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In Vitro Binding of *N*-Acetoxy-*N*-2-acetylaminofluorene to DNA in Chromatin

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SUMMARY

The binding of *N*-acetoxy-*N*-2-acetylaminofluorene to DNA in native and partially dehistonized chicken erythrocyte chromatin was studied. The amounts of carcinogen bound to DNA were measured, after removal of proteins with phenol, by using the absorption ratio A_{305}/A_{260} or by counting the radioactivity of ^{14}C-labeled carcinogen. Measurements of uncovered zones of DNA in chromatin were made by comparison of results obtained with free DNA and with chromatin at increasing ratios of carcinogen/nucleotide. The proportion of DNA accessible to the carcinogen was found to be 15% in native chicken erythrocyte chromatin and about 22% in native calf thymus chromatin. The amount of accessible DNA increases to 55% in chicken erythrocyte chromatin depleted of histones H1 and H5.

Formaldehyde unwinding performed on DNA extracted from chromatin after modification showed an increasing number of defects in the double helix with the amount of DNA-fixed carcinogen.

At high ratios of carcinogen/nucleotide, the recoveries of DNA (by phenol method) and of histones (by acidic extraction) decreased with increasing ratios. This suggests a covalent linkage between proteins and DNA.

INTRODUCTION

It has been shown (11, 12, 21, 22) that carcinogenic aromatic amides such as AAF[2], when administered *in vivo*, are transformed into strong electrophilic metabolites that bind to liver tRNA, rRNA, DNA, and proteins. The active derivative has been identified as an ester of N-OH-AAF. It was demonstrated that N-AcO-AAF reacted mainly with the C_8 of guanine in DNA (12) and, to a lesser extent, with NH_2 of this base and with adenine (9, 11). For this reaction the base must be pulled out of the double helix to be accessible to the carcinogen (9). Physical studies of DNA modified by N-AcO-AAF showed a destabilizing effect with the formation of loops in the double helix; also, cross-linkages are formed between the 2 strands by an unknown mechanism (3, 4, 6).

The reaction of N-AcO-AAF with proteins is not well understood. *In vitro* studies of RNase showed an acetylation of the protein by N-AcO-AAF (1), whereas *in vivo* N-OH-AAF was found to cause preferential arylamidation of liver histones H4 and H2A (8). We have studied *in vitro* the reaction of N-AcO-AAF on native and partially dehistonized chromatin from calf thymus and chicken erythrocytes. The existence and the extent of "breathing zones" [as defined by Printz and von Hippel (23)] of DNA in chromatin have been shown, and the effect of nonhistone proteins associated with chromatin was observed. We found that covalent attachment of proteins to DNA occurred when high concentrations of carcinogen were used.

MATERIALS AND METHODS

All chemicals were reagent grade (Merck, Darmstadt, Germany). DNA with a protein content of less than 0.8% by weight was prepared from calf thymus and chicken erythrocytes according to the method of Kay *et al.* (10); hypochromicity of the 2 DNA's at 260 nm was 40 and 38%, respectively.

Native calf thymus and chicken erythrocyte chromatin were prepared by the method of Zubay and Doty (28) and were dialzyed 1st against twice-distilled water and then against 0.1 mM sodium citrate buffer at pH 7. The protein/DNA ratio was 1.60 (w/w). In order to remove the nonhistone proteins from calf thymus chromatin before dialysis, repeated washings by 0.35 M NaCl, buffered in 1 mM sodium phosphate, pH 7, were performed as described by Goodwin and Johns (7). In this case, one-half of the nonhistone proteins were removed, and the protein/DNA ratio was 1.35 (w/w). Removal of histones H1 and H5 from chicken erythrocyte chromatin was performed by the method of Bolund and Johns (2). The selectivity of the extraction was checked by polyacrylamide gel electrophoresis (16). In this case, the protein/DNA ratio was 0.8 (w/w). The N-AcO-AAF was synthesized as previously described (17), with some modifications. It gave a single spot by thin-layer chromatography on silica plate (F254 Merck) with elution solvent 95% $HCCl_3$, 5% acetone (v/v). The labeled carcinogen [9-^{14}C]N-AcO-AAF was made in the same way and had a specific activity of 55.5 mCi/mole.

The reaction of the carcinogen with DNA in the chromatin was performed in a way similar to that described by

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[2] The abbreviations used are: AAF, *N*-2-acetylaminofluorene; N-OH-AAF, *N*-hydroxy-*N*-2-acetylaminofluorene; N-AcO-AAF, *N*-acetoxy-*N*-2-acetylaminofluorene; [9-^{14}C]N-AcO-AAF, *N*-acetoxy-*N*-2-acetylaminofluorene with ^{14}C in position 9; C/D, carcinogen/nucleotide ratio during reaction (molecule/residues).

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Miller *et al.* (20), in 0.1 mM sodium citrate buffer, pH 7, except when indicated. The carcinogen, dissolved in a volume of ethanol equal to 10% of the volume of DNA or chromatin solution, was added rapidly under argon atmosphere. The mixture was kept at 37 ± 0.2° for 3 hr, and then the excess of fluorene derivative was removed by 3 successive extractions with ethyl ether.

The percentage of modified bases in DNA was measured by counting radioactivity and by the A_{305}/A_{260} ratio according to Fuchs and Daune (4) when the amount of modified bases was high enough to give a shoulder at 305 nm in the absorption spectrum. For lower values, radioactive counting was used. In the latter case, the DNA was extracted from chromatin once by phenol in 10 mM $MgCl_2$:10 mM Tris-HCl, pH 7.4. After elimination of phenol with diethyl ether, the DNA was precipitated with 2 volumes of cold ethanol and redissolved in 0.1 mM sodium citrate buffer, pH 7. A given quantity of DNA was precipitated by addition of 1 N HCl to yield a final concentration of 0.25 N HCl, and the precipitate was collected on a Millipore filter (MF-VCWP 02500; Millipore Corp., Bedford, Mass.) The filter was put in vials containing 10 ml of scintillation liquid (toluene:PPO 5 g/liter) and counted in an Intertechnique liquid scintillation counter SL 30 (Intertechnique S.A., Plaisir, France).

Measurements of defects in the DNA helix were made by using the formaldehyde unwinding rate (15, 24, 25). For this reaction, the DNA was prepared by means of 3 phenol extractions in the $MgCl_2$:Tris medium described above. After removal of phenol, the DNA solution was dialyzed against 42 mM borate buffer, pH 9. This DNA contained 1 to 2% protein in weight and its hypochromicity was 38% at 260 nm. The formaldehyde method permits determination of the number of defects in DNA produced by the carcinogen, since it induces local opening of base pairs in the double helix (6). The reaction was performed in a Cary 15 spectrophotometer (Varian Instrument Division, Palo Alto, Calif.) in a way similar to that described by Fuchs and Daune (5). The temperature was kept constant and equal to 42.8 ± 0.2°, and the absorbance at 251 nm was recorded as a function of time. This variation of absorbance with time was S-shaped, which permitted the application of the method of Lazurkin *et al.* [for further details, see Ref. (15)]. The calculations were made on a Univac 1108 computer with a program written in Fortran language. The absorbance melting curves were measured at 260 nm by means of a technique designed by Neimark and described by Wilhelm *et al.* (26). The DNA content of solutions was determined spectrophotometrically by absorbance at 260 nm using a molar extinction coefficient/nucleotide residue of 6700. Protein content was determined by the method of Lowry *et al.* (18).

RESULTS

Reaction with Chicken Erythrocyte Chromatin. In order to compare the reactivity of free DNA with that of DNA in chromatin depleted of nonhistone proteins, the reaction was 1st conducted with increasing values of N-AcO-AAF over nucleotide (C/D) ratio in a medium such that the melting temperatures were the same. In 0.1 mM sodium citrate buffer, pH 7, the chicken erythrocyte chromatin melts at 83° and, by adding 10% ethanol, the melting temperature drops to 70.8°. In order to maintain a melting temperature of 83° with free DNA, 0.12 M NaCl must be added to the 0.1 mM sodium citrate buffer; further addition of 10% ethanol gives the same lowering of 12.2° (Chart 1).

Under these experimental conditions, the modification rate was higher for the DNA in chromatin than for the free DNA. By radioactivity counting, 0.035% ± 0.005% of modified bases was found after reaction of free DNA at high ionic strength when using the higher C/D ratio of 0.03. For the DNA extracted from chromatin, radioactivity counting gave 0.195% ± 0.005% of modified bases for the same C/D ratio. The higher rate of modification for DNA in chromatin than for free DNA can be explained by assuming that, in the medium used for DNA, "breathing" is inhibited by the ionic strength, whereas inside chromatin, uncovered zones of DNA behave as if they were in a medium of low ionic strength.

The carcinogen was reacted with DNA, with chromatin depleted of histones H1 and H5, with nonhistone protein-depleted chromatin, and with native chromatin. All reactions were performed in the same medium (0.10 mM sodium citrate buffer, pH 7, plus 10% ethanol). In this case, the modification was least for native chromatin and increased progressively with the removal of proteins, being the highest for free DNA.

There was a linear relationship between the percentage of

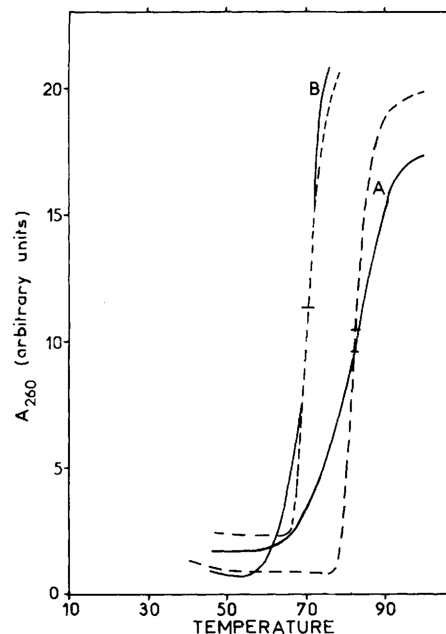

Chart 1. Melting curves of chicken erythrocyte DNA and native chromatin. For *Curves A*, the experiment was made in 0.1 mM sodium citrate buffer, pH 7, for chromatin, and in the same buffer plus 0.12 M NaCl for DNA. The melting temperatures were then the same (83°). For *Curves B*, DNA and chromatin were in the same medium as above, but 10% ethanol was added. The same lowering of melting temperatures was observed (12.2°). — — —, DNA; ——, chromatin.

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modifications and the C/D ratio, both for DNA and for DNA extracted from either native chromatin or from chromatin that was partially dehistonized or depleted of nonhistone protein (Chart 2). Calculated intercepts (Table 1) were negligible, and each straight line was considered to be passing through the origin. The ratios of the slopes were then equal to the respective ratios of reactivities. It was therefore possible to determine the proportion of free DNA that was breathing inside chromatin. The values were 15.1 ± 0.5% for chicken erythrocyte native chromatin, 16.7 ± 0.5% for nonhistone protein-depleted chromatin, and 55.1 ± 0.5% for chromatin without histones H1 and H5. The removal of these 2 histones exposed an additional 40% of the total DNA to the carcinogen. Formaldehyde unwinding performed on free DNA after reaction with the carcinogen at high ionic strength did not show any detectable number of defects, whereas for DNA extracted from chromatin after modification, an increasing number of defects with an increasing percentage of modified bases was observed. This latter result was also found with free DNA modified at low ionic strength. We can thus conclude that the binding of carcinogen to guanine residues induces defects in the double helix of DNA inside chromatin as well as in the free state.

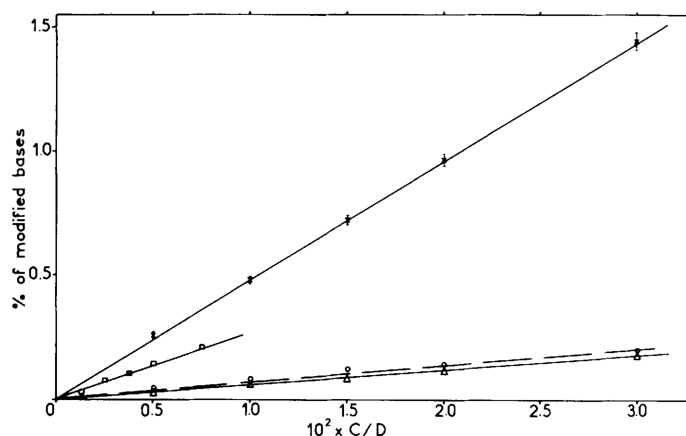

Chart 2. Plot of percentage of modified bases in native chromatin, nonhistone protein-depleted chromatin, partially dehistonized chicken erythrocyte chromatin, and in free DNA after reaction with increasing quantities of carcinogen per nucleotide. Reaction with [9-^{14}C]N-AcO-AAF was made in 0.1 mM sodium citrate buffer, pH 7, + 10% ethanol (v/v). See "Materials and Methods" for details. ×, free DNA; □, chromatin without histones H1 and H5; O, nonhistone protein-depleted chromatin; Δ, native chromatin.

Reaction on Calf Thymus Chromatin. It has been shown that the removal of nonhistone proteins from calf thymus chromatin caused a reappearance of premelting that was absent in native chromatin (27). To see whether this removal of nonhistone proteins exposed DNA regions, the reactivities of native and nonhistone-depleted chromatin were compared with N-AcO-AAF. The modifications were measured by counting the radioactivity. Results are shown in Table 2; a plot of the percentage of modification *versus* C/D ratio (Chart 3) gives 2 slightly different straight lines for the 2 chromatins. We can thus conclude, within the limits of experimental accuracy, that at most an additional 3% of DNA was exposed by washing off about one-half of the nonhistone proteins in 0.35 M NaCl. This result is similar to that observed with chicken erythrocyte chromatin (Chart 2; Table 1), where an additional 2% of DNA became accessible to the carcinogen.

DISCUSSION

The dynamic state of the DNA, as studied by formaldehyde unwinding, is strictly dependent on the difference between the temperature at which the rate is measured and the melting temperature of the DNA under the same conditions. One would thus expect the same dynamic state for DNA and chromatin when comparison is made in such a medium that their melting temperatures are equal. In fact, the dynamic accessibility to the carcinogen, which acts as a chemical probe of the dynamic state of the DNA, is greater in chromatin than in free DNA when both have the same melting temperature. It appears, therefore, that in chromatin the DNA offers uncovered regions where breathing may take place in a medium of low ionic strength. On the other hand, no melting, as followed by the absorbance change at 260 nm, can be detected before the cooperative melting takes place at around 83° in 0.1 mM sodium citrate. The

Table 1

Accessibility of DNA in native and partially dehistonized chicken erythrocyte chromatin

The percentage of accessible DNA was determined by the ratios of the slopes of percentage of modified bases *versus* C/D for the different chromatins and the free DNA (see text for details).

	Slope of % of modified bases *vs.* 10^2 C/D[a]	Intercept of % of modified bases *vs.* 10^2 C/D[a]	% of accessible DNA
Chicken erythrocyte free DNA	0.479	0.001	100
Native chromatin	0.060[b]	0.001[b]	15.1 ± 0.5[c]
Nonhistone protein-depleted chromatin	0.066[b]	0.005[b]	16.7 ± 0.5
Chromatin without histones H1 and H5	0.264[b]	0.005[b]	55.1 ± 0.5

[a] Determined by the least square method.
[b] Mean of 2 experiments.
[c] Mean ± S.D.

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Table 2

Accessibility of DNA in native and nonhistone protein-depleted calf thymus chromatin

The accessibility of DNA was determined in the same manner as in Table 1. In each experiment, the modifications of the chromatin without nonhistone proteins were 3 to 4% higher than modifications of native chromatin.

	Slope of % of modified bases *vs.* 10^2 C/D[a]	Intercept of % of modified bases *vs.* 10^2 C/D[a]	% of accessible DNA
Calf thymus free DNA	0.412	0.021	100
Native chromatin	0.077[b]	0.002[b]	18.6 ± 2.8[c]
Chromatin depleted of nonhistone proteins	0.094[d]	0.006[d]	22.8 ± 1.8

[a] Determined by the least square method.
[b] Mean of 4 experiments.
[c] Mean ± S.D.
[d] Mean of 3 experiments.

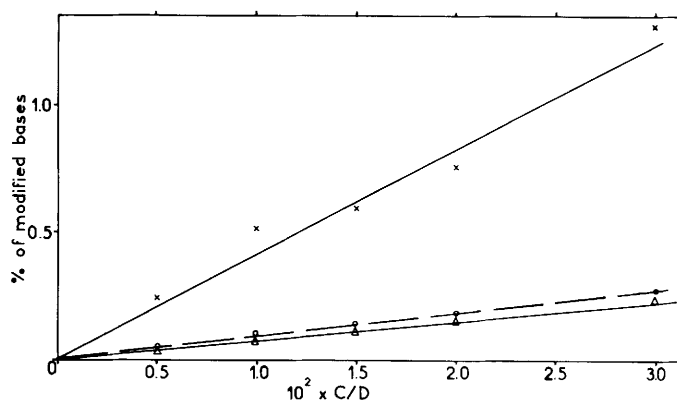

Chart 3. Plot of percentage of modified bases in native and nonhistone protein-depleted calf thymus chromatin and in free calf thymus DNA after reaction with increasing quantities of [9-^{14}C]N-AcO-AAF/nucleotide. See legend of Chart 2 for additional details. ×, free DNA; Δ, native chromatin; O, nonhistone protein-depleted chromatin.

unprotected zones of DNA must therefore be longer than a "breathing unit" (25) made of 8 to 10 base pairs but short enough to be protected against melting by "locked" ends. These short pieces of protein-free DNA represent one-fifth of the DNA length in calf thymus chromatin, a result in agreement with values found by using intercalated ethidium bromide (14, 19) or proflavine (13). Less of the DNA (16%) was accessible in chicken erythrocyte chromatin, in which more than one-half of the usual amount of histone H1 has been replaced by the erythrocyte-specific histone H5. Since this histone is known to interact more strongly with DNA, it is probable that, in chicken erythrocyte chromatin, the DNA is less accessible.

The difference observed between native and nonhistone-depleted chromatin is significant, since in all the experiments the accessibility of the washed chromatin was always higher than that of the native one. However, in view of the small value of this difference and of the accuracy in the determination of histone content, it is difficult to decide whether the increase of accessibility is due only to the removal of a large amount of nonhistone protein or to the loss of a small percentage of histone H1, the bulk of which is extracted at higher ionic strength. On the other hand, formaldehyde unwinding demonstrated defects caused by binding of the carcinogen to DNA, even inside chromatin, with a correlation observed between the number of defects and the amount of DNA-fixed carcinogen.

After removal of histones H1 and H5 from chicken erythrocyte chromatin, 40% more DNA became accessible (Table 1), to net a total accessibility of 55%.

In addition to these results dealing with the accessibility of DNA inside chromatin, it was found that, at high values of C/D ratio, linkages formed between DNA and proteins (unpublished results). These linkages are strong enough to be resistant to both phenolic extraction of DNA and HCl extraction of histones. This suggests that a covalent binding must take place between the molecules of DNA and protein. This linkage remains unexplained for the moment. Work is in progress to see whether this covalent binding occurs between DNA and any histone or nonhistone molecule or whether it involves only 1 or 2 specific types of proteins.

The apparent linkage between DNA and proteins may also be present with low C/D ratios, but the low amount of nonrecovered DNA after phenol extraction may not be detectable at this level of experimental accuracy. At the present state of our knowledge, one cannot say whether this covalent linkage is only a side effect or whether it is indeed involved in tumorigenesis.

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