherichia coli*, and *Citrobacter* was reported earlier (Jann & Westphal, 1975), but to date the structure of their O-specific polysaccharides is unknown. The structures of capsular antigens

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