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The Effect of Epoxide Hydratase on Benzo[*a*]pyrene Diol Epoxide Hydrolysis and Binding to DNA and Mixed-Function Oxidase Proteins

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SUMMARY

GOZUKARA, E. M., G. BELVEDERE, R. C. ROBINSON, J. DEUTSCH, M. J. COON, F. P. GUENGERICH, AND H. V. GELBOIN. The effect of epoxide hydratase on benzo[*a*]-pyrene diol epoxide hydrolysis and binding to DNA and mixed-function oxidase proteins. *Mol. Pharmacol.* **19**:153–161 (1981).

A reconstituted purified mixed-function oxidase (MFO) system converts ^{3}H(−)*t*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene [^{3}H(−)*t*-7,8-dihydrodiol BP] to *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (diol epoxide I) and *r*-7,*t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (diol epoxide II). The addition of DNA to the incubation mixture results in covalent binding of metabolites to DNA. The binding of metabolites to DNA is a linear function of cytochrome P-450 LM_4 content. The addition of epoxide hydratase reduces the binding of metabolites to DNA by 35%–43%. Human monocytes, lymphocytes, and Fisher rat liver cells (TRL-2) also catalyze the binding of ^{3}H(−)*t*-7,8-dihydrodiol BP to added DNA. Cells that have been induced by benzanthracene (BA) exhibit greater levels of DNA binding. The addition of epoxide hydratase to the medium reduces the amount of reactive metabolites binding to DNA by 54% and 31% in control and induced monocytes, 45% and 26% in control and induced lymphocytes, and by 18%–28% in control and 13%–24% in BA-preinduced rat liver TRL-2 cells. Thus, with both purified reconstituted MFO systems or with whole cell systems of either human monocytes, lymphocytes, or rat cells, the addition of purified epoxide hydratase reduces the binding of ^{3}H(−)*t*-7,8-diol metabolites to DNA. These results indicate that epoxide hydratase functions in the hydrolytic inactivation of the diol epoxides. When heat-inactivated epoxide hydratase was used, no alteration in DNA binding was detected. The MFO system autocatalyzes the binding of ^{3}H(−)*t*-7,8-dihydrodiol BP to the two protein components of the MFO enzyme system. The metabolites are bound to cytochrome P-450 LM_4 to the greatest extent both with respect to specific activity and total binding and to a lesser extent to cytochrome *c* (P-450) reductase. DNA can compete for reactive metabolite since the addition of DNA considerably reduces the binding of metabolites to the protein components. Added bovine serum albumin is also covalently bound to metabolites but does not reduce the binding to either DNA or the purified proteins of the enzyme system. Added epoxide hydratase is also covalently bound and reduces the binding to the other proteins. Diol epoxide I and II hydrolysis was measured by high-pressure liquid chromatography of the four tetrol products. The addition of DNA reduced the formation of tetrols I-2 (7/8,8,10), II-1 (7,9/8,10), and II-2 (7,9,19/8) and had no effect on the major I-1 (7,10/8,9) tetrol, indicating a stereoselective effect of DNA on diol epoxide hydrolysis. The addition of epoxide hydratase reduced the formation of three of the four tetrols by 11%–44% and increased tetrol II-1 formation, indicating a hydratase-

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induced alteration in the ratio of hydrolysis products. The latter results and the inhibition of DNA binding by hydratase indicate that the hydratase has a stereospecific interaction with diol epoxides, binding them covalently, altering their mode of hydrolysis, and apparently reducing their activity as carcinogenic intermediates that bind to DNA. Thus, epoxide hydratase may play an important role in diol epoxide detoxification.

INTRODUCTION

It is widely accepted that many chemical carcinogens, including PAH,[3] are converted to detoxified metabolites or to ultimate carcinogens by microsomal MFO system(s) (1). BP is the most common PAH in our environment (2). Microsomal MFO system(s) metabolize benzo[*a*]pyrene to five phenols (3), three quinones, four dihydrodiols (3), two triols, four tetrols (4, 5), several simple epoxides, two diol epoxides, and water-soluble conjugates (3). Previous studies showed that epoxide hydratase added to cell-free MFO systems increased the formation of 4,5-, 7,8-, 9,10-, and 11,12-dihydrodiols and decreased the formation of 7-OH, 9-OH, 4,5-oxide, 6,12-, and 11,12-quinones (6). BP epoxides are generally very reactive metabolites and can covalently bind to cellular macromolecules (6). Epoxide hydratase catalyzes the conversion of simple epoxides to dihydrodiols. All of the dihydrodiols formed, except (−)*t*-7,8-dihydrodiol BP [(−)*t*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene], appear to be on detoxification pathways. The (−)*t*-7,8-dihydrodiol BP, however, is further epoxidized at the 9,10-position of the molecule by the P-450 containing microsomal MFO system(s) and results in the formation of *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [diol epoxide I], and *r*-7,*t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [diol epoxide II] (3). Diol epoxides I and II are the most mutagenic (7, 8) and carcinogenic (9–11) intermediates of BP. These reactive metabolites bind covalently to DNA *in vivo* (12), *in vitro* (6, 12–15), in tissue culture (16), in isolated rat liver, and lung nuclei (17). They also have been shown to bind to RNA (17, 18) and proteins (17–19). This covalent binding can lead to cell death, mutations or transformation to a cancer cell (7, 8). The binding of chemical carcinogens or their metabolically reactive products to DNA is believed to be an essential step in chemical carcinogen action. Since diol epoxides are believed to be the major reactive carcinogenic metabolites of BP, it is important to determine whether they are susceptible to detoxification by enzymatic mechanisms. In this study we have examined the role of epoxide hydratase in the hydrolysis of diol epoxides I and II and its effect on the binding of the diol epoxides to DNA and protein. Furthermore, we have found and studied an enzymatic autocatalytic binding of ^{3}H(−)*t*-7,8-dihydrodiol BP to components of the MFO system.

[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; MFO, mixed-function oxidase; BP, benzo[*a*]pyrene; (−)*t*-7,8-dihydrodiol BP, (−)*t*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; diol epoxide I, *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; diol epoxide II, *r*-7,*t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; in the latter two abbreviations, *r*-7 indicates that the substituent at position 7 is the reference, and *t* and *c* indicate that the substituents are *trans* and *cis*, respectively, to the reference substituent; PB, sodium phenobarbital; HPLC, high-pressure liquid chromatography; BA, benz(*a*)anthracene; MEM, minimal essential medium; HBSS, Hanks' balanced salt solution; FCS, fetal calf serum; BSA, bovine serum albumin; P-450 LM_4, liver microsomal cytochrome P-450; SDS, sodium dodecyl sulfate; AHH, aryl hydrocarbon hydroxylase; TRL-2 cells, Fisher rat liver cells; DMSO, dimethyl sulfoxide.

MATERIALS AND METHODS

Chemicals. (±)*t*-7,8-Dihydrodiol BP was synthesized on the National Cancer Institute Contract No. NO1.CP-33387. ^{3}H(−)*t*-7,8-dihydrodiol BP (specific activity 240 to 371 nCi/nmole) was prepared in our laboratory according to published procedures (20, 21). NADPH and NADH were obtained from Calbiochem, San Diego, Calif. Dilauroylglyceryl 3-phosphocholine was purchased from Serdary Research Laboratories, Ontario, Canada. Acrylamide *N,N'*-methylene-bis-acrylamide, ammonium persulfate, and Coomassie blue were obtained from Bio-Rad, Richmond, Calif. Fetal calf serum, HBSS, phytohemagglutinin, and pokeweed mitogen were obtained from Grand Island Biological Company, Grand Island, N. Y. Minimal essential medium was purchased from Microbiological Associates, Inc., Bethesda, Md. Calf thymus DNA and nicotinamide were obtained from Sigma Chemical Company, St. Louis, Mo.; dimethyl sulfoxide and 8-hydroxyquinoline were obtained from Fisher Scientific Company, Fair Town, N. J. *m*-Cresol was purchased from Aldrich Chemical Company, Milwaukee, Wis. Spectroquality glass-distilled methanol, ethyl acetate, tetrahydrofuran, and acetone were purchased from Burdick and Jackson Laboratories, Muskegon, Mich.

Highly purified cytochrome P-450 LM_4 (14.5 nmole/mg of protein) and NADPH-cytochrome *c* (P-450) reductase (13.1 moles of cytochrome *c* reduced/min/mg of protein) were isolated from PB-induced rabbit liver microsomes, as previously described (22). Epoxide hydratase was purified from PB-treated rat liver (Fraction A) and had a specific activity of 650 nmoles of styrene-7,8-oxide hydrated/min/mg of protein (23), as judged by estimation of protein content using the method of Lowry *et al.* (24).

Cell cultures. Human peripheral blood was supplied from the Regional Blood Program of the American Red Cross in Washington, D. C. The monocytes and lymphocytes were isolated and induced with BA according to previously published methods (25). Twenty-five milliliters MEM were added into 150 cm^2 plastic tissue culture flasks and 20% FCS and 50 μg/ml of gentamicin were included. Isolated monocyte and lymphocyte mixtures were suspended in 8 ml of MEM containing Eagle's salt solution and L-glutamine. One milliliter of cell suspension was added to each flask and cells were incubated at 37°C for 1.5 h in 5% CO_2-95% humidified air. Medium containing nonadherent lymphocytes was poured into one large flask. Adherent monocytes were washed once with MEM and twice with HBSS; finally, 20 ml of fresh complete

medium were added to each flask. The lymphocyte suspension was adjusted to 1.5×10^6 cells/ml with complete MEM containing 1% phytohemaglutinin and 1% pokeweed mitogen 24 h later, 0.1% DMSO was added to control cells, and 0.1% DMSO containing 2 μg/ml of BA were added to the other flasks to induce the cells. Lymphocytes were incubated for 48 hr and then induced with BA as above.

Epithelial-like TRL-2 cells (Fisher rat liver) were isolated from livers of 10-day-old Fisher (F-334) strain rats (NIH) by G. M. Williams and E. Weisburger at the National Cancer Institute (26). These cells were grown in monolayer culture in William's D medium, supplemented with 10% FCS and 50 μg/ml of gentamicin. Cells were allowed to grow in monolayer for 6 days. Cells were induced with BA for 24 hr before harvesting. The cells were collected by centrifugation and then twice washed with HBSS.

DNA binding assay with purified MFO system. All experiments were performed under yellow light. The standard reaction mixture contained the following in a final volume of 0.5 ml: 50 μmoles of Tris-HCl buffer (pH 8.5), 0.1 mg of NADPH, 0.15 nmole of P-450 LM_4, 0.3 nmole of NADPH-cytochrome *c* (P-450) reductase, 20 μg of dilauroylglyceryl 3-phosphocholine, 0.5 mg of calf thymus DNA, and 5 nmoles of ^{3}H(−)*t*-7,8-dihydrodiol BP. Where indicated, 50 μg of epoxide hydratase and 50 μg of BSA were included. Incubation was performed in air at 37° in a shaking water bath for 20 min. At the end of the incubation period the samples were placed on ice and extracted three times with 3.0 ml of ethyl acetate to remove unbound substrate and metabolites. The other treatments and DNA binding activity were determined as in the following section.

DNA binding assay with tissue culture cells. The reaction mixture contained the following in a final volume of 1 ml: 50 μmoles of Tris-HCl (pH 8.5), 0.2 mg of NADPH, 0.1 mg of NADH, 0.2 mg of nicotinamide, 0.5 mg of calf thymus DNA, 1–20 × 10^6 cells, and 10 nmoles of ^{3}H(−)*t*-7,8-dihydrodiol BP. Where indicated, 130 μg of epoxide hydratase and goat γ-globulin or inactivated enzyme were included. The reaction was started by the addition of 10 nmoles of ^{3}H(−)*t*-7,8-dihydrodiol BP. The incubation was performed in a shaking bath at 37° for 25 min and DNA binding activity was determined as described (27).

The reaction was terminated by the addition of 3 ml of ice-cold ethyl acetate. The mixture was extracted three times with 3 ml of ethyl acetate to remove unbound and unmetabolized substrates. The remaining aqueous phase was brought to 2 ml with water, and 2 ml of Kirby's phenol reagent (27) was added. The mixture was agitated and centrifuged at 2000 rpm for 20 min. The upper phase containing DNA and water was removed. One milliliter of chloroform was added, the mixture was centrifuged, and the upper phase was collected. First, 30 μl of 0.1 M $MgCl_2$, then 5 ml of cold ethanol were added to the upper phase and DNA was precipitated. The precipitate was redissolved in 250 μl of 0.2 M Tris-HCl (pH 8.5) and 50 μl of 0.1 M $MgCl_2$ and brought to about 0.7 ml by addition of water. The mixture was extracted three times with 2 ml of ethyl acetate. A 0.5-ml aliquot of the third extract of ethyl acetate was removed, and 5 ml of Aquasol were added and counted. The values obtained from these samples were considered as background. DNA was precipitated by the addition of 5 ml of cold ethanol and the precipitate was collected by centrifugation. The DNA was dissolved in 0.6 ml of 1 N $HClO_4$ (perchloric acid) and 0.6 ml of water and hydolyzed at 85° in a water bath for 30 min. A 0.5-ml aliquot was removed from this hydrolysate and 5 ml of Aquasol were added. The samples were counted in a Beckman LS 8100 liquid scintillation counter. For DNA determination, another 0.5 ml of solution was placed in test tubes and 2.5 ml of water were added. The DNA content was measured on a Gilford 2400 spectrophotometer by its absorption at 260 nm and using the calf thymus DNA as a standard.

Protein binding assay. Reaction mixtures were prepared as indicated in the DNA binding studies, except that only 0.1 mg of DNA was added. The mixture was incubated at 37° for 20 min. The reaction was terminated by the addition of 3 ml of cold ethyl acetate and the extraction was repeated three times to remove unbound metabolites and unmetabolized substrates. The extracts were pooled for HPLC analysis. The remaining aqueous phase was dried under nitrogen and precipitated proteins were dissolved in sample buffer containing SDS and β-mercaptoethanol, and this solution was boiled 3 min before application to the gel. Proteins were separated by SDS-polyacrylamide slab gel electrophoresis according to the method of Laemmli (28), using a 5%–15% gradient separating gel and a 3% stacking gel. NADPH-cytochrome *c* (P-450) reductase, BSA, cytochrome P-450 LM_4, and epoxide hydratase were identified after Coomassie blue staining. Gels were sliced in 1-mm thickness, dissolved in 0.3 ml of H_2O_2 at 65° for 1 or 2 hr, and 10 ml of Aquasol were added and counted in a Beckman LS 8100 liquid scintillation counter. For fluorographic determinations, gels were treated as previously described (29) and exposed to Kodak X-Omat R film at −80° for 25 days.

HPLC analysis. For tetrol analysis, HPLC was carried out on a Spectra physics Model 3500 liquid chromatograph by using a Dupont Zorbax octadecyltrimethoxysilane (ODS) 119 column (6.2 mm inner diameter × 0.25 m) (20, 21). Two milliliters of sample were taken from pooled ethyl acetate extract, and 100 μl of nonradioactive tetrol standard were added and the mixture was dried under nitrogen. The dried sample was dissolved in 50 μl of 10% tetrahydrofuran in 100% methanol. Tetrols were separated with a linear gradient from 60% methanol in water to 100% methanol at a sweep time of 50 min. Flow rate was 0.8 ml/min, the monitoring wavelength was 248 nm, 20- to 40-drop fractions were collected, 5 ml of Aquasol were added, and fractions were counted in a Beckman LS 8100 liquid scintillation counter.

RESULTS

Purified reconstituted MFO systems, human monocytes and lymphocytes, and rat TRL-2 cells convert ^{3}H(−)*t*-7,8-dihydrodiol BP to diol epoxide I and diol epoxide II. The latter are reactive metabolites that bind covalently to DNA and proteins. Table 1 shows the binding of ^{3}H(−)*t*-7,8-dihydrodiol BP to DNA catalyzed

TABLE 1

Effect of epoxide hydratase on P-450 LM_4-catalyzed DNA binding of $^3H(-)t$-7,8-dihydrodiol BP

Incubation mixtures are as described under Materials and Methods. The numbers are averages of four experiments.

Expt.	Additions to incubation mixture	DNA binding	% Control
		pmoles/min/nmoles P-450/mg DNA	
I	None	42	100
	Epoxide hydratase	24	57
II	None	42	100
	BSA	44	105
	Epoxide hydratase	26	62

by a purified reconstituted MFO system containing cytochrome P-450 LM_4, NADPH-cytochrome *c* reductase, dilauroylglyceryl 3-phosphocholine, and NADPH. In this system, 1 nmole of P-450 LM_4 catalyzes the binding of 42 pmoles of reactive metabolites per milligram DNA/min. The addition of epoxide hydratase to the incubation mixture reduces the binding of reactive metabolites to DNA by 38%–43%. When albumin (BSA) was used as a control protein, no alteration in DNA binding was observed. Thus, it appears that the binding of metabolites to DNA is specifically reduced by epoxide hydratase. DNA binding activity was found to be a function of cytochrome P-450 LM_4 content. Figure 1 shows the direct linear dependence of DNA binding with P-450 LM_4 concentration over a 4-fold range of enzyme. At each of the three concentrations of P-450 LM_4, the addition of epoxide hydratase reduced the level of DNA binding. This reduction was between 24% and 45%.

We have also found that cultured human monocytes, lymphocytes, and TRL-2 Fisher rat liver cells catalyze the binding of $^3H(-)t$-7,8-dihydrodiol BP to exogenous DNA added to the culture medium. Figure 2 shows the

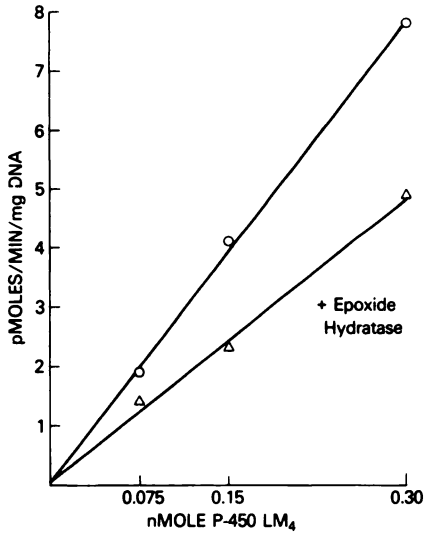

FIG. 1. *Effect of P-450 LM_4-catalyzed DNA binding of $^3H(-)t$-7,8-dihydrodiol BP*

A purified, reconstituted MFO system was used. DNA binding activity was determined in the absence (O——O) and presence (Δ——Δ) of 100 μg of epoxide hydratase.

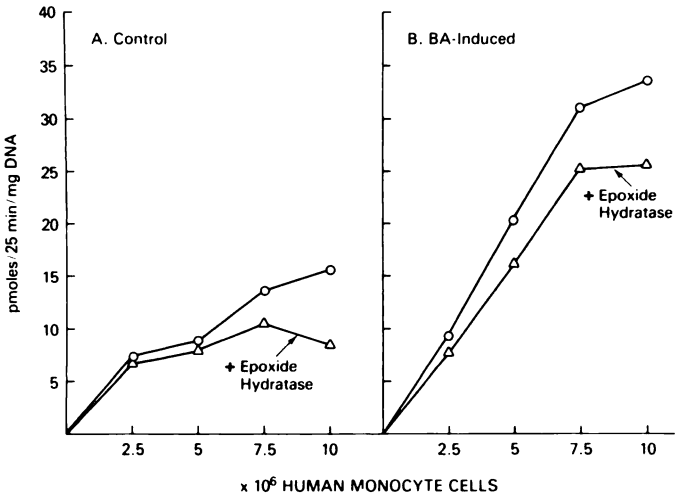

FIG. 2. *Effect of epoxide hydratase on human monocyte-catalyzed binding of $^3H(-)t$-7,8-dihydrodiol BP to DNA*

The reaction mixture was as described under Materials and Methods. Control and BA-induced human monocytes were measured in the absence (O——O) and presence (Δ——Δ) of 130 μg/ml of epoxide hydratase.

binding of $^3H(-)t$-7,8-dihydrodiol BP to exogenously added DNA catalyzed by various levels of control and BA-preinduced monocytes. Preinduced cells exhibit a linear relationship of binding with cell content up to 7.5 × 10^6, whereas control cells show a nonlinear increase with increasing cell concentration (Fig. 2A). The preinduced cells also exhibit a greater capacity to catalyze DNA binding than do the control cells.

The addition of epoxide hydratase at low cell concentration has only negligible effects on binding. However, at cell concentrations of 7.5 × 10^6 cells or greater, the hydratase causes a significant reduction of DNA binding ranging from 30% to 50%. Table 3 shows similar effects with lymphocytes. Preinduction of the MFO by incubating the BA increased the level of binding, and the addition of epoxide hydratase reduced the DNA binding by 45% in the control and 26% in the induced lymphocytes. When the epoxide hydratase was first inactivated by boiling, no reduction in binding was observed. TRL-2 cells also catalyzed the binding of $^3H(-)t$-7,8-dihydrodiol BP to DNA. Figure 3 shows that BA-pretreatment of TRL-2 cells increased the binding of metabolites to DNA. The addition of epoxide hydratase to the incubation inhibited DNA binding to 18%–28% in control and 13%–24% in BA-induced cells. Adding goat γ-globulin as a

TABLE 2

Induction of AHH activity by BA in cultured human monocytes, lymphocytes, and TRL-2 Fisher rat liver cells

Type of cell	AHH activity[a]		
	Control	BA-preinduced	BA/control
	pmoles/10^6 cells/30 min		
Monocytes	2.13	21.19	10.28
Lymphocytes	0.16	0.40	2.50
TRL-2 cells	16.30	138.00	8.46

[a] The numbers represent the formation of picomoles of product equivalent to 3-OH-BP produced per 10^6 cells/30 min.

TABLE 3

Effect of epoxide hydratase on lymphocyte-catalyzed binding of ^{3}H(−)t-7,8-Dihydrodiol BP to DNA

The standard reaction mixture is as described under Material and Methods.

Type of cells	Added to incubation mixture	Bound	% Control
		pmoles/20 × 10^6 cells 25 min/mg DNA	
Control	None	16.11	100
	Inactivated epoxide hydratase	16.91	105
	Epoxide hydratase	8.96	66
BA-preinduced Lymphocyte	None	20.68	100
	Inactivated epoxide hydratase	19.26	93
	Epoxide hydratase	17.48	84

control protein caused no significant alteration in DNA binding (Fig. 3A and B). Table 2 shows that the AHH activity was increased by treatment of cells with BA. These results are consistent with our previous report on human monocyte and lymphocyte catalyzed metabolism of benzo[*a*]pyrene as reflected in AHH activity and the metabolism of ^{3}H(−)*t*-7,8-dihydrodiol BP to diol epoxides. The preincubation of the cells with BA stimulates AHH activity to a much greater extent than diol metabolism, suggesting that the particular form of P-450 largely responsible for diol metabolism is different from the form active in BP metabolism. These results are also consistent with our studies on the stereoselectivity of purified P-450s (27). Table 2 indicates that the induction of each of these cell types with respect to AHH activity is much greater than the relative increase in diol metabolism resulting in DNA binding.

We have found that the metabolic products of ^{3}H(−)*t*-7,8-dihydrodiol BP bind covalently to the protein components of the purified reconstituted MFO system. Since

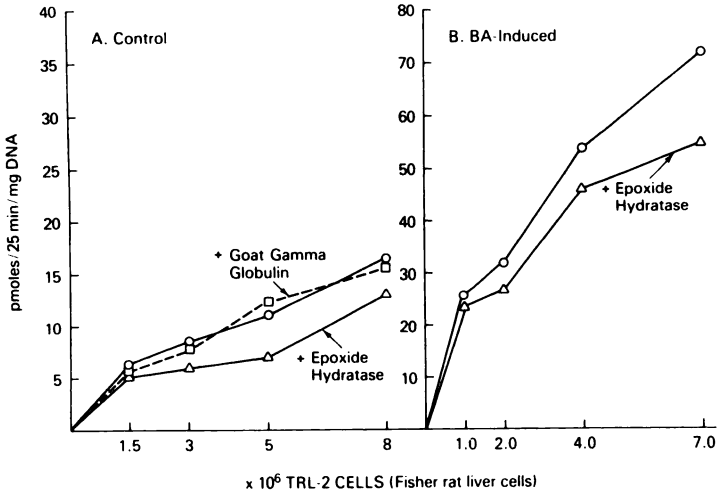

FIG. 3. *Effect of epoxide hydratase on TRL-2 Fisher rat liver cell-catalyzed binding of ^{3}H(−)t-7,8-dihydrodiol BP to DNA*

The reaction mixture was as described under Materials and Methods. Control and BA-induced TRL-2 cells in the absence (○——○) and presence of epoxide hydratase (△——△), and in the presence of goat γ-globulin (□——□).

the known product, diol epoxides I and II, are known to react with DNA and protein, it is likely that these are the protein-bound metabolites. The quantitative measurement of radioactivity showed that cytochrome P-450 LM_4 was bound at a 14%–39% higher specific activity and greater total binding than NADPH-cytochrome *c* (P-450) reductase. We have also found that the reactive metabolites bind covalently to epoxide hydratase. Table 4 shows that on a specific activity basis the cytochrome P-450 LM_4 bound reactive metabolite to a greater extent than the NADPH-cytochrome *c* (P-450) reductase. Values for total binding to the reductase are greater. This is due to more than 2½ times the concentration of reductase present in the incubation mixture in comparison with P-450. When hydratase was added to the reaction mixture, there was only a slight reduction in P-450 LM_4 binding (8% in specific activity), but a larger reduction in reductase binding (34% in specific activity). The P-450 protein is believed to be the terminal oxidase for the diol epoxide formation, and the amount of diol epoxide bound may be more related to the amount formed rather than to its disposition by the hydratase.

It is possible that the diol epoxide binding to the reductase requires diffusion of the diol epoxides from the P-450 and thus is more susceptible to inactivation by the added hydratase. The hydratase added is also covalently bound to the reactive metabolites. The addition of BSA results in a large amount of binding of the metabolite to the BSA, but does not reduce the binding to either the P-450 or reductase. The addition of hydratase in the presence of BSA reduces binding to the P-450, reductase, and the BSA. Thus it appears that the hydratase effectively reduces the binding of the reactive metabolites, presumably diol epoxides, to protein as well as to DNA. Table 5 shows that the addition of DNA to the incubation reduces the binding of reactive metabolites to all the protein components of the reaction mixture. This is true both in the presence and absence of hydratase.

The separation of proteins by SDS-polyacrylamide slab gel electrophoresis and autoradiography of the same gel also indicates that DNA is reducing the binding of metabolites to all proteins. This is shown in Fig. 4B. After incubation, a clear separation of reductase, BSA, P-450 LM_4, and epoxide hydratase was achieved by electrophoresis. These proteins were identified by staining with Coomassie blue (Fig. 4A). In this gel, track 1 is identical with 2, tracks 4 and 5 with 7, and track 6 with 8, except that 0.1 mg of DNA was added in the mixture of tracks 2, 7, and 8. The binding intensity to all proteins in all DNA-containing samples was considerably reduced by DNA and this is clearly seen in Fig. 4B. This autoradiographic illustration is in very good agreement with the quantitative measurement of radioactivity as shown in Table 5. These results indicate that DNA and proteins were competing for reactive metabolites, and DNA shows a similar affinity for the reactive metabolites.

When ^{3}H(−)*t*-7,8-dihydrodiol BP is incubated with the reconstituted MFO system, diol epoxides I and II are formed. Each of these hydrolyzes spontaneously to two distinct tetrols and are also converted to the other unknown products. Table 6 shows the effect of hydratase addition on these proteins. Tetrols I-1 (7,10/8,9), I-2 (7/

TABLE 4

Effect of epoxide hydratase on reconstituted MFO system catalyzed protein binding of ^{3}H(−)t-7,8-dihydrodiol BP

Standard incubation mixtures are as described under Materials and Methods. All values represent an average of five experiments.

Addition to standard incubation mixture	Protein covalent binding: specific activity							
	Reductase	% Control	P-450 LM_4	% Control	BSA	% Control	Epoxide hydratase	% Control
None	7.9	100	9.3	100				
Epoxide hydratase	5.2	66	8.6	92			4.9	100
BSA	8.4	106	9.8	105	13.4	100		
BSA + epoxide hydratase	5.2	66	10.1	108	8.3	62	4.6	94

Addition to standard incubation mixture	Protein covalent binding: total binding			
	Reductase (30 μg[a])	P-450 LM_4 (11.7 μg)	BSA (50 μg)	Epoxide hydratase (50 μg)
	pmoles/20 min			
None	4.74 ± 0.15	2.18 ± 0.60		
Epoxide hydratase	3.12 ± 0.86	2.02 ± 0.80		4.9 ± 1.4
BSA	5.04 ± 0.30	2.30 ± 0.26	13.4 ± 1.5	
BSA + epoxide hydratase	3.12 ± 0.70	2.37 ± 0.50	8.3 ± 2.6	4.6 ± 0.24

[a] Protein added to each reaction mixture.

8,9,10), and II-2 (79,10/8) are reduced 34%, 44%, and 11%, respectively, in the presence of the hydratase, and Tetrol II-1 (7,9/8,10) formation was increased by 23% by epoxide hydratase (Table 6). Thus, *trans* addition of —OH group to position C-10 in tetrol II-1 may be catalyzed by epoxide hydratase. Thus, diol epoxide I hydrolysis is inhibited by the hydratase with no change in the ratio of tetrol products. The hydrolysis of diol epoxide II, however, appears to be specifically altered, resulting in a slight change in the ratio of the products. This tetrol II-1 amount is increased by hydratase presence. The reduction of hydrolysis of the diol epoxide I to tetrols is likely due to covalent binding of the substrate to the hydrase. The separation profiles of tetrol products of diol epoxides are illustrated in Fig. 5.

The formation of tetrols is also largely affected by DNA. The addition of DNA to the reaction mixture reduced the formation of tetrols I-2 (7/8,9,10), II-1 (7,9/8,10), and II-2 (7,9,10/8) by 20%–58% and had no effect on the major I-1 (7,10/7,9) tetrol (Table 6). These results indicate that DNA has a stereoselective effect on the diol epoxide hydrolysis. Our experiments further revealed that DNA diol epoxide binding is also stereoselective.

TABLE 5

Effect of epoxide hydratase and added DNA on cytochrome P-450 LM_4-catalyzed protein binding of ^{3}H(−)t-7,8-dihydrodiol BP

Standard incubation mixtures are as described under Materials and Methods. All values represent an average of five experiments.

Addition to standard reaction mixture	Protein covalent binding: specific activity					
	Reductase	% Control	P-450 LM_4	% Control	Epoxide hydratase	% Control
None	7.9	100	9.3	100		
DNA	5.9	74	6.4	68		
Epoxide hydratase	5.2	65	8.6	92	4.9	100
DNA + epoxide hydratase	3.4	43	7.2	77	3.6	74

Addition to standard reaction mixture	Protein covalent binding: total binding		
	Reductase (30 μg[a])	P-450 LM_4 (11.7 μg)	Epoxide hydratase (50 μg)
None	4.74 ± 0.15	2.18 ± 0.60	
DNA	3.54 ± 0.70	1.5 ± 0.70	
Epoxide hydratase	3.12 ± 0.86	2.02 ± 0.80	4.9 ± 1.4
DNA + epoxide hydratase	2.04 ± 0.20	1.69 ± 0.30	3.6 ± 0.70

[a] Protein added to each reaction mixture.

DISCUSSION

In this study we have examined the role of epoxide hydratase on the disposition of reactive metabolites formed from ^{3}H(−)*t*-7,8-dihydrodiol BP by a purified MFO enzyme system, human monocytes, lymphocytes, or rat TRL-2 cells. This was measured by examining the binding of reactive metabolites to DNA and to purified P-450 LM_4, reductase, and the hydrolysis of the diol epoxides. The diol epoxide II was found to be more mutagenic in strains TA 98 and TA 100 of *Salmonella typhimurium* and in cultured Chinese hamster V79 cells than BP, 4,5-epoxide (30). However, the diol epoxide I was found to be more mutagenic in mammalian cells than diol epoxide II, 4,5-epoxide and 13 other BP derivatives tested, including all of the known BP metabolites (8). Diol epoxides are very unstable compounds in aqueous media and quickly hydrolyzed to a pair of tetrahydroxytetrahydrobenzo[*a*]pyrenes (tetrols) (3, 4, 5, 8, 20), and are nonenzymatically reduced to two triols by NADH or NADPH (4, 5). Thus, diol epoxides have very short half-lives in aqueous media, approximately 2.5 min for diol epoxide I and 30 sec for diol epoxide II (31). We therefore used ^{3}H(−)*t*-7,8-dihydrodiol BP as a substrate to generate diol epoxides I and II in the incubation medium. Thus, the mechanism and molecular interactions of the diol epoxides with DNA and proteins and also analysis of hydrolysis products could be examined. Diol epoxides I and II were generated either by purified MFO system or cultured human monocytes, lymphocytes, and rat TRL-2 cells. The binding of these extremely reactive metabolites to DNA and cytochrome P-450 LM_4, NADPH-cytochrome *c* (P-450) reductase, BSA, epoxide hydratase, and the hydrolysis products of diol epoxides was investigated.

As shown in Table 1 and Fig. 1, the binding of reactive metabolites to DNA was dependent on P-450 LM_4 con-

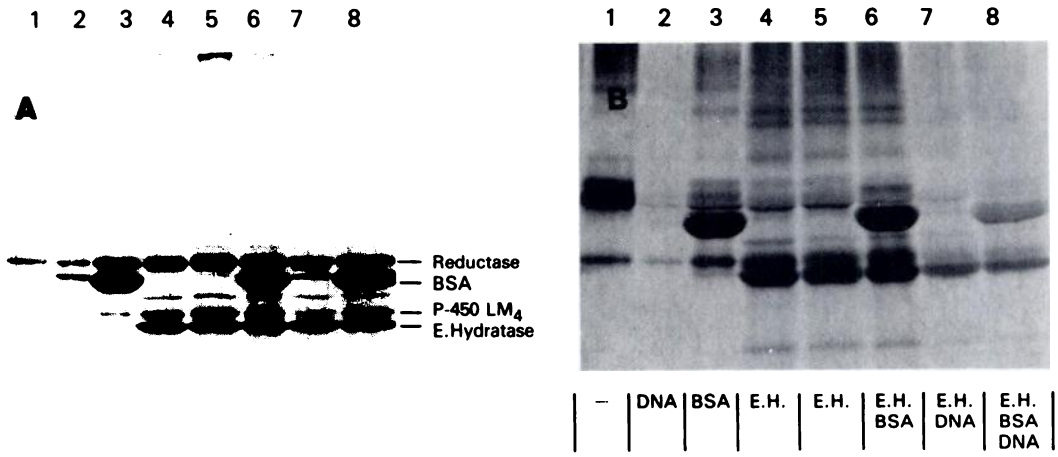

FIG. 4. *Binding of ^{3}H(−)t-7,8-dihydrodiol BP metabolites to proteins in the reconstituted MFO enzyme system, containing P-450 LM$_4$, NADPH-cytochrome c (P-450) reductase, and dilauroylglyceryl 3-phosphocholine. Where indicated, epoxide hydratase, DNA, and BSA were included*

A. After a 20-min incubation and three ethyl acetate extractions, the remaining aqueous phase was dried under nitrogen and the resulting precipitate was submitted to SDS-polyacrylamide slab gel electrophoresis.

B. Autoradiographic pattern of the same gel. Track 1 is identical with Track 2, Tracks 4 and 5 are identical with Track 7, and Track 6 is identical with Track 8 except that only 0.1 mg of DNA was added to the incubation mixtures of Tracks 2, 7, and 8.

centration and inhibited by epoxide hydratase. Our results showed that human monocytes, lymphocytes, and TRL-2 cells catalyze the binding of ^{3}H(−)5-7,8-dihydrodiol BP to DNA. When BA-preinduced cells were used, binding of reactive metabolites to DNA was increased in monocytes, lymphocytes and in TRL-2 cells. AHH was found to be present and inducible by PAH in human blood monocytes, lymphocytes, and rat liver cells (32). In the present study we found that, when BA-induced cultured cells were added to the incubation mixture, DNA binding activity was also increased, but to a much lesser extent than AHH activity. This suggests that different forms of P-450 are responsible for AHH activity and DNA binding. Diol epoxide-DNA binding (12–16, 25) and induced microsome-mediated DNA binding (17, 19) have also been shown by others. This binding to DNA can lead to heritable cellular damage, most directly if the target is DNA. If such DNA damage is not properly repaired before the cell division, it may lead to cell death, mutation, or transformation to a cancer cell (33). It has also been shown that the carcinogenicity of a number of polycyclic hydrocarbons to mouse skin was correlated with covalent binding to skin DNA (34). The addition of epoxide hydratase to the incubation mixture reduces binding of metabolites to DNA by 13%–54% under different experimental conditions. When albumin (BSA), goat γ-globulin, or inactivated epoxide hydratase was used as control, no alteration in DNA binding was detected. This

TABLE 6

Effect of epoxide hydratase and DNA on P-450-catalyzed metabolism of ^{3}H(−)t-7,8-dihydrodiol BP to tetrols (via diol epoxides)

Incubation mixtures are as described under Materials and Methods. The values obtained without added epoxide hydratase were set at 100% control. All numbers represent an average of four experiments.

Metabolite	Tetrols Formed after addition to incubation mixture				
	None	Epoxide hydratase	% Control	DNA	% Control
			pmoles/20 min		
I-1 (7, 10/8, 9)	220	144	66	220	100
I-2 (7/8, 9, 10)	71	39	56	29	42
II-1 (7, 9/8, 10)	27	36	123	13	50
II-2 (7, 9, 10/8)	19	17	89	15	80
Aqueous phase	102	35		49	
Unknown	670	599		439	
Total	1107	866		766	

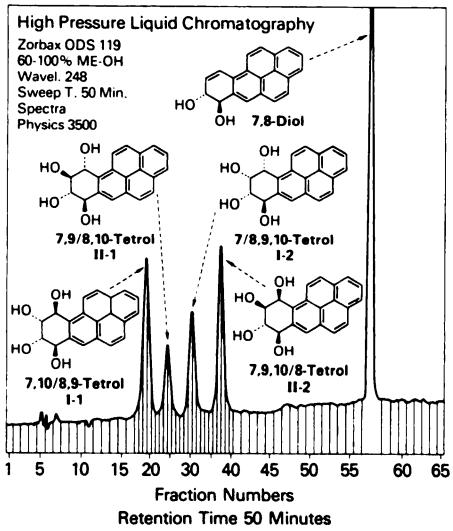

FIG. 5. *HPLC separation of tetrol hydrolysis products of diol epoxides*

Samples were treated and prepared as described under Materials and Methods. Tetrol I-1 (7,10/8,9) indicates that 8—OH and 9—OH are *trans* and 10—OH is *cis* to the 7—OH group, respectively. Tetrol I-2 (7/8,9,10) indicates that 8—OH, 9—OH, and 10—OH are in *trans* position to the 7—OH group; tetrol II-1 (7,9/8,10) indicates that 8—OH and 10—OH are *trans* and 9—OH is *cis* to 7—OH group; tetrol II-2 (7,9,10/8) indicates that 8—OH is *trans* and 9—OH and 10—OH are *cis* to 7—OH group.

suggests that the inhibition of DNA binding is specific to epoxide hydratase, and suggests a role for epoxide hydratase in the deactivation of diol epoxides. The mutagenic activity of the highly reactive diol epoxides was shown in histidine-deficient *S. typhimurium* tests (9, 35), in mammalian cells (9, 11, 36), and their tumorigenic activity has been demonstrated in mouse skin (9, 10). A previous study showed that the addition of 32 units of epoxide hydratase to *S. typhimurium* TA 1537 strain media reduced mutagenic activity of BP to background level in microsomal preparations. When 2.3 μmoles of 1,1,1-trichloropropene 2,3-oxide (an epoxide hydratase inhibitor) was added to microsomal preparations, the mutagenic activity of BP was increased 100% with respect to control (37). It has also been shown that the addition of 75 units of epoxide hydratase to *S. typhimurium* TA 98 strain media reduced mutagenic frequency of BP 9,10- and 7,8-oxide to 75%–90%, respectively. *S. typhimurium* TA 1537 and TA 98 strains detect frameshift mutations (38). In all of the latter studies, however, the hydratase effect is due to inactivation of the simple epoxides which may be mutagenic. In a previous report the addition of large amounts (300 units) of epoxide hydratase to TA 98 strain media lowered the mutagenic activity of diol epoxides I and II by only 7% and 28%, respectively (35). This relatively small effect may relate to the high reactivity of the diol epoxides when added as such to the medium. It is possible that diol epoxides generated *in situ*, as in the experiments we described are sufficiently stabilized by binding to cell or enzyme components to make them more accessible to hydratase action.

Protein binding studies showed that cytochrome P-450 LM_4 was bound at a higher specific activity and greater total binding than NADPH-cytochrome *c* (P-450) reductase. Previous studies show that the interaction appears to be highly selective, and the diol epoxides bind predominantly to P-450 LM_4 rather than P-450 LM_2 (39). The present report shows that the reactive metabolites also bind covalently to epoxide hydratase. The addition of epoxide hydratase to the incubation mixture reduces the covalent binding to all purified proteins. BSA added as a control was covalently bound to reactive metabolites, but did not reduce the binding of metabolites to other proteins. The present studies show that diol epoxides formed from $^3H(-)t$-7,8-dihydrodiol BP react directly with enzymes such as P-450 LM_4, reductase, and epoxide hydratase. The addition of DNA to the incubation mixture considerably reduced the binding of metabolites to all proteins (27, 39). These results indicate that DNA and proteins were competing for the same reactive metabolites.

The DNA and protein binding was inhibited by epoxide hydratase. In order to understand the mechanism of inhibition of DNA and protein binding by epoxide hydratase, the diol epoxide I and II hydrolysis products were analyzed by HPLC (Fig. 5). The addition of epoxide hydratase to the incubation mixture reduced the formation of tetrols I-1, I-2, and II-2 by 34%, 44%, and 11%, respectively. Tetrol II-1 (7,9/8,10) formation was increased by 23% by epoxide hydratase. Previously our laboratory and others reported that epoxide hydratase catalyzes the *trans* addition of labeled water to position C-8 when BP 7,8-oxide was used as a substrate (9, 21). The diol epoxides were reported to be poor substrates for epoxide hydratase (9, 40, 41). However, these experiments did not rule out the possibility that diol epoxides generated *in situ* may be used as substrates by epoxide hydratase. In this report a slight change in tetrol products was observed, and a 23% increase in *trans* addition of —OH group to position C-10 in tetrol II-1 may be catalyzed by epoxide hydratase. It has also been shown that diol epoxide II (1) was converted to *trans*-1 (tetrol II-1) by the P-448-containing MFO system, and this conversion was increased by 68 units of pure epoxide hydratase (41). We have used highly purified epoxide hydratase A from rat liver. Recent studies have shown that epoxide hydratase also has multiple forms (23). This result may be related to one of the different forms and different stereospecific activities of epoxide hydratase. Our result is in good agreement with the conclusion of a study indicating that the epoxide hydratase can have both very high positional effect and stereospecificity toward arene oxides of difference polycyclic aromatic hydrocarbons (9).

The addition of DNA to the incubation mixture reduced the formation of tetrols I-2, II-1, and II-2 by 20%–58% and had no effect on the major I-1 tetrol, indicating an effect of DNA on the stereoselectivity of diol epoxide hydrolysis. Thus, the DNA-diol epoxide interaction is likely to be highly stereoselective. The binding of diol epoxides I and II to quanine, adenine, and, to a lesser extent, cytosine bases of DNA has been identified by others (13–15, 18). It has also been shown that diol epoxides are attached from position C-10 to the 2-amino group of guanine, and this addition was found to be *trans* rather than *cis* (13, 14). In addition, two minor adducts were found which could represent (+) and (−)BP diol epoxide reacting by the *cis* addition of guanine base (15, 18). It may that the hydratase plays an important role *in vivo* where the diol epoxide may be more susceptible to hydratase action. The inhibition of DNA and protein binding by epoxide hydratase indicates that the hydratase has some stereospecific interaction with diol epoxides, binding them covalently, altering their mode of hydrolysis, and thus may reduce their activity as carcinogenic intermediates. These results indicate that epoxide hydratase may play an important role in diol epoxide detoxification.

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