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Histone-Histone Propinquity by Aldehyde Fixation of Chromatin

(chromatin structure/chromosome/formaldehyde/glutaraldehyde/DNA)

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ABSTRACT **Histones have been fixed within the chromatin complex using either formaldehyde or glutaraldehyde. Evidence is presented which argues that in short time periods formaldehyde fixation leads to the formation of reversible covalent bonds between histone and DNA. On the other hand, fixation of chromatin with glutaraldehyde leads initially to the formation of polymers of F1 histone, and at a later stage to multiple small oligomers of the remaining histones. These oligomers then increase in size until they become too large to detect by polyacrylamide gel electrophoresis. Exclusive formation of histone dimers or tetramers was not observed. The simplest model for histone distribution on DNA which encompasses these observations is one in which histones are organized as a fairly extensive linear overlapping array.**

Considerable interest is now being focused on the structure of chromatin. A critical role in the development of our understanding of chromatin structure will most likely come from increased knowledge of the way in which histone molecules are organized with respect to one another in their interaction with DNA. The development of bifunctional crosslinking reagents (1) and their application to ribosomal material (2, 3) has already been described. Histones can be fixed within the chromatin structure by reaction with formaldehyde (4–6, 13), and it appears likely that at least short-term exposure to this agent gives a substantial degree of covalent histone-DNA interactions (6). Olins and Wright (7) have utilized glutaraldehyde to explore histone-histone proximity in avian erythrocyte nuclei, where they analyzed in detail the fixation of the lysine-rich histones (F1 and F2c). They showed that it is possible to demonstrate the formation and isolation of polymers rich in the lysine-rich histones.

We have extended their studies to isolated chromatin and have also observed the formation of polymers of the other histone fractions. The differences in nature of histone fixation by formaldehyde and glutaraldehyde have been analyzed.

MATERIALS AND METHODS

Isolation of Nucleohistone. Calf thymus was collected from the slaughterhouse, carried to the laboratory on ice, and rapidly frozen. Samples of tissue (2–3 g) were homogenized, and nucleohistone was isolated by standard procedures (9). Nucleohistone is operationally defined as chromatin that has been vigorously sheared and centrifuged. Control fixation studies with unsheared chromatin revealed an identical pattern of fixation. The nucleohistone was stored at 4° in water before use. In general, most experiments were performed within an hour of preparation; however, storage in H_2O totally prevents proteolysis, and identical results are obtained on material stored for as long as 48 hr.

Fixation by Aldehydes. The nucleohistone was adjusted to A_{260} about 10, and to the required ionic strength with a stock solution of triethanolamine·HCl, pH 7.0. The fixatives used were 2% formaldehyde or 0.6% glutaraldehyde. Stock solutions were prepared by adding 2.0 ml of 38% formaldehyde or 1.5 ml of 50% glutaraldehyde (purchased from Fischer Scientific) to 5.5 ml of H_2O. The pH was adjusted to 7.0 ± 0.2 with 0.1 M NaOH. Immediately before use, the glutaraldehyde was diluted 3-fold with water. The fixatives were then added to the nucleohistone solution (0.2 ml of diluted fixative per 1.0 ml of nucleohistone) at 4°.

The fixation reactions were terminated by adding a 1/10 volume of 2 M H_2SO_4. After centrifugation (14,000 rpm/75 min) the supernatant (extracted histones and soluble polymers) was dialyzed against 2 liters of 0.2 M H_2SO_4 (4–6 hr minimum) and, finally, the histones were precipitated by dialysis against ethanol (6–12 hr). The histones were collected by centrifugation and were dissolved in either 0.9 M acetic acid, 20% sucrose, 0.5 M 2-mercaptoethanol for acid-urea electrophoresis or in 0.1% sodium dodecyl sulfate, 0.01 M glycine, 4 M urea, pH 10.5, for high pH sodium dodecyl sulfate electrophoresis.

Fractionation of Histones and Histone Polymers. Histones were fractionated into three groups, F1, F2b, and (F2A + F3), by the modified procedures of Johns (8).

Electrophoresis of Histones in low pH-urea (9) or in high pH-sodium dodecyl sulfate systems (10) followed described methods. After the gels were destained, they scanned with a Beckman Acta III microdensitometer.

RESULTS

Fixation of Histones by Formaldehyde and Glutaraldehyde. A comparison of the nature and rates of fixation of nucleoprotein by formaldehyde and glutaraldehyde is shown in Fig. 1. At the ionic strength used (5×10^{-4}), formaldehyde fixation of histones to the nucleoprotein proceeds quite rapidly so that about 80% of the original histone content is not extractable in acid after 30 min. As shown previously, histone becomes wholly bound after an additional 90 min (6). All histone fractions are bound at essentially the same rates. In contrast, the rate of fixation of different histone fractions varies during glutaraldehyde treatment. As shown in Fig. 1, the lysine-rich (F1) histone is fixed (and therefore not extracted into acid) most rapidly, in accord with similar observations made by Olins and Wright, who studied fixation in isolated nuclei (7). In fact, most of the F1 histone has been fixed before significant inroads have been made into the other

TABLE 1. *Reversibility of fixation by formaldehyde or glutaraldehyde*

Substrate	% Reversal	
	Formaldehyde	Glutaraldehyde
Nucleohistone	90–100	<2
Free histone	<2	<2

Material was fixed at low ionic strength for 2 hr. The fixative was removed by dialysis against 5×10^{-4} M triethanolamine (pH 7.0). The material was then dialyzed for 48 hr at 37° with three changes of water (500-fold excess, each change). Histones were extracted in 0.2 M H_2SO_4, and the material was analyzed quantitively on polyacrylamide gels. The % reversal is measured relative to an identical sample that had undergone all the above procedures except that the appropriate fixative was omitted.

fractions, and in observations described below we will argue that the F1 is mostly converted into polymers of F1 histone. Histone F2b is then fixed at a greater rate than the remaining three histone fractions until about 50% of this fraction has been removed, after which time all four histone fractions F2b, F3, F2a1, and F2a2 are fixed at approximately equal rates.

Reversal of Fixation. Recent observations (6) have shown that histones fixed to DNA by minimal exposure to cold formaldehyde, can be reversed efficiently if the nucleoprotein is dialyzed against water for 48 hr at 37°. In order to try to assess the various types of crosslinking involved in formaldehyde and glutaraldehyde fixation, we have reacted either free histone or nucleohistone with these agents and assayed for efficiency of reversal by the techniques previously developed (6). The results are shown in Table 1. Free histones, whether crosslinked by formaldehyde or by glutaraldehyde, show no tendency to reverse these new bonds when treated in this way. Likewise, nucleohistone fixed with glutaraldehyde is totally irreversible. Thus, we conclude that the bonds linking histone and DNA in formaldehyde-fixed nucleohistone are completely different from those linking histones in glutaraldehyde-fixed material.

The nature of the linkage between histone and DNA can be inferred from the following experiment. If formaldehyde-fixed nucleohistone is thermally denatured, a relatively normal melting profile is observed with no more than usual renaturation of DNA upon cooling (at these elevated temperatures the histone-DNA linkage is also rapidly reversed). If fixed nucleohistone is denatured by adding NaOH at 20°, a typical hyperchromic shift is observed. However, upon titration to pH 7.0 a total return to the original A_{260} is observed, occurring almost instantaneously. If the histone in the fixed nucleoprotein is removed with Pronase, the DNA behaves in an identical fashion upon alkali denaturation and subsequent neutralization. The conclusion drawn is that crosslinks that are reversible at high temperature had been formed between DNA strands by the action of formaldehyde. Such a linkage presumably links the bases themselves, as there are no active groups on deoxyribose. Since histones also form a reversible crosslink to DNA, it is not unreasonable to surmise that the histone molecules become bound to the bases of the DNA by a similar bond to that involved in crosslinking DNA strands. Since lysine is probably involved in the formaldehyde crosslinking interaction between histone and DNA, a feasible

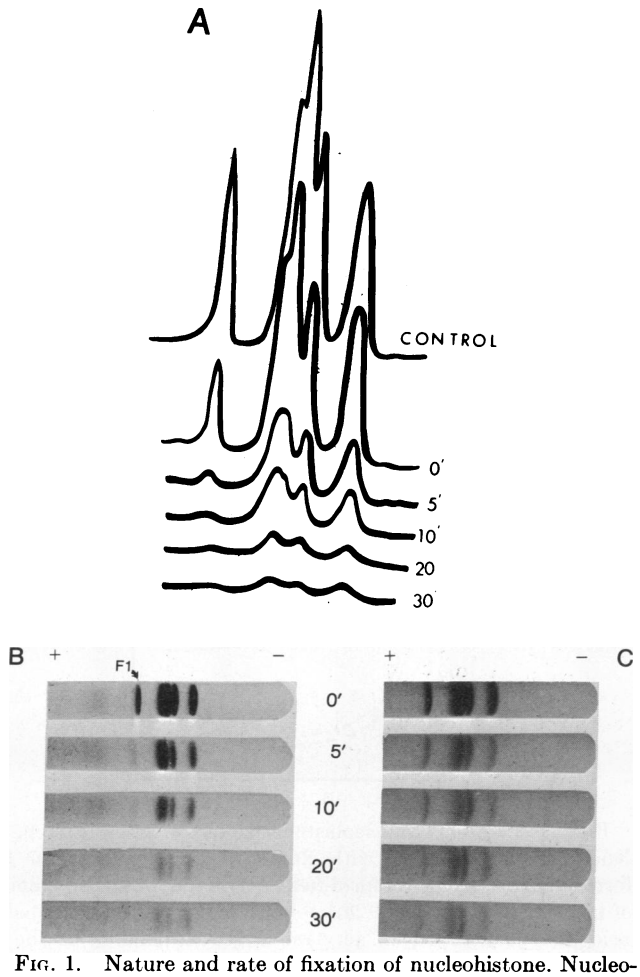

FIG. 1. Nature and rate of fixation of nucleohistone. Nucleohistone was fixed with glutaraldehyde (*Materials and Methods*). Histones were extracted with acid and analyzed on acid-urea polyacrylamide gels (panel B) at the times indicated. Densitometer scans are shown in panel A. Histone patterns obtained during fixation with formaldehyde are shown in panel C. Time is in min.

reaction scheme would be the initial formation of the hydroxymethyl derivative of the ε-amino group of lysine, followed by hemiketal formation with a free carboxyl group on the C_2 of thymine. Hemiketal bonds are easily broken.

Effect of Ionic Strength on Rate of Fixation. An increase in ionic strength increases the rate of fixation of histones with either formaldehyde or glutaraldehyde (Fig. 2). The data are presented in terms of the fixation of all histones other than F1 which is fixed too rapidly upon glutaraldehyde treatment for precise measurement. The glutaraldehyde fixation shows incremental increases in rate at higher ionic strengths, whereas the formaldehyde fixation appears to reach a constant rate at an ionic strength of about 0.01. In glutaraldehyde the relative rates of fixation of individual histones are the same at all ionic strengths studied; however, at higher ionic strengths the formaldehyde fixation changes in character and F1 is fixed much more rapidly than the other histone fractions (Fig. 2), and in this regard formaldehyde fixation at higher ionic strength more nearly resembles that occurring in glutaraldehyde.

Fixation with Glutaraldehyde (but not Formaldehyde) Produces Histone Polymers. Samples of nucleohistone were fixed

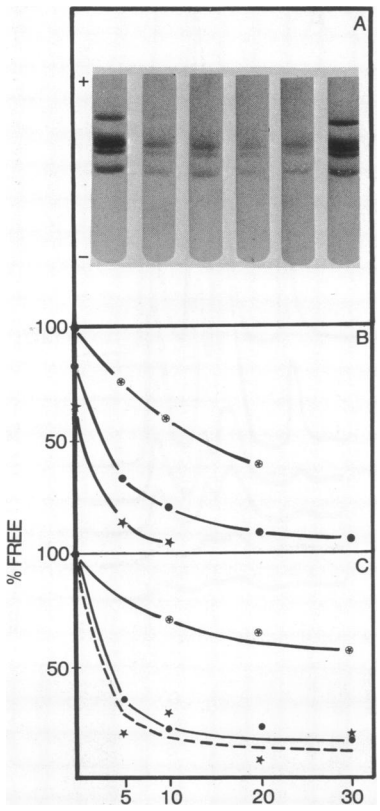

FIG. 2. Fixation of nucleohistone as function of ionic strength. Nucleohistone was fixed with glutaraldehyde (panel B) or 1% formaldehyde (panel C) for different time periods. The amount of histones F3 + F2b + F2a2 + F2a1 that could be extracted in acid and quantitated on acrylamide gels is denoted as the % free histones on the ordinate (relative to a control, unfixed sample of nucleohistone). Fixation was in triethanolamine·HCl (pH 7.0) at concentrations of buffer 5×10^{-4} M (✱); 1×10^{-2} M (●), or 0.10 M (★). Panel A shows the time course of abnormal fixation of different histone fractions in formaldehyde at the highest ionic strength used (0.10). The times of incubation are, from left to right, 0, 5, 10, 20, and 30 min and unfixed control.

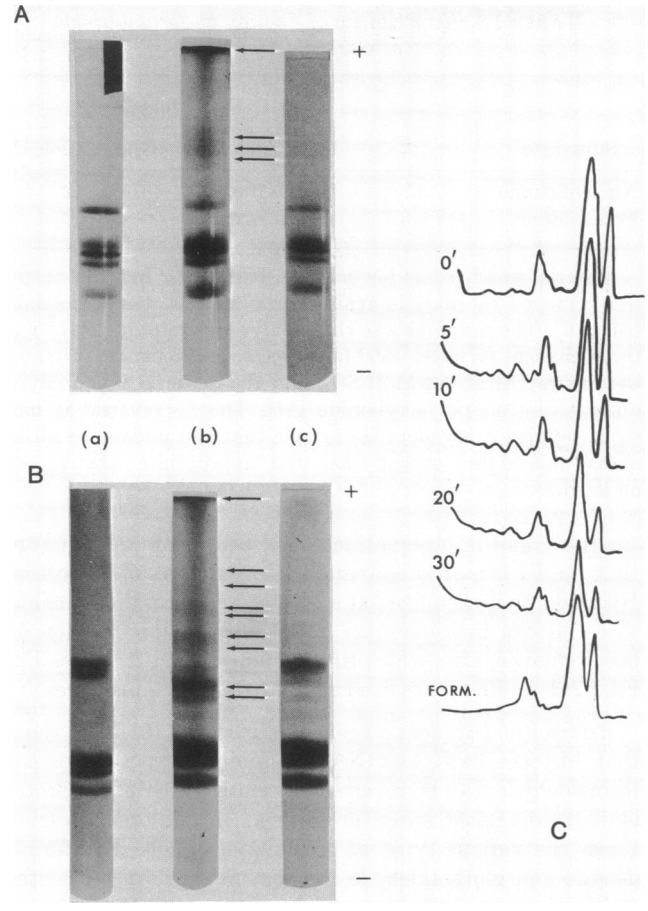

FIG. 3. Analysis of polymers of histone produced during fixation. Histones were fixed in glutaraldehyde or formaldehyde until approximately 50% of the histone was rendered nonextractable into acid. The acid-extractable material was analyzed on (A) acid-urea gels and (B) on sodium dodecyl sulfate gels. The gels are: (a) control histones from nucleohistone not exposed to fixative; (b) histones from nucleohistone fixed with glutaraldehyde for 3 min in 5×10^{-4} M triethanolamine·HCl (pH 7.0) for 5 min; (c) histones from nucleohistone fixed with formaldehyde. Polymers are indicated by *arrows*. The time course of fixation by glutaraldehyde is shown in panel C, in which densitometer traces of sodium dodecyl sulfate gels are presented. The bottom-most tracing is that of histone from a 5 min fixation in formaldehyde.

with formaldehyde or glutaraldehyde at low ionic strength until fixation was about 50% complete. Those histones (or their derivatives) that could be extracted into sulfuric acid were collected and analyzed in either the acid-urea (9) or the sodium dodecyl sulfate system (10). The results so obtained are presented in Fig. 3. In the acid-urea electrophoretic system (Fig. 3A), we see that glutaraldehyde fixation produces a set of bands with about 50% of the mobility of histones and that a substantial amount of material unable to enter the gel is also present. Formaldehyde fixation does not yield material with these electrophoretic properties. This is shown more dramatically in sodium dodecyl sulfate gels (Fig. 3B), with Coomassie blue as a stain. In contrast to formaldehyde, glutaraldehyde treatment produces a series of bands moving more slowly than the normal histones. The mobilities of these bands are related logarithmically (Fig. 4), and we suspect they represent polymers of histones of increasing molecular weight. Only glutaraldehyde fixation also gives rise to polymeric material that cannot enter the gel.

A time course of the production of the histone polymers of intermediate size is shown in Fig. 3C. The intermediate polymers are produced rapidly (although mostly after F1 fixation is complete), and the amount of the intermediate polymers decreases along with that of the monomer histone as fixation nears completion. Initially the amount of very high-molecular-weight polymers is quite large, but this decreases with extended fixation, presumably due to either its binding to DNA or to an increased insolubility in acid as its molecular weight increases.

Source of the Glutaraldehyde-Induced Histone Polymers. The chemical fractionation procedure of Johns (8) provides a rapid and convenient means for separating histones into three main groups. These are histone F1, histone F2b, and histones (F3 and F2A). If the polymeric material retained properties of the histones of which it was composed, we reasoned that they could be at least partially separated by the same approach. After a short period of glutaraldehyde fixation, histones were isolated, fractionated by the above procedures, and analyzed electrophoretically in sodium dodecyl sulfate

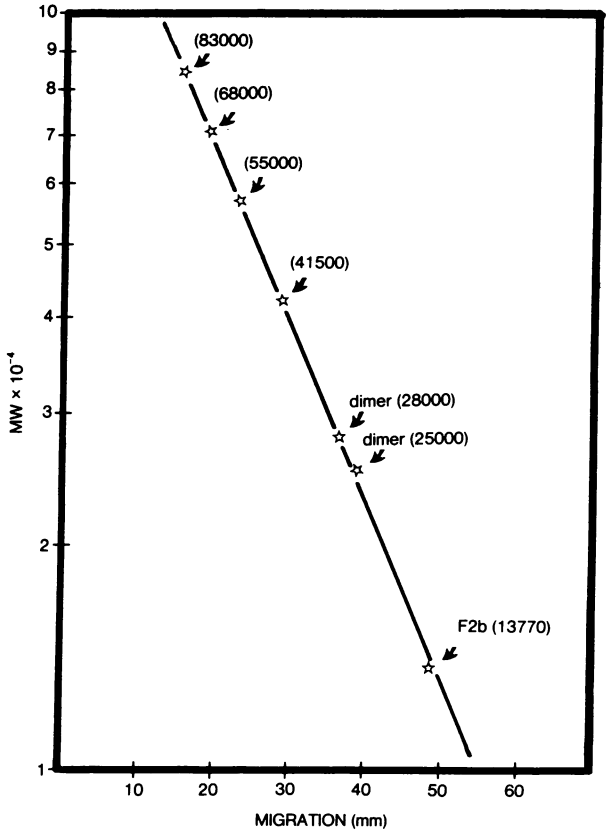

FIG. 4. Migration of polymer bands on logarithmic scale. The bands appearing in Fig. 3B (gel b) were analyzed in the following way. The bands moving slightly faster than F1 and slower than F2b were designated dimer bands (see *text*) and were assigned a molecular weight of 28,000, corresponding to (F2b − F3) for the slower band and 25,000 corresponding to (F2b − F2a1) for the faster band. A line was then drawn through the two points and the point for F2b. The additional points for the polymers (arrows in Fig. 3B, gel b) were then plotted onto this line depending on their migration in the gel, and attendant molecular weights were then calculated directly.

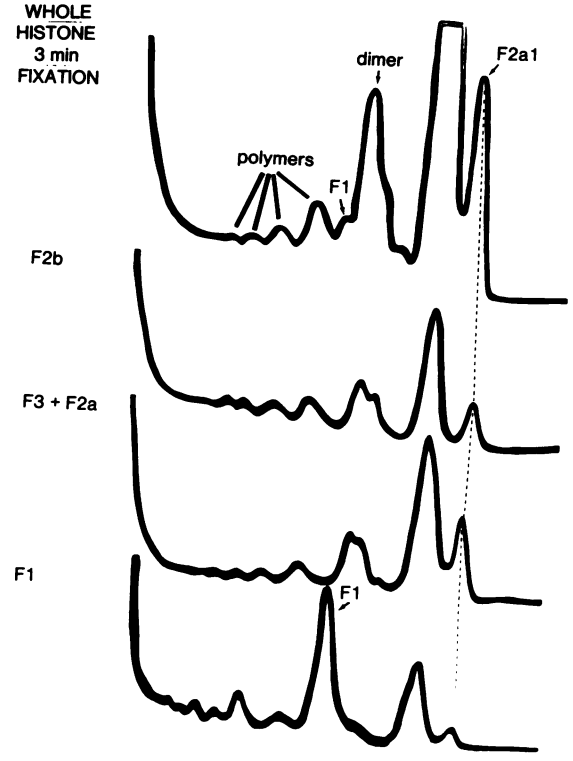

FIG. 5. Division of histone polymers into various subfractions. Nucleohistone was fixed with glutaraldehyde in 5×10^{-4} M triethanolamine·HCl (pH 7.0) for 3 min. Acid extraction yielded the material designated "whole histone 3 min fixation." A sample of the whole histone was fractionated by a modified procedure of Johns (8) into F2b, (F3 and F2A), and F1. Each sample was analyzed on sodium dodecyl sulfate gels at pH 10.5.

gels (Fig. 5). Material that is rich in lysine is soluble in 5% perchloric acid, and F1 and its polymers are extracted in these solutions. Highly characteristic polymer bands are shown along with the monomer F1 histone. The overall contribution of these polymer bands to the whole histone sample at this stage of fixation is small. This is because there is relatively little F1 remaining at this stage (7 min fixation), as documented above. Curiously, after partial fixation by glutaraldehyde, this means of fractionation (normally giving very pure F1) loses some of its selectivity and a small fraction of F2b and F2a2 are co-extracted into the F1 fraction (see Fig. 5).

Histone F2b, which is pure except for the F2a1 as a marker, shows the intermediate polymer bands characteristic of partially fixed whole histone. Interestingly enough, fraction (F2A + F3) also shows a good yield of the intermediate polymers in a much higher proportion than would be expected if they were simply a reflection of F2b contamination. In fact, F2b contamination was very small (<5%) when assayed in acid-urea gels. Further recycling and purification of the (F2A + F3) fraction effectively removed all F2b, and the polymer bands were still present. Thus, we conclude that the material migrating as "intermediate polymers" fractionates into both the F2b fraction and into the (F3 + F2A) fraction, indicating that the polymers have properties in common between these two groups, most probably reflecting their composition. The band that we have designated as a dimer band moves quite distinctly and separately from F1 (Fig. 4).

DISCUSSION

Histones are defined as fixed when they, or their polymer derivatives, can no longer be extracted from the nucleoprotein by 0.2 M sulfuric acid. Fixation by formaldehyde differs radically from that by glutaraldehyde. These differences are: (*i*) at low ionic strength formaldehyde causes all five histone fractions to be fixed at the same rates, whereas glutaraldehyde gives rise to differential rates of fixation. (*ii*) The two processes of fixation respond in somewhat different ways to increasing ionic strength. (*iii*) Formaldehyde fixation of histones, when they are a part of the nucleoprotein complex, is reversible. In contrast, glutaraldehyde fixation of nucleoprotein and both formaldehyde and glutaraldehyde fixation of free histones gives an irreversible product. (*iv*) Partial fixation with formaldehyde yields no polymeric material, whereas fixation with glutaraldehyde gives a range of polymers of varying complexity. By analogy with the reversible cross-strand fixation of DNA and for the above reasons we conclude that formaldehyde generates bonds directly between the histone molecules and the bases of the DNA, and that glutaraldehyde generates bonds primarily between histones

(though the presence of a few histone-DNA bonds cannot be excluded).

The differential fixation of histones by glutaraldehyde was particularly revealing. The lysine-rich histone (F1) is rapidly polymerized to give a product that has the solubility behavior of pure F1 histone, and we conclude that although additional histones may be present, these rapidly produced polymers are highly enriched in F1. Although polymers of intermediate molecular weight were observed, the polymers were mostly of sufficiently high molecular weight that they did not enter 15% polyacrylamide gels. Apparently F1 is organized in groups in such a way that large (tetramers or larger) F1 polymers are rapidly formed. Obviously such "groups" could be in the form of globular complexes or extended overlapping linear arrays. Since the F1 molecule is particularly easily attacked by proteolytic enzymes, the latter suggestion would appear to be more attractive.

We do not think it likely that the polymers are due to interstrand crosslinking for two reasons. (*i*) Examination of fixed chromatin in urea-sucrose gradients indicates that less than 20% of the chromatin is in units larger than those containing one DNA duplex, and (*ii*) the polymer products of fixation at higher ionic strengths are quite different from those obtained at the lower ionic strengths used in this analysis. The products obtained at the higher ionic strength may well reflect secondary interactions of importance, whereas the low ionic strength polymers presumably mirror the possibilities for histone-histone interaction along the backbone of single DNA molecules.

The polymerization of the remaining four histones follows an unexpectedly simple pattern. The intermediates in the polymerization consist of a pair of (putative) dimer bands [the mobility of F3 disulfide dimers is close to this region in this gel system (10)] together with higher polymers which are related logarithmically and which also appear to be split into at least two components. It seems unlikely that all possible contributions of dimer are formed, but rather that this process is highly specific. Both dimer bands are extracted into the F2b fraction and equally into the (F2A + F3) fraction, indicating that they resemble both F2b and (F2A + F3) components. Coupled with the observation that monomer F2b is lost more rapidly than the other fractions at early stages of fixation, this leads us to suggest that a significant quantity of the dimers consist of F2b complexed with each of the three remaining histones such that (F2b − F2a2) and (F2b − F3) migrate together and are distinct from (F2b − F2a1), a prediction based on the relative mobilities of F2a2, F3, and F2a1 in sodium dodecyl sulfate gels. These dimers might then act as nucleation sites for the production of more complex polymers until the complexity is such that more than six histones are covalently bound together. Hexamers are probably the most complex unit we can distinguish before the larger polymers appear as a continuum of staining in the high-molecular-weight region of the gels. Certainly, the final products of glutaraldehyde fixation are very high-molecular-weight polymers of the histone molecules. Again, the most convincing account for this behavior might be found in an extended array of partially overlapping histone molecules along the DNA. Globular sets of histone molecules such as those suggested by Kornberg (11, 12) are not excluded if one demands that they are in close contact with additional histone molecules; however, major yields, specifically of discrete dimers and tetramers (F2a1 and F3 on the one hand, and F2b and F2a2 on the other), were not found in the partially polymerized material. Obviously a contribution of some homologous or heterologous F2a1 and F3 dimers is not excluded and indeed is highly likely. The point we wish to make is that they are not converted exclusively to small, discrete oligomers.

Thus, we conclude (*i*) that based on the formaldehyde fixation studies, all the five histone fractions possess the capacity to interact intimately with the bases of DNA; (*ii*) that based on the glutaraldehyde data, extended overlapping arrays of all histone fractions are present; (*iii*) that F1 histones are often arranged contiguously with few other histones interspersed; and finally (*iv*) that F2b is arranged so that it is next to F3, F2a2, or F2a1 with roughly equal frequency.

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