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BINDING OF ISOMERS OF BENZO[A]PYRENE DIOL-EPOXIDE TO CHROMATIN*

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Summary. Both the carcinogenic B[a]P diol-epoxide (anti) and its relatively non-carcinogenic isomer, B[a]P diol-epoxide (syn), when reacted with chromatin in vitro, bind more extensively to the internucleosomal region of chromatin than to nucleosomes. These results suggest that the increased binding of B[a]P diol-epoxide (anti) to the internucleosomal region may have little relevance to the process of carcinogenesis.

In vitro studies with DNA and B[a]P 7,8-diol-9,10-epoxide, produce adducts which appear to be identical to those obtained from in vivo experiments; consequently, it has been suggested that the B[a]P diol-epoxide is the ultimate carcinogenic form of B[a]P (1–7). Two diastereoisomers must be considered: B[a]P diol-epoxide (anti), and the B[a]P diol-epoxide (syn). Both isomers are potential chemical mutagens in bacterial systems; in mammalian cells the B[a]P diol-epoxide (anti) shows exceptional mutagenicity but the syn isomer does not (8–11). The carcinogenic potency of the isomers has also been tested in vivo, and the data obtained suggest that the B[a]P diol-epoxide (anti), rather than the B[a]P diol-epoxide (syn), is the ultimate carcinogenic form (8–11). These results suggest that the underlying mechanism of initiation of the tumor response may well be stereo-specific in nature. It could be argued that the increased chemical reactivity of the syn isomer (12, 13) may result in the absence of bound adducts to the target molecules, and that this, then,

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Abbreviations: B[a]P, benzo[a]pyrene; B[a]P diol-epoxide (anti), (±) 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; B[a]P diol-epoxide (syn), (±) 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; PMSF, phenylmethanesulfonylfluoride

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would result in the virtual lack of the carcinogenic response of B[a]P diol-epoxide (syn). However, experiments with human alveolar cells in culture have clearly demonstrated that both isomers react with cellular DNA (14). Furthermore, recent experimental evidence (15) has shown that in vivo both isomers form adducts with DNA, although the binding to DNA for the syn isomer is approximately three times lower than that of the anti isomer of B[a]P diol-epoxide. In vivo experiments, in which an increase dose range of the syn isomer was used to compensate for the threefold less binding to DNA, showed no change in relative tumor yields (unpublished observation).

Taking into account these factors and the observation that the (+) and (-) enantiomers of B[a]P diol-epoxide (anti) are markedly different in their carcinogenic activity but similar with respect to their chemical reactivity (16, 17), we suggest that the virtual inactivity of the syn isomer of B[a]P diol-epoxide with respect to the carcinogenic response is probably due to a stereo-specific nature in the binding to the target molecule (that is, chromatin).

When chromatin (for review, see ref. 18) was reacted in vitro with B[a]P diol-epoxide (anti), analyses of the distribution of adducts showed that the internucleosomal region bound more carcinogen per unit DNA than the nucleosomal core particles (19, 20). The biological significance of this increase, however, was not clear. We have therefore reacted chromatin with B[a]P diol-epoxide (anti) and its relatively noncarcinogenic isomer B[a]P diol-epoxide (syn) to establish whether the stereospecificity of binding might reside in the internucleosomal region of chromatin. Our results show that both isomers of B[a]P diol-epoxide bind to a greater extent to the internucleosomal region under in vitro conditions.

MATERIALS AND METHODS

Chicken erythrocyte nuclei were isolated as described previously (20). The nuclei were resuspended in 10 mM Tris (pH 7.2), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P40, 0.1 mM PMSF to a final concentration of 1.7×10^9 nuclei ml^{-1}. The suspension was made 1.0 mM $CaCl_2$ and digested with micrococcal nuclease (63 units ml^{-1}) at 37°C. The reaction was terminated after 10 min by addition of EDTA (pH 7.2), 10 mM final concentration. The digest was dialyzed for 12 h at 4°C with three changes of 10 mM Tris (pH 7.2), 0.1 mM PMSF. The lysed nuclear suspension was then centrifuged at 10,000 g for 10 min, and the supernatant solution, containing solubilized chromatin, was concentrated to 7.9 A_{260} units ml^{-1} by ultrafiltration on an Amicon PM10 membrane. Aliquots of the chromatin solutions were equilibrated at 37°C. B[a]P diol-epoxide (anti) and (syn) (specific activity, 29.4 and 29.6 mCi $mmol^{-1}$, respectively) were added slowly to the chromatin solutions. The final ratio of either B[a]P diol-epoxide concentration to that of chromosomal DNA was 1:100 (w/w). The reaction mixture was incubated for 30 min after which the solutions were dialyzed against 0.2 mM EDTA (pH 7.2), 0.1 mM PMSF at 4°C until radioactivity was constant. The solutions were concentrated (see above) to 22.0 A_{260} units ml^{-1}. Oligonucleosomes were obtained by ultracentrifugation through 5%–25% linear sucrose gradients containing 0.2 mM EDTA (pH 7.2), 20 mM NaCl by use of an SW 25.2 rotor at 23,000 r.p.m. for 16 h at 4°C. The gradients were fractionated with an Isco gradient fractionator. The

fractions containing carcinogen-labeled oligonucleosomes were pooled as indicated and dialyzed against 10 mM Tris (pH 7.2), 0.1 mM PMSF.

Labeled oligonucleosomal fractions were concentrated to 13.0 A_{260} units ml^{-1}, made 1.0 mM $CaCl_2$, and redigested to nucleosomal core particles by micrococcal nuclease (200 units ml^{-1}) for 20 min at 37°C. The reaction was terminated with EDTA (pH 7.2), 10 mM final concentration. The nucleosomal core particles were obtained by ultracentrifugation through sucrose gradients containing 20 mM NaCl as described above except that 10 A_{260} units were loaded and an SW 41 Ti rotor was used at 37,000 r.p.m. for 12 h at 4°C. The fractions containing nucleosomal core particles were pooled and dialyzed against 10 mM Tris (pH 7.2), 0.1 mM PMSF.

Proteins were analyzed on 15% SDS-polyacrylamide slab gel electrophoresis essentially as described by Laemli (21). Nucleosomes (2.0 A_{260} units) were dissolved in 50 µl of sample application buffer and 2 to 4 µl per slot were electrophoresed at 90 V for 5 h. The gels were stained in 10% acetic acid, 32% isopropanol, 0.1% Coomassie blue for 15 min, then rinsed with water (at this point the protein bands are clearly visible). They were then further destained with 10% acetic acid for 12 h and photographed. Nucleosomal DNA was treated and subjected to electrophoresis in 10% polyacrylamide containing 0.1% SDS, as described by Lutter (22). The gels were stained with toluidine blue for 30 min, destained in water, and photographed.

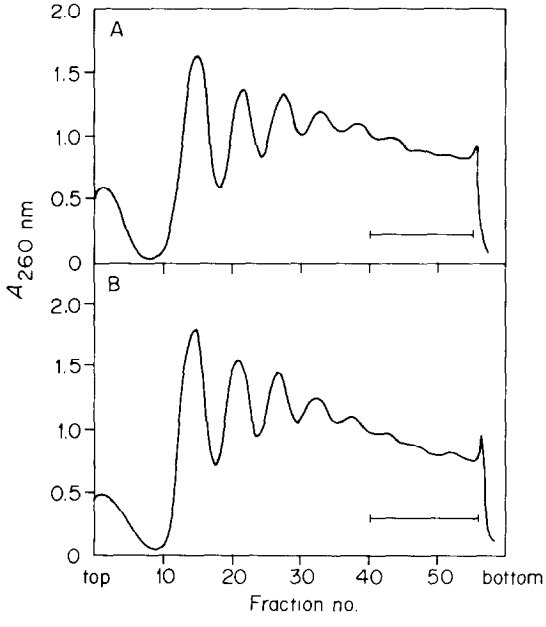

Fig. 1. Optical density profiles of sucrose gradients. Chromatin solubilized by short micrococcal nuclease digestion was loaded on linear 5–25% sucrose gradients and subjected to ultracentrifugation in a SW 25.2 rotor. The gradients were fractionated with an ISCO density gradient collector (Model 640) equipped with an ISCO UV-5 absorbance monitor. Oligonucleosomes were collected as indicated. A and B are representative profiles obtained from chromatin reacted with B[a]P diol-epoxide (anti) and (syn), respectively.

Table 1. Distribution of labeled carcinogen in chromatin

Fraction	Specific activity (c.p.m. per A_{260} unit)	
	B[a]P diol-epoxide (anti)	B[a]P diol-epoxide (syn)
Oligonucleosomes	14,190	10,478
Mononucleosomes	5,662	3,846

The specific activity of the internucleosomal region for B[a]P diol-epoxide (anti) was obtained by subtracting the specific activity of mononucleosomes from that of oligonucleosomes. Since the internucleosomal DNA is approximately half the length of the nucleosomal core particle, the internucleosomal region contained 3.0 times more bound carcinogen than the nucleosomal core particle per unit DNA or base pair. The case of B[a]P diol-epoxide (syn) was similar; 3.4 times the amount was bound per unit DNA to the internucleosomal region. Repeat experiments carried out under identical conditions have shown that the variability of these values was 5–6%.

RESULTS AND DISCUSSION

The carcinogen-labeled oligonucleosomes were purified by ultracentrifugation through linear sucrose gradients (Fig. 1). Specific activities of the oligonucleosomal fractions are shown in Table 1; they indicate that the B[a]P diol-epoxide (anti) is more effective in binding to oligonucleosomes than the chemically more active syn isomer. The amounts of B[a]P diol-epoxide associated with oligonucleosomes were 7% and 5.1% of the total input of B[a]P diol-epoxide (anti) and (syn), respectively.

Analysis of the purified nucleosomal core particles (Fig. 2), which contained approximately 140 base pairs of DNA and all four core histones (H2A, H2B, H3, and H4)

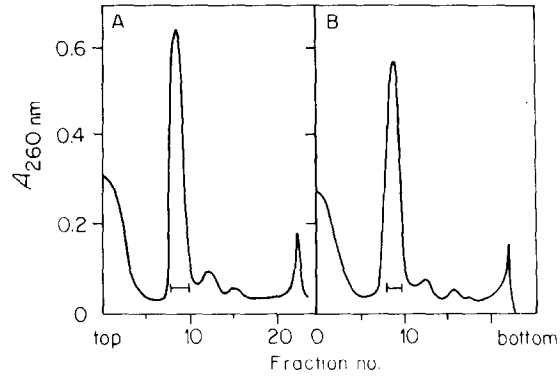

Fig. 2. Nucleosomal core particles were purified and isolated as described in Fig. 1 and in the section Materials and Methods using a SW 41 Ti rotor. A and B refer to condition shown in Fig. 1.

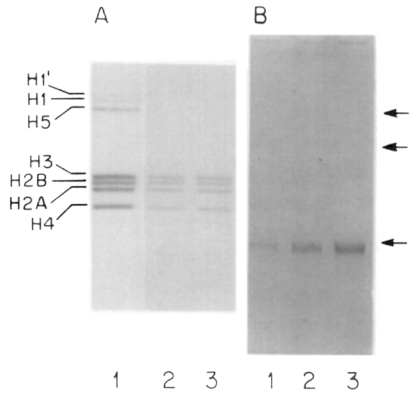

Fig. 3. A, 15% SDS polyacrylamide gels of histone proteins from (1) oligonucleosomes, (2) mononucleosomes containing B[a]P diol-epoxide (anti) adducts, and (3) mononucleosomes containing B[a]P diol-epoxide (syn) adducts. B, 10% polyacrylamide gels of DNA fragments from (1) standard nucleosomal core particles containing approximately 140 base pairs of DNA (courtesy of Dr. G. J. Bunick), (2) nucleosomal core particles containing B[a]P diol-epoxide (anti) adducts, and (3) mononucleosomal core particles containing B[a]P diol-epoxide (syn) adducts.

(Fig. 3), showed that the ratios of specific activities of chromatin to nucleosomal core particle were 2.5 and 2.7 for chromosomal material treated with B[a]P diol-epoxide (anti) and (syn), respectively (Table 1). This indicates that in the case of the syn isomer, the internucleosomal region contained 3.4 times more carcinogens per unit DNA than was found in the nucleosomal core particle; in the case of B[a]P diol-epoxide (anti) this value was 3.0. These results are comparable with previously published values (20) and suggest that both isomers of B[a]P diol-epoxide have a greater affinity to bind to the internucleosomal region. If the initiating events in the process of malignant transformation involve a stereo-specific interaction with chromatin to exert their carcinogenic effect, the data presented here suggest that the increased binding of both isomers of B[a]P diol-epoxide to the internucleosomal region of chromatin *in vitro* is not subject to stereo-specific constraints, and that the stereo-specific nature of the carcinogenic interaction may lie elsewhere. Extrapolating our *in vitro* findings to the nature of the carcinogenic process, we suggest that the increased binding of B[a]P diol-epoxide to the internucleosomal region of chromatin may have little relevance to initial events that lead to malignant transformation.

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