

Properties of Formaldehyde-Treated Nucleohistone*

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ABSTRACT: When native nucleohistone is treated with formaldehyde, the resulting soluble complex does not dissociate in solutions of high ionic strength and may be banded in a CsCl gradient.

The buoyant density of the complex, 1.411 g/cc, is that calculated from the ratio of deoxyribonucleic acid to histone in nucleohistone. Formaldehyde treatment of nucleohistone cross-links histone molecules to deoxyribonucleic acid.

Thus the histone of the formaldehyde-treated complex is no longer soluble in acid. Treatment with pronase removes 96% of the protein from deoxyribonucleic acid, leaving 4% firmly bound, an amount which is in approximate stoichiometric proportion to the formaldehyde cross-links between deoxyribonucleic acid and protein.

When histones are extracted from nucleohistone with salt and then banded in a CsCl equilibrium density gradient, the width of the resulting band indicates that the banding material consists of aggregates of 10–20 histone molecules rather than of single histone molecules (Huang and Bonner, 1965). In order to determine whether such histone aggregates occur natively in nucleohistone, an attempt was made to attach each histone to its neighbor by treatment of nucleohistone with formaldehyde. We then found that the histones of formaldehyde-treated nucleohistone are not dissociable from DNA by salt. By such treatment histone molecules are firmly bound to DNA. The formaldehyde-stabilized histone–DNA complex is thus similar in its behavior in CsCl to that of formaldehyde-stabilized ribosomes, first studied by Spirin (Spirin *et al.*, 1965). Romakov has studied the reaction of formaldehyde with nucleohistone and has reported on the precipitation properties of the product of the reaction as compared with those of native nucleohistone (Romakov, 1968). In this paper we consider the buoyant density and other properties of formaldehyde-treated nucleohistone.

Materials and Methods

Preparation of Nucleohistone. Chromatin was prepared from pea buds according to the method of Bonner *et al.* (1968) except that the initial grinding media contained 0.25 M sucrose, 0.01 M Tris (pH 8.0), and 0.002 M MgCl₂. The chromatin pellets from the sucrose purification step were resuspended in 40 ml of 0.01 M triethanolamine (pH 7.8; CalBiochem, heavy metal free) by homogenizing in a Potter-Elvehjem homogenizer. The resulting chromatin solution was dialyzed for 4 hr *vs.* 0.01 M triethanolamine (pH 7.8). The chromatin was then sheared in the Virtis homogenizer for 90 sec at 40 V, centrifuged at 10,000g for 15 min, and the supernatant was dialyzed overnight *vs.* 0.01 M triethanolamine (pH 7.8). The resulting nucleohistone usually had a concentration of 20–40 OD₂₆₀, a ratio OD₂₃₀/OD₂₆₀ of about 0.7, and showed no turbidity in the 320–360-mμ range.

Histone to DNA ratio of the complex was 1.30 as determined by chemical analysis (Lowry *et al.*, 1951).¹ Such nucleohistone constituted our starting material. Preparation of chromatin in the presence of triethanolamine buffer throughout rather than of Tris for the initial steps was unsuccessful in that the chromatin aggregated more readily with contaminating nonchromosomal protein.

The Formaldehyde Reaction. The reaction with formaldehyde was performed at various concentrations of formaldehyde, but always at a nucleohistone concentration of 10 OD₂₆₀. The nucleohistone was first diluted to 11.1 OD₂₆₀ and then 9 ml of this dilution was added rapidly and with vigorous stirring to 1 ml of a solution of formaldehyde at 10 times the final concentration, buffered pH 7.8 with 0.01 M triethanolamine. The reaction was allowed to proceed for 24 hr at 0°. The solution was then dialyzed *vs.* 0.01 M triethanolamine (pH 7.8) for 24 hr with at least four changes of buffer.

Melting Procedure. The nucleohistone solutions were dialyzed extensively *vs.* 2.5 × 10^{−4} M EDTA (pH 8.0), diluted to 1 OD₂₆₀ with dialysate, and then melted in a Gilford Model 2000 multiple-sample absorbance recording apparatus adapted for the recording of melting profiles. The rate of temperature increase was 0.5°/min. All of the melting profiles are normalized to 1.0 OD₂₆₀ and are plotted on the same scale for ready comparison. They are not corrected for thermal expansion which is negligible for the present purposes.

Buoyant Density Determination. All buoyant density experiments were carried out in the Spinco Model E analytical ultracentrifuge and brought to equilibrium at 44,770 rpm, 25°, for at least 16 hr. The results were recorded using absorption optics employing the Spinco scanner, multiplex, and recorder. The buoyant density was calculated by a computer program, written for the IBM 7094, and based on the method of Ifft *et al.* (1961), which determines the distribution of CsCl throughout the entire cell. This method allows accurate calculation of buoyant density regardless of the isoconcentration density (in the range 1.100–1.800 g/cc) and regardless of the position of the band in the liquid column. The initial concentration of the band in the liquid column was 1.0 g/cc.

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¹ Care must be taken to eliminate triethanolamine from any sample to be tested with the Lowry reaction as it interferes very strongly even at a concentration of 0.01 M.

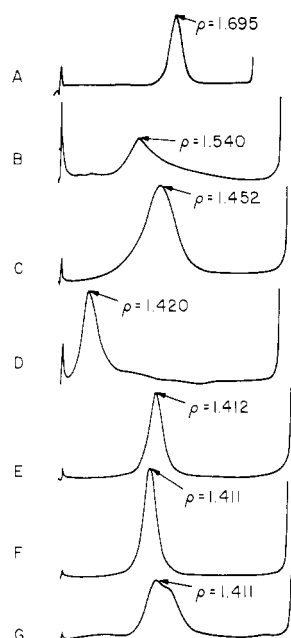


FIGURE 1: Equilibrium buoyant density banding patterns of nucleohistone in CsCl after treatment with various concentrations of formaldehyde. (A) Untreated, buoyant density = 1.695; (B) treated with 0.05% formaldehyde, buoyant density = 1.540; (C) with 0.1% formaldehyde, density 1.452; (D) with 0.2% formaldehyde, density 1.420; (E) with 0.5% formaldehyde, density 1.412; (F) with 1.0% formaldehyde, density 1.411; (G) with 10% formaldehyde, density of main peak is 1.411.

tration of nucleohistone was 0.1–0.2 OD_{260} if the isoconcentration density was 1.400 g/cc or higher, and it was 4–5 OD_{260} if the isoconcentration density was in the histone density range.

Determination of Sedimentation Coefficients. Sedimentation velocity measurements were made using the band sedimentation method in self-forming density gradients in the analytical centrifuge (Vinograd *et al.*, 1963). D_2O was used to form the gradients and all sedimentation coefficients were corrected to $s_{20,w}$. The partial specific volume of nucleohistone was taken to be 0.650 cc/g as determined by a weight average of the partial specific volumes of DNA (0.555 cc/g) (Brunner and Vinograd, 1965) and of histones (0.745 cc/g).²

Disc Gel Electrophoresis of Histones. The histone samples were prepared for electrophoresis by adding 0.5 ml of 1 N H_2SO_4 to 2 ml of nucleohistone at a concentration of 10 OD_{260} . The solution was mixed vigorously, allowed to stand at 0° for 15 min, and then centrifuged at 10,000g for 15 min. The supernatant was decanted and mixed with two volumes of cold 100% ethanol and allowed to stand for 30 min. The histones were then precipitated by centrifugation for 15 min at 10,000g. The pellet was washed with 100% ethanol and then air dried. The protein was dissolved in the proper amount of 10 M urea to make the final solution 1 mg/ml. In some cases the amount of acid-extractable protein was determined by the Lowry reaction (Lowry *et al.*, 1951). The standard bovine serum albumin fraction V (Sigma Chemical Co.) was also dis-

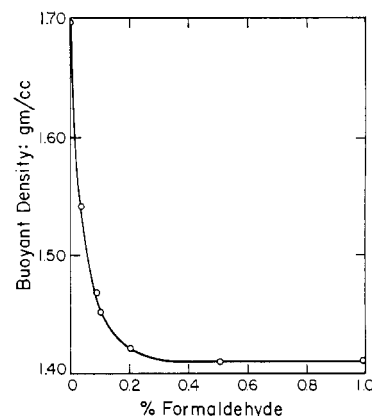


FIGURE 2: Buoyant density of nucleohistone complexes as a function of treatment formaldehyde concentration.

solved in 10 M urea. These samples were then electrophoresed on 15% polyacrylamide gels as previously described (Bonner *et al.*, 1968).

Pronase Treatment. Nucleohistone samples at a concentration of 1 OD_{260} in 0.01 M triethanolamine (pH 7.8) were treated with pronase for 60 min at 37° by the addition of 20 μ l/ml of sample of pronase solution (CalBiochem, grade B), 1 mg/ml, which had been predigested for 90 min at 37°. Sequential pronase treatment was done by adding further 20 μ l/ml of sample of freshly predigested pronase 1 hr after the preceding treatment.

DNase Treatment. For treatment with DNase, the nucleohistone samples were 1 OD_{260} , 0.01 M triethanolamine (pH 7.8), and 0.001 M $MgCl_2$. These samples were cloudy before, during, and after the reaction. To the turbid solution was added 50 μ l of DNase II (Worthington, electrophoretically pure), 1 mg/ml, and incubation at room temperature for 60 min.

Results

When native pea bud nucleohistone is centrifuged to equilibrium in a CsCl density gradient, it yields a homogeneous band of DNA with a mean density of 1.695 g/cc (Figure 1A). The released protein forms a skin at the meniscus. Nucleohistone treated with concentrations of formaldehyde greater than 0.2% forms a complex whose buoyant density approaches 1.410 g/cc (Figure 2). Using the buoyant density of free histone (1.245 g/cc) in the density relation, $\bar{V} \cong 1/\rho; (1 + X)\bar{V}_c = \bar{V}_{DNA} + X\bar{V}_{Histone}$, where \bar{V}_c is the \bar{V} of the complex, \bar{V}_{DNA} of the DNA, $\bar{V}_{Histone}$ of the histone, and X the weight ratio of histone to DNA in the complex; the ratio X for the complex can be calculated and is 1.27³ in agreement with chemical analysis. The influence of formaldehyde on the density of the complex is therefore slight. Figure 2 shows the buoyant density of the complex as a function of the formaldehyde concentration during treatment. From these densities, the amount

² Partial specific volume calculated from the amino acid composition of histone.

³ This relation ignores differences in hydration of histone and DNA due to variation in the activity of water at different CsCl concentrations and it ignores the difference between the buoyant density and the reciprocal of the partial specific volume. It is accurate enough for the present purpose. See Hearst and Vinograd (1961) and Meselson *et al.* (1957).

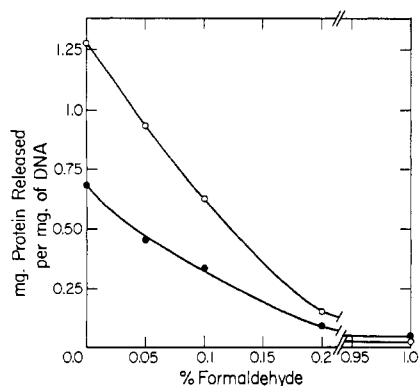


FIGURE 3: Amounts of protein bound in formaldehyde-treated complexes expressed as per cent of total protein present in the original nucleohistone. (A) Open circles: protein released as calculated from the buoyant density of the complex; (B) closed circles: amount of protein released by acid extraction as determined by chemical analysis. Scale $2\times$ that of A.

of protein bound to DNA in a form resistant to dissociation by CsCl may be calculated (Figure 3). The amount of protein bound in acid-nonextractable form was determined by chemical analysis of nucleohistone treated with varied concentrations of formaldehyde. Interestingly, the proportion of protein not dissociable by CsCl is in close agreement with that not acid extractable. The protein dissociated in CsCl from nucleohistone was also banded in the density range 1.2–1.3 g/cc and was observed to decrease in quantity with increasing formaldehyde treatment concentration.

The buoyant band shapes of formaldehyde-treated nucleohistone are also a function of formaldehyde concentration (Figure 1). The band formed by pure DNA (Figure 1A) is very symmetrical and the band width indicates an apparent molecular weight⁴ of 2.0×10^6 . After treatment with 0.05% formaldehyde, 30% of the chromosomal protein remains bound to and bands with the DNA. The band is, however, broad and skewed (Figure 1B). Treatment with higher concentrations of formaldehyde causes sharpening of the peak and an increase in its symmetry as well as a decrease in its density (Figure 1C–F). Treatment with formaldehyde concentration higher than 1% causes the band to separate into two bands, the lighter having a buoyant density of 1.410 g/cc. Neither the band shape nor buoyant density of purified DNA is changed by treatment with 1% formaldehyde under the present conditions (Table I).

The sedimentation coefficient of nucleohistone is altered by formaldehyde treatment. Thus, the $s_{20,w}$ of the original nucleohistone was 26 S; after treatment with 0.1% formaldehyde, 17 S; with 0.5% formaldehyde, 19 S; and with 1% formaldehyde, 21 S. In general the sedimenting bands of the formaldehyde-treated complex although single are broader than those of untreated nucleohistone.

Nucleohistone treated with formaldehyde shows both a

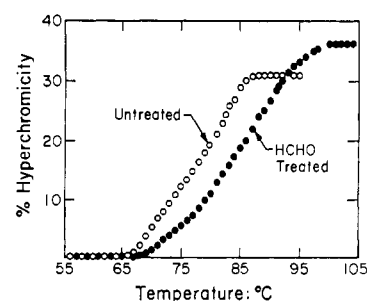


FIGURE 4: Normalized melting profiles of native nucleohistone and of nucleohistone treated with 1% formaldehyde.

greater thermal stability and a greater hyperchromicity upon melting than does the native material (Figure 4). The melting profile of treated nucleohistone is also broader. The increase in T_m as a function of formaldehyde concentration parallels the amount of protein bound in nondissociable form (Figure 5). Treatment of purified DNA with 1% formaldehyde as described above and after removing the formaldehyde by dialysis alters neither its T_m nor its hyperchromicity (Table I).

The protein which remains acid extractable from nucleohistone after formaldehyde treatment was subjected to examination by disc electrophoresis to determine the kinds of histone present (Figure 6). Treatment with even the lowest concentration of formaldehyde (0.05%) causes all of the lysine-rich histones Ia and Ib to become non-acid extractable. Treatment with 1% formaldehyde causes all but 3% of the protein of nucleohistone to become non-acid extractable. The 3% which is extractable is mainly histone II. There are thus substantial differences in reactivity with formaldehyde of the several species of histone.

The complex formed by reaction of nucleohistone with 1% formaldehyde was used as a substrate for DNase II or for pronase, and the digestion products banded in CsCl. The turbid solutions from the DNase reaction became clear immediately upon addition of CsCl. After incubation of nucleohistone with DNase II, no band of material formed in the density range 1.4–1.75 g/cc. Rather, the optically absorbing material remained dispersed throughout the entire CsCl gradient (Figure 7A). The DNA is thus degraded to small molecular weight pieces which are not held together by the proteins. Treatment with pronase (60 min) results in a complex of density 1.634 g/cc and of asymmetric band shape (Figure 7B).

TABLE I: Properties of DNA as a Function of Treatment with Formaldehyde.

	Purified Pea Bud DNA	1% Form- aldehyde- Treated DNA
Buoyant density	1.695	1.697
T_m (deg)	45	45
Hyperchromicity (%)	38	38

⁴ The apparent molecular weights differ from the expected values of about 10^7 for the DNA and the nucleohistone (based on sedimentation coefficients) because of band broadening due to minor heterogeneities in molecule weight and buoyant density (due to the G + C content).

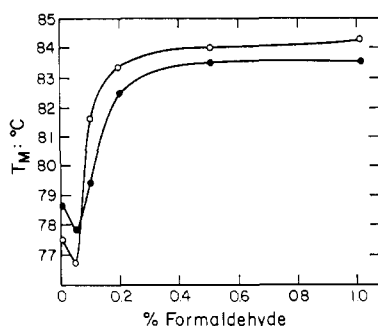


FIGURE 5: The T_m of treated nucleohistone as a function of treatment formaldehyde concentration. Each curve represents the results obtained from one preparation of nucleohistone.

Repeated (four times) pronase treatment of formaldehyde-treated complex resulted in material which banded at a density of 1.669 g/cc and from which therefore 96% of the protein had been removed (Figure 7C).

To determine the number and nature of the cross-links introduced, nucleohistone was treated in the standard manner with 1% ^{14}C -labeled formaldehyde (sp act. 1 mCi/mmole). After reaction, a portion was precipitated and washed with 10% trichloroacetic acid and counted to determine the total amount of formaldehyde which had reacted. A further portion was subjected to repeated pronase treatment as described above. The extensively pronased sample was next precipitated and washed with 10% trichloroacetic acid and then counted to determine the number of pronase-resistant cross-links. The results are given in Table II. The treated nucleohistone contained approximately 1 formaldehyde cross-link per 2 base pairs or per 15 amino acid residues. Pronase treatment removed 96% of the protein and also approximately two-thirds of the formaldehyde cross-links. These must then represent interprotein cross-links. The protein-DNA cross-links are represented by the formaldehyde which remains attached to the complex after pronase treatment. The ratio of amino acids to formaldehyde in the pronased material, 1.67, indicates the presence of 1-2 amino acids/cross-link. Pronase therefore removes all of the protein down to a stub of this length through which the protein was originally cross-linked to DNA.

Discussion

The treatment of nucleohistone with formaldehyde results in complexes in which protein-DNA interaction is no longer mainly ionic as it is in native nucleohistone. The treated complexes are dissociated neither by 4 M CsCl nor 0.2 N H_2SO_4 . Reaction with formaldehyde results in other forms of DNA-protein interaction. Formaldehyde, used as a fixative, causes the formation of methylene bridges between two neighboring amino groups. The amino groups in purified DNA are not cross-linked under the conditions employed here. However, association of DNA with histone may encourage the formation of such bonds as well as of DNA-histone cross-links. The existence of the latter is indicated by the residual 4% bound protein which pronase treatment cannot remove and which bands with DNA in CsCl. This amount of protein corresponds to one amino acid for each four base pairs.

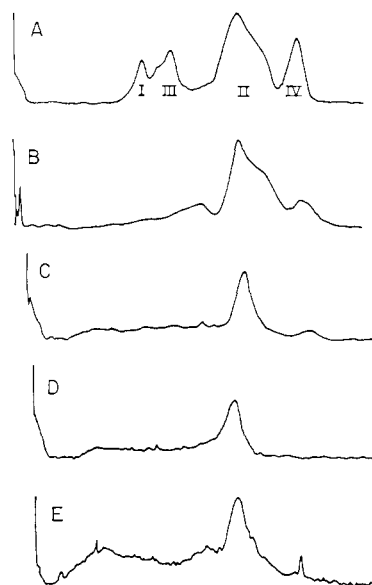


FIGURE 6: Microdensitometer traces showing disc gel electrophoresis patterns obtained from the acid-extractable proteins of formaldehyde-treated nucleohistone. (A) Untreated; (B) 0.05% formaldehyde; (C) 0.1% formaldehyde; (D) 0.5% formaldehyde; (E) 1.0% formaldehyde. Roman numerals refer to classes of histone: I, lysine rich; II, slightly lysine rich; III and IV, arginine rich.

Since the sedimentation coefficient does not change markedly, the buoyant band broadening caused by treatment with very low formaldehyde concentrations must be due to a distribution of histone/DNA ratios, the denser species being those containing less bound protein. Treatment with higher formaldehyde concentrations causes more of the total protein to be bound and the symmetry of the buoyant bands indicates that the complexes formed under these conditions are very homogeneous from molecule to molecule with respect to histone/DNA ratio. The apparent molecular weight⁴ of the material in such bands is 3.0×10^6 . Treatment with formaldehyde concentrations above 1% causes a new band of greater

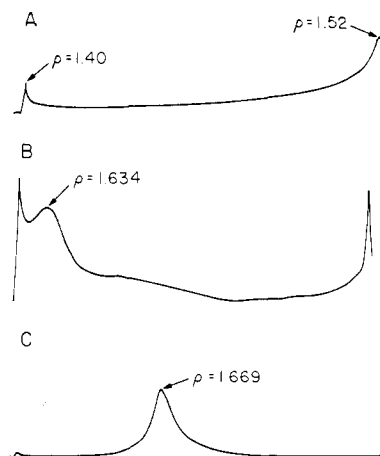


FIGURE 7: Buoyant density band patterns of the complex formed by the reaction of nucleohistone and 1% formaldehyde after treatment with (A) DNase II, (B) pronase, and (C) pronase four times.

TABLE II: Quantities of Formaldehyde Reacted with and Bound in Acid-Insoluble Form in Nucleohistone before and after Removal of Protein by Pronase.

Sample	Treatment	Buoyant Density	Protein Content (Amino Acid Base Pair)	Formaldehyde Reaction Product (Formaldehyde/ Base Pair)
Formaldehyde-treated nucleohistone	None	1.410	6.6	0.43
Formaldehyde-treated nucleohistone	Four-times-repeated pronase	1.669	0.25	0.15

density to appear. High concentrations of formaldehyde must therefore result in secondary alterations of nucleohistone structure or composition.

The increased T_m of formaldehyde-treated nucleohistone indicates that the DNA of the complex is more effectively stabilized by protein as a result of the more intimate binding between histone and DNA caused by the treatment. Such a stabilizing effect could result either from histone-histone methylene bridges or from histone-DNA bridges as described above.

The fact that histone I is completely missing from the acid-extractable proteins after treatment with very low concentrations of formaldehyde indicates that this histone is most susceptible of all histones in nucleohistone to attack by formaldehyde and is most readily bound to DNA. A portion of histone II is, on the contrary, acid extractable even after treatment with even the highest concentrations of formaldehyde. This histone must therefore be the least susceptible to attack by formaldehyde, indicating that it is more protected from solvent than are other histones of nucleohistone.

The fact that chromosomal proteins can, by treatment with formaldehyde, be joined to DNA and to each other in such a way that they do not dissociate from DNA in CsCl would seem to have many potential applications in chromosomal biology and chemistry. Thus, the method might be used to separate, on the basis of their buoyant densities, regions of DNA complexed with one class of protein from the DNA of other regions complexed with either other classes of proteins or with none at all.

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