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EFFECT OF BENZO[*a*]PYRENE ON DNA SYNTHESIS AND DNA POLYMERASE ACTIVITY OF RAT LIVER NUCLEI*

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Abstract—When benzo[*a*]pyrene (B[*a*]P) was administered intraperitoneally to rats 48 hr before they were killed, the DNA-synthesizing capability of isolated rat liver nuclei was decreased as compared with control animals. B[*a*]P also inhibited *in vitro* DNA synthesis in nuclei purified from control animals; this effect was enhanced by NADPH. DNA polymerases solubilized from purified nuclei of B[*a*]P-treated animals were less active than those of control animals. DNA polymerase α was more inhibited than DNA polymerase β. Purified rat liver nuclei devoid of cytoplasmic contamination possess an NADPH-dependent B[*a*]P hydroxylase activity. The observed inhibition of DNA synthesis in nuclei isolated from B[*a*]P-treated rats was increased by NADPH. Moreover, there was an increased inhibition of DNA polymerase activity by nuclear membranes obtained from B[*a*]P-treated animals when the incubations were performed in the presence of NADPH. Also, the derivative B[*a*]P-*trans*-9,10-dihydrodiol was a potent inhibitor of DNA polymerase α under conditions where DNA polymerase β was less affected. These results suggest that nuclear B[*a*]P hydroxylase might be involved in the inhibition of DNA synthesis probably at the level of DNA polymerase α. As in the *in vivo* studies, the nuclear polymerase most affected by the hydrocarbon *in vitro* was DNA polymerase α.

Polynuclear aromatic hydrocarbons are environmental contaminants that have been shown to cause cancer in laboratory animals as well as inducing mutations and cell death in cultured animals cells [1, 2]. These substances are metabolically activated to a state of greater biological potency [2]. Their damaging effects result from the binding of the activated hydrocarbon to nuclear macromolecules. The most thoroughly studied target of these drugs is DNA although significant amounts of the activated substance can be found covalently linked to nuclear histones and non-histone proteins [3].

The carcinogen B[*a*]P‡ is metabolized by the microsomal aryl hydrocarbon hydroxylase system (AHH) (EC 1.14.14.2). The activity of this system is modulated by inducers as well as by hormonal and nutritional states [4]. The action of this mixed-function oxidase system leads to the activation of B[*a*]P to oxides, phenols, dihydrodiols, diolepoxides and quinones [1]. Although microsomes are thought to be the main site of B[*a*]P activation, the drug is also metabolized in the nucleus [5, 6]. It is tempting to think that the nuclear metabolic activity is responsible for the *in vivo* transformation of B[*a*]P which leads to the effects observed at the nuclear level.

DNA synthesis is inhibited after administration of hydrocarbons; this effect has been ascribed to the modification of nuclear DNA [7]. DNA synthesis is carried out by a very complex multienzymatic system including DNA polymerases (EC 2.7.7.7). Three types of DNA polymerases have been described in animal cells [8, 9]. DNA polymerase α, which seems to be responsible for the replication of the nuclear genome, has a high molecular weight and is strongly inhibited by *N*-ethylmaleimide and aphidicolin [10]. DNA polymerase β, the repair enzyme, has a low molecular weight, is inhibited by the analog dideoxyTTP (ddTTP), and is very resistant to *N*-ethylmaleimide [11]. DNA polymerase γ, the enzyme that replicates the mitochondrial DNA [12], is strongly inhibited by ddTTP and ethidium bromide [11, 13].

In this paper we have investigated the possibility that the inhibition of rat liver nuclear DNA synthesis by B[*a*]P, both *in vivo* and *in vitro*, is caused, at least in part, by the modification of nuclear proteins involved in nuclear DNA replication or repair. We have focused our attention on the effect of B[*a*]P on nuclear DNA polymerases. Moreover, we have studied the possible involvement of the nuclear membrane B[*a*]P hydroxylase activity in the inhibition of DNA synthesis by B[*a*]P.

MATERIALS AND METHODS

Calf thymus DNA (Sigma Chemical Co.) was activated as a DNA polymerase template with pancreatic deoxyribonuclease (EC 3.1.4.5) (Sigma) as

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‡ Abbreviations: B[*a*]P, benzo[*a*]pyrene; ddTTP, 2′,3′-dideoxy-thymidine-5′-triphosphate; PMSF, phenylmethylsulfonyl fluoride; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(2-(5-phenyloxazoyl))-benzene; DTE, dithioerythritol; and DNase, pancreatic deoxyribonuclease (EC 3.1.4.5).

described previously [14]. Synthetic polynucleotides were purchased from Sigma and Boehringer-France. The annealing of primers to templates was performed as previously described [13]. Nucleotides and deoxynucleotides were obtained from Sigma. DideoxyTTP was from Boehringer-France, ethidium bromide and *N*-ethylmaleimide were from Sigma, and aphidicolin was the gift of Dr. A. H. Todd of the Imperial Chemical Industries, England. Radioactive precursors were purchased from New England Nuclear, Germany. Labeled B[*a*]P was obtained from the Radiochemical Centre, Amersham. B[*a*]P derivatives were gifts of Dr. J. N. Keith, Chemical Repository, IIT Research Institute, Chicago, IL, U.S.A.

Animal treatment

Thirty five-day-old female rats of the Wistar strain (80–100 g) were used. B[*a*]P at a dose of 40 mg/kg body weight was injected intraperitoneally with corn oil. Control animals were injected with corn oil. Animals were killed 48 hr after injection.

Purification of rat liver nuclei

Animals (four rats for each group) were decapitated and exsanguinated. Livers were removed, pooled by groups (control or B[*a*]P-treated), weighed, dissected into small pieces, and washed in buffer A (250 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5, 3 mM $CaCl_2$ and 0.5 mM PMSF). This tissue was homogenized at 4° in 5 vol. of buffer A in a Potter homogenizer (glass–Teflon) driven by a low speed motor. The homogenate was centrifuged at 3000 *g* for 10 min. The supernatant fraction can be used for preparing mitochondria, microsomes and high speed supernatant. The pellet was resuspended in 50 ml of buffer B (2.3 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5, 3 mM $CaCl_2$ and 0.2 mM PMSF) and centrifuged at 39,000 rpm (142,000 *g*) at 4° for 40 min using an SW-50 rotor in a Spinco ultracentrifuge. The supernatant fraction was discarded and the nuclear pellet was resuspended in buffer A. Triton X-100 was added to a final concentration of 0.25% (v/v). The suspension was gently vortexed and centrifuged at 39,000 rpm as above. The final pellet, containing purified nuclei, was resuspended in buffer A containing 30% glycerol, and was stored at −80°. Purity was checked by direct observation in a phase contrast microscope. After several months of storage at −80°, the nuclei were morphologically intact. The DNA synthetic activity was stable after several months of storage under these conditions.

Nuclear DNA polymerase solubilization

Triton X-100 was added to the nuclear suspension to a final concentration of 0.5% (v/v), and the pH was equilibrated to pH 7.5 by adding potassium phosphate to a final concentration of 200 mM. The mixture was vortexed and then homogenized in a mini Potter homogenizer (0.4 ml glass–glass). The homogenate was frozen at −20°, thawed, and centrifuged for 2 min in a Beckman Microfuge. The supernatant fraction was dialyzed against a buffer containing 20 mM Tris–HCl, pH 8.0, 5 mM 2-mercaptoethanol, 1 mM EDTA, 20 mM KCl and 20% glycerol and kept at −80°.

DNA synthesis assays

Using intact nuclei, in a final volume of 0.1 ml, the incubation mixture contained: 25 mM Tris–HCl, pH 8.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 40 mM KCl, 10 mM $MgCl_2$, 3 mM ATP, 50 μM dATP, dCTP and dGTP, 5 μM [^{3}H]dTTP (sp. act. 500–1000 cpm/pmole), 25% glycerol and various amounts of nuclear suspension. The reaction was incubated at 37° with occasional stirring. The reaction was stopped at various times by the addition of 2 vol. of a cold solution of 10% trichloroacetic acid and 1% sodium pyrophosphate. The samples were kept on ice for 10 min and subsequently filtered through nitrocellulose membranes (Sartorius, Millipore or Schleicher and Schuell, 0.45 μm). These filters were washed with cold 2% trichloroacetic acid, dried, and counted in 3 ml of a scintillation mixture containing 0.4% PPO and 0.02% POPOP in toluene.

Using solubilized DNA polymerases, in a final volume of 0.05 ml, the incubation mixture contained: 50 mM Tris–HCl, pH 8.0, 2 mM DTE, 5 mM $MgCl_2$, 50 μM dCTP, dGTP and dATP each, 5 μM [^{3}H]-dTTP (sp. act. 500–1000 cpm/pmole), 10 μg bovine serum albumin, 4 μg activated calf thymus DNA, and various amounts of enzyme. Incubation conditions and radioactivity determination were carried out as in the case of intact nuclei.

The specific assays for DNA polymerases α and β were performed as previously described [13].

When B[*a*]P or B[*a*]P-*trans*-9,10-dihydrodiol were utilized in the *in vitro* assays for DNA synthesis or DNA polymerase activities, the hydrocarbons were added and the solvent was evaporated under N_2 stream until dryness prior to the addition of the reaction mixture.

Sucrose gradient centrifugation

Linear gradients between 5 and 20% sucrose (w/v) in a buffer containing 20 mM Tris–HCl, pH 8.0, 5 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mg/ml of bovine serum albumin and 200 mM KCl were done in 5-ml tubes in a SW-50 rotor in a Spinco ultracentrifuge or an AH-650 rotor with an OTD-65 Sorvall ultracentrifuge and centrifuged at 49,000 rpm (224,000 *g*) at 4° for 16 hr. About thirty-five fractions were collected, and DNA polymerase activity was determined in two tubes each. The linearity of sucrose gradient was monitored with a refractometer at 25°.

Extraction of nuclear DNA

Nuclear suspensions from control or B[*a*]P-treated animals were made 1% in sodium dodecyl sulfate and treated with 1 vol. of water-saturated phenol. The samples were vortexed for 5 min at room temperature and centrifuged at 5000 rpm for 5 min. The phenol layer was extracted once more with 1 vol. of a buffer containing 20 mM Tris–HCl, pH 7.2, 1 mM EDTA and 5 mM $MgCl_2$. The aqueous fractions were pooled, and DNA was precipitated with 2 vol. of 95% ethanol at −20° for 120 min. DNA was pelleted, resuspended in the same buffer as above, and incubated for 15 min at 37° with pancreatic ribonuclease which had been heated at 100° for 10 min (1 μg of ribonuclease per unit of A^{260}). After ribonuclease treatment, DNA was extracted with phenol

Table 1. Benzo[a]pyrene hydroxylase activity in liver nuclei and microsomes from normal and B[a]P-treated rats*

	[pmoles · B[a]P metabolized hr^{-1} · (mg protein)$^{-1}$]	
In vivo treatment	Microsomes	Nuclei
Control (6)	6,795 ± 374	345 ± 23
B[a]P (5)	26,345 ± 4,200	3,248 ± 122

* The hydrocarbon was injected in one single dose i.p. (40 mg/kg body wt) 48 hr before the animals were killed. Activity was determined as described by DePierre *et al.* [15]. Values are the mean ± S.D. in different subcellular preparations. The number of animal groups is indicated in brackets (four animals in each group).

and precipitated as above. DNA activation was performed as described before [14].

Preparation of microsomes

The supernatant fraction of 3000 *g* was centrifuged at 10,000 *g* for 10 min. Microsomes were obtained by the centrifugation of the 10,000 *g* supernatant fraction at 100,000 *g* for 60 min in an L2-65B Beckman Ultracentrifuge. The microsomal pellet was suspended in 0.1 M Tris–HCl, 5 mM EDTA at pH 7.5 for determination of B[a]P hydroxylase activity.

Determination of B[a]P hydroxylase activity

The method of DePierre *et al.* [15] using tritiated B[a]P was used.

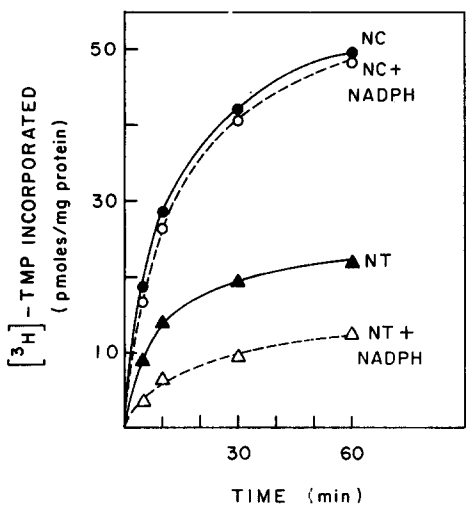

Fig. 1. Kinetics of DNA synthesis in intact nuclei purified from B[a]P-treated (NT) and control rats (NC) in the absence and presence of NADPH. Incubation conditions are described in Materials and Methods. NADPH concentration was 1.2 mg/ml. DNA values of 0.48 mg DNA/mg protein (NC) and 0.51 mg DNA/mg protein (NT) were obtained. As in Figs. 2 and 3, the data represent the mean of four experiments in four different nuclear preparations with duplicate determinations in each experiment. Standard deviation of the mean was always less than 10% of the values.

Preparation of nuclear membranes

Nuclear membranes were purified from nuclei of control and B[a]P-treated animals by the method of Kay *et al.* [16].

Miscellaneous

DNA concentration was determined by the diphenylamine method [17]; protein concentration was determined with Folin reagent [18].

RESULTS

Rat liver nuclear B[a]P hydroxylase activity

Table 1 shows that nuclei purified from livers of control animals had marked B[a]P hydroxylase activity. In animals treated with the drug, a strong stimulation of the hydroxylase activity was observed. The increase of B[a]P hydroxylase activity after hydrocarbon injection was higher in the nuclear membranes as compared with the microsomal fraction. The activity of the enzyme was completely dependent on the presence of NADPH (not shown).

In vivo *effect of B[a]P on DNA synthesis*

As seen in Fig. 1, purified nuclei from animals treated with B[a]P (NT) showed a decreased level of DNA synthesis as compared with nuclei from control animals (NC).

To study whether the nuclear B[a]P hydroxylase activity was related to the decreased ability of nuclei from B[a]P-injected rats to synthesize DNA, nuclei from control and treated animals were incubated under optimal conditions for DNA synthesis in the presence of NADPH, a cofactor required for hydroxylase activity. The same figure shows that DNA synthesis in control nuclei was barely affected by NADPH while the incorporation of TMP into DNA was diminished in nuclei from B[a]P-injected rats in the presence of NADPH, suggesting that the latter animals may still have some unmodified drug. The activation of this drug in the presence of NADPH may lead to the low level of DNA synthesis observed in the nuclei of rats treated with B[a]P.

In vitro *inhibition of DNA synthesis by B[a]P*

The effect of B[a]P on DNA synthesis in intact nuclei of control rats was studied in the presence or absence of NADPH. The drug alone caused a weak inhibition of TMP incorporation as seen in Fig. 2. In

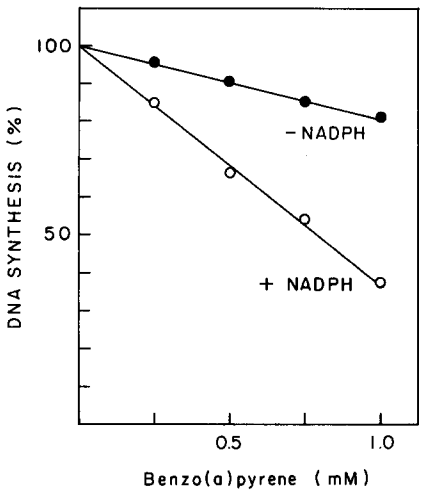

Fig. 2. *In vitro* effect of B[*a*]P on DNA synthesis in nuclei purified from control rats in the presence and absence of NADPH (1.2 mg/ml). The [^{3}H]TMP incorporation in the absence of B[*a*]P (100% activity) was 51 pmoles × (mg protein)$^{-1}$ × h^{-1}.

the presence of NADPH, a marked inhibition of DNA synthesis was observed. Similar results were obtained when the synthesis of DNA with nuclei from control animals was studied as a function of the preincubation time with B[*a*]P. The preincubation of nuclei with B[*a*]P in the presence of NADPH gave a higher inhibition of DNA synthesis than the nuclei preincubated without NADPH (not shown).

Effect of B[a]*P on DNA polymerase activity*

When DNA polymerase activity solubilized from control and treated rats was assayed with activated calf thymus DNA as template, the results shown in Fig. 3 were obtained. The activity from B[*a*]P-

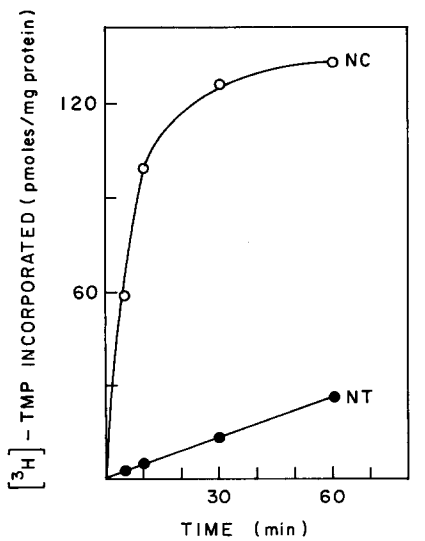

Fig. 3. DNA polymerase activity solubilized from nuclei of B[*a*]P-injected (NT) and control animals (NC). Activated calf thymus DNA was used as template.

Table 2. Nuclear DNA polymerase activity in the presence of microsomes and nuclear membranes of control and B[*a*]P-treated animals*

Conditions	Basal activity pmoles / mg protein	Basal activity % Activity	Activity plus NADPH pmoles / mg protein	Activity plus NADPH % Activity	Activity plus NADPH plus B[*a*]P pmoles / mg protein	Activity plus NADPH plus B[*a*]P % Activity
DNA polymerase alone	100	100	93	92	87	86
Plus nuclear membranes from control animals	118	100	99	84	77	65
Plus nuclear membranes from treated animals	115	100	63	54	51	44
Plus microsomes from control animals	118	100	97	82	87	74
Plus microsomes from treated animals	114	100	82	72	74	65

* DNA polymerase was solubilized from purified nuclei of control animals. The incubation was carried out at 37° for 60 min as described for the polymerase assay except that 10 μg of nuclear membrane or microsomes was added. The results represent the average of three separate experiments within 10% of the mean.

injected animals was lower than that released from nuclei of control animals.

Microsomes and nuclear membranes were prepared from the liver of B[a]P-treated and control rats. Nuclear DNA polymerases were solubilized from control animals as above, and enzyme activity was determined in the presence of microsomes and nuclear membranes of control and B[a]P-treated animals. We observed practically no inhibition of DNA polymerase activity in the presence of nuclear membranes from control rats, unless B[a] was present, while the inhibition with nuclear membranes from treated rats, even in the absence of B[a]P, was very marked (Table 2). As expected, a higher degree of inhibition was obtained when NADPH plus B[a]P were present in the incubation mixture. Similar results were obtained when microsomes from control and B[a]P-treated animals were used.

The experiment described in Table 3 indicates that the preincubation of DNA polymerase α (the enzyme corresponds to the pooled fractions 7–13 of Fig. 4A, extensively dialyzed against 50% glycerol in buffer Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, and 2 mM 2-mercaptoethanol) with DNA purified from nuclei of B[a]P-treated rats was not blocked by the modified template, since the enzyme was still active in a further incubation with poly dC-oligo dG. The same table shows that DNA from treated animals had a lower template capacity than DNA from control animals. Polymerase activity was 35% of control with native DNA but was 61% of control with activated DNA. Since these data suggested that B[a]P modified bases in DNA are being removed during the activation of DNA with DNase, we measured the radioactivity released during activation of calf thymus DNA modified by [^{14}C]B[a]P-7,8-dihydrodiol-9,10-oxide (isomer I). We found that 23.8% of the radioactivity was released from DNA under our experimental conditions (not shown).

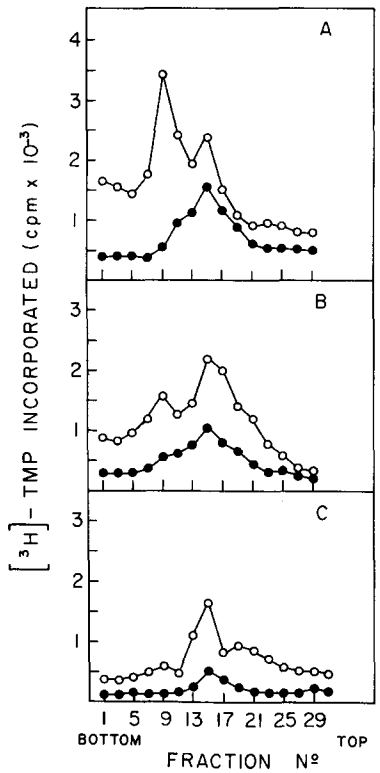

Fig. 4. Determination of DNA polymerase activity after separation by sucrose gradient centrifugation of DNA polymerases solubilized from nuclei from control animals (4A), nuclei from *in vivo* B[a]P-treated animals (4B), and nuclei from control animals incubated *in vitro* with 1 mM B[a]P and 1.2 mg/ml NADPH for 60 min at 37° (4C). Key: (○) DNA polymerase α assay, and (●) DNA polymerase β assay. Other conditions are described in Materials and Methods.

Characterization of the DNA polymerases inhibited by B[a]*P*

DNA polymerases were solubilized from purified nuclei of control and B[a]P-treated animals as described in Materials and Methods, and separated by centrifugation in a 5–20% sucrose gradient overnight. The DNA polymerase activity was assayed in

Table 3. DNA polymerase activity after preincubation with normal and B[a]P-modified DNA*

Template in the preincubation	dGMP incorporated (pmoles/30 min)		Difference
	Minus poly dC-dG	Plus poly dC-dG	
Native DNA (control animals)	2.6	9.6	7.0
Native DNA (B[a]P-treated animals)	0.9	7.7	6.8
Activated DNA (control animals)	13.5	21.0	7.5
Activated DNA (B[a]P-treated animals)	8.2	15.4	7.2

* Nuclear DNA was isolated from control (NC) and B[a]P-treated animals (NT) as described in Materials and Methods. The pooled fractions 7–13 from the high molecular weight peak of the sucrose gradient (in Fig. 4A) was used as the source of DNA polymerase α from control animals. The preincubation of the enzymatic fraction with native or activated DNA (5 μg) was performed in the presence of Tris–HCl, DTE, $MgCl_2$ and bovine serum albumin as the assay for DNA polymerase described in Materials and Methods. After 30 min at 37°, 1 μCi each of [^{3}H]dGTP (sp. act. 1578 cpm/pmoles), dCTP dATP and TTP at a final concentration of 50 μM were added and the incubation was carried out at 37° for 30 min in the absence or presence of poly dC-oligo dG_{12}. We have performed previous experiments to measure the degree of DNA modification. [^{3}H]B[a]P (2 μCi) was injected intraperitoneally. After 48 hr nuclei and DNA were isolated from rat liver as described in Materials and Methods. DNA was labeled at about 3500 cpm/μg (not shown).

Table 4. Effects of inhibitors on DNA polymerase activity solubilized from nuclei of control and B[*a*]P-treated animals*

Inhibitor	% Inhibition of DNA polymerase activity	
	Control	B[*a*]P-treated
Aphidicolin	54	24
Ethidium bromide	40	11
ddTTP	68	7
N-Ethylmaleimide	66	19

* Incubation was carried out at 37° for 60 min. The final amounts of inhibitors were 6 μg aphidicolin, 20 μM (ddTTP/TTP = 2) ddTTP, 10 μM ethidium bromide and 10 mM *N*-ethylmaleimide. DNA polymerase was preincubated with *N*-ethylmaleimide for 10 min at 4° prior to the incubation at 37°. When aphidicolin was used, dCTP concentration was lowered to 10 μM. Aphidicolin was dissolved in dimethyl sulfoxide. No effect of this solvent on DNA synthesis was observed. The data correspond to the average of three individual experiments which were within 12% of the mean. The activity in the absence of inhibitors (0% inhibition) was 90 pmoles × (mg protein)$^{-1}$ × hr^{-1} for control and 14.8 pmoles × (mg protein)$^{-1}$ × hr^{-1} for B[*a*]P-treated.

two different ways, one that is optimal but not specific for DNA polymerase α, and a second one that is specific for DNA polymerase β [13]. As seen in Fig. 4 DNA polymerase α was more affected by the *in vivo* (4B) and *in vitro* (4C) treatment with B[*a*]P than was DNA polymerase β. Although DNA polymerase γ was present at low levels when assayed in whole nuclei, this enzyme activity seemed less stable than the two other polymerases, since we were not able to detect DNA polymerase γ in gradients of control or treated rat liver nuclei as judged by the lack of activity with a poly rA-dT$_{12}$ template, in the region of the gradient where polymerase γ is supposed to migrate.

Another approach shown in Table 4 to determine which polymerase is more affected *in vivo* by treatment with B[*a*]P was to use specific inhibitors of the different DNA polymerases. Aphidicolin, a specific inhibitor of DNA polymerase α, more strongly inhibited DNA polymerase of nuclei from control animals than from drug-treated animals, suggesting that this enzyme has a lower activity in nuclei from drug-treated animals. A similar effect was observed with ethidium bromide. With *N*-ethylmaleimide the polymerase of treated rats showed a greater resistance to this reagent, indicating that the major activity present was DNA polymerase β which is highly resistant to this SH-reacting substance. Finally,

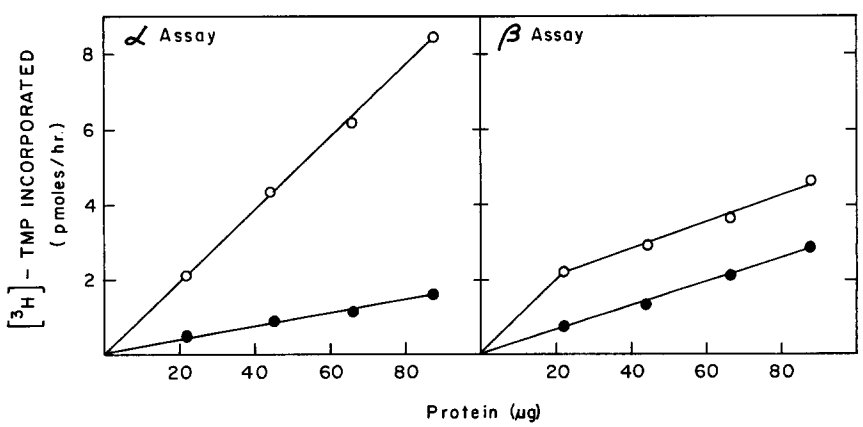

Fig. 5. *In vitro* effect of *trans*-9,10,-dihydroxy-9,10-dihydro-B[*a*]P on DNA polymerase activity. Nuclei from control animals were incubated in the absence and presence of 140 μM B[*a*]P-*trans*-9,10-dihydrodiol at 37° for 15 min. DNA polymerase was solubilized and assayed using the α and β assays. Key: (○) DNA polymerase activity solubilized from control nuclei incubated in the absence of B[*a*]P-metabolite, and (●) DNA polymerase activity solubilized from control nuclei incubated with 140 μM B[*a*]P-*trans*-9,10-dihydrodiol. Each point represents the mean of three separate experiments with duplicate determinations in each experiment. Standard deviation of the mean was less than 8% of the values.

ddTTP strongly inhibited DNA polymerase in control animals. This analog is a potent inhibitor of DNA polymerases β and γ.

Effect of B[a]*P-trans-9,10-dihydrodiol on DNA polymerase activity*

The B[*a*]P derivative *trans*-9,10-dihydroxy-9,10-dihydro-B[*a*]P was also assayed as an inhibitor of nuclear DNA polymerases solubilized from control rats. As seen in Fig. 5, this B[*a*]P metabolite was a strong inhibitor of DNA polymerase under the α-assay conditions (left panel) while the incubation in the presence of *N*-ethylmaleimide (β-assay conditions) showed a lower degree of inhibition (right panel).

DISCUSSION

It is usually accepted that the effects observed at the nuclear level, after the *in vivo* administration of B[*a*]P, are caused by the covalent linking of the activated hydrocarbon to the nuclear or mitochondrial DNA. Less attention has been paid to the fact that macromolecules other than nuclei acids can bind the activated forms of B[*a*]P. In this article, we described both the *in vivo* and *in vitro* effects of B[*a*]P on rat liver nuclear DNA synthesis. B[*a*]P is partially soluble in water but the presence of proteins greatly increases its solubility. The concentration of B[*a*]P used in our *in vitro* experiments (1 mM) is in good agreement with the recently reported K_m value for B[*a*]P hydroxylase (16 μM) [19].

Our results indicate that DNA synthesis is clearly diminished in rat liver nuclei from animals that have been injected with B[*a*]P 48 hr before being killed. This decrease in DNA synthesis can be explained, at least partially, by an effect of the drug metabolites on nuclear DNA polymerase activity, since in the presence of unmodified activated DNA the enzyme activity released from control nuclei is higher than that from the nuclei of treated animals.

The *in vivo* effect of B[*a*]P can also be explained by a role of the drug metabolites on the regulation of the DNA polymerase activity. A possibility that can be discarded is that the observed inhibition is mediated by the blocking of polymerase α when it is in contact with the modified residues of DNA since the preincubation of DNA polymerase with DNA purified from B[*a*]P-treated nuclei does not preclude the recognition of a normal template by the enzyme.

It may be pointed out that our results are in agreement with the idea that the B[*a*]P hydroxylase activity present in purified nuclei [devoid of cytoplasmic contamination as determined by light microscopy and the search of cytoplasmic specific enzymes (not shown)] might be involved in the inhibition of DNA synthesis by B[*a*]P. This is supported by the more pronounced inhibition of DNA synthesis observed in nuclei from drug-treated rats when the incubation was performed in the presence of NADPH, while this cofactor of hydroxylase activity had no effect on nuclei from control animals. These results suggest that nuclei from treated animals may have some residual, unmodified hydrocarbon. As seen in the *in vitro* experiments, the incubation of nuclei from control animals with B[*a*]P alone gave a small inhibition of DNA synthesis as compared with the incubation in the presence of the hydrocarbon plus NADPH. Thus, the residual unmodified B[*a*]P can be activated by nuclear aryl hydrocarbon hydroxylase and associated enzymes; the metabolites produced would lead to a lower level of TMP incorporation into DNA. DNA polymerase α purified through several chromatographic steps was unaffected by incubation with B[*a*]P (not shown). Moreover, solubilized DNA polymerase activity was strongly inhibited when incubated with nuclear membranes from B[*a*]P-treated rats, in the absence or presence of the hydrocarbon, while the inhibition in the presence of nuclear membranes from control animals was less dramatic. Microsomes of B[*a*]P-treated rats inhibited DNA polymerase activity more than those from control animals. It is clear from the data presented that the DNA polymerase activity is inhibited to a greater extent by the nuclear membranes as compared to microsomal membranes. This difference is not due to a higher activity of cytochrome P-450 reductase in the nuclear membranes of rats injected with B[*a*]P, since we have determined that the specific activity of this enzyme in nuclear membranes of treated animals is 80% lower than that of microsomes from the same animal. It is possible, however, that the nuclear membranes preferentially produce active metabolites and/or these metabolites are detoxified at a lower rate. This idea is supported by the studies of Lesko *et al.* [20], which clearly demonstrated that nuclear membranes produce a ratio B[*a*]P-quinones/B[*a*]P-phenols equal to 3.9, whereas this ratio is only 0.5 in microsomes. It has been suggested that the microsomal activation system is involved mainly in the process of drug detoxification whereas the nuclear hydroxylase activity may be related to the effect of polynuclear hydrocarbons on the expression of the nuclear genome [1]. Our results support this idea.

Part of our work was devoted to studying whether there is a specific inhibition of one of the cellular DNA polymerases. We found that DNA polymerase β was the most resistant activity in nuclei from B[*a*]P-injected animals. Similar results were obtained when nuclei from control rats were incubated with the drug. Different approaches were used to obtain these results. Both sucrose gradient centrifugation and the use of specific inhibitors indicated that DNA polymerase α is more affected than DNA polymerase β. Furthermore, we have checked the amount of DNA polymerases alpha and beta (using the appropriate differential assays) in crude nuclei and after Triton treatment and we have not seen a preferential release of one of these polymerases by the detergent, either in treated or control nuclei. For this reason we can discard the possibility that a preferential release from nuclei of B[*a*]P-treated rats resulted in an apparently lower activity of DNA polymerase α.

The importance of B[*a*]P metabolism on the interaction of polycyclic hydrocarbons with DNA polymerases is clearly demonstrated since the 9,10-dihydrodiol derivative of B[*a*]P was also a potent inhibitor of DNA polymerase α activity *in vitro*. It is well known that this B[*a*]P metabolite is found preferentially bound to nuclear proteins [3].

The results with the inhibitors aphidicolin, ethi-

dium bromide and *N*-ethylmaleimide support the idea that DNA polymerase α is more affected than β. However, the effect obtained with ddTTP is difficult to explain since this is a strong inhibitor of DNA polymerase β and the inhibition observed with the polymerase from treated animals was very low as compared with that of control rats. We can speculate that *in vivo* B[*a*]P treatment may affect DNA polymerase β sensitivity to ddTTP while not affecting the enzyme activity assayed *in vitro*.

Polymerase β is involved in DNA repair and the high level of this enzyme in B[*a*]P-treated animals may reflect a high resistance to the hydrocarbon or an increased biosynthetic rate. However, as the same effect has been observed *in vitro*, this would rule out an effect on the synthesis of the enzyme and favor the idea that DNA polymerase β is less affected than polymerase α. A possible explanation of this higher resistance may be related to the small size of polymerase β which would provide a smaller target to B[*a*]P or its metabolites.

We have also studied the behavior of mitochondrial DNA polymerase in normal and B[*a*]P-treated rats. As reported recently [21], the DNA synthetic ability of mitochondria from B[*a*]P-treated animals is diminished as compared with the organelles from control rats. However, the mitochondrial DNA polymerase activity in B[*a*]P-treated animals is clearly higher than the enzyme activity of control organelles. This difference with the nuclear system reported in this article can be related to the fact that only one DNA polymerase is found in mitochondria. This enzyme must be involved in both the replication and repair of the mitochondrial genome. These results may indicate that the inhibition of DNA synthesis in mitochondria, after *in vivo* administration of B[*a*]P, is not mediated by the inactivation of the mitochondrial DNA polymerase.

As the effect of B[*a*]P on DNA synthesis is transient [7], it remains to be established if the effect of this hydrocarbon on nuclear DNA polymerases, described in this article, is related to the carcinogenic activity of the drug. Currently we are investigating the behavior of purified rat liver DNA polymerases in the presence of DNA templates modified with B[*a*]P or its metabolites.

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