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Carcinogen-Binding Proteins

High-Affinity Binding Sites for Benzo[a]pyrene in Mouse Liver Distinct from the *Ah* Receptor

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SUMMARY

Mouse liver cytosol contains saturable, high-affinity binding sites for the aromatic carcinogen benzo[*a*]pyrene that are distinct from the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-binding aryl hydrocarbon receptor. Specific binding parameters determined by an equilibrium binding assay indicate a dissociation constant of 7.7 nM and a binding capacity of 4.7 pmol of benzo[*a*]pyrene per milligram of cytosolic protein. Although the data best fit a single class of binding sites by Scatchard and Hill analyses, discrete 4 S and 9 S [^{3}H]benzo[*a*]pyrene peaks are identified on sucrose density gradients comprising about 98% and 2% of the specific binding, respectively. Steroid hormones and other ligands for previously described binding proteins have no effect on the specific carcinogen binding when present in the cytosol incubation at saturating levels. The glutathione *S*-transferases, shown in rat and human liver to possess carcinogen-binding properties, were also found not to be responsible for this receptor-like benzo[*a*]pyrene binding in mouse liver cytosol. Competition binding studies indicate that other aromatic hydrocarbon compounds of a structure similar to that of benzo[*a*]pyrene are equipotent in affinity for this major carcinogen-binding site.

INTRODUCTION

Carcinogen-binding proteins can be broadly classified into two major categories: (a) those that covalently bind carcinogen metabolites after *in vivo* treatment and (b) those that participate in a reversible, noncovalent binding. Pretreatment of rats and mice with chemical carcinogens that are metabolized to electrophilic products has led to the isolation of such covalently modified species as the "*h*-proteins" (1–3). Their abundance relative to other protein adducts produced was substantial. While *in vivo* covalent modification is generally considered a deleterious consequence, it has been suggested that some specific proteins may serve as scavenging agents, thereby affording protection (4). In this regard, the glutathione *S*-transferases, isolated from rat liver, have been shown to be one major protein alkylated *in vivo* (5, 6), and for which a protective role has been considered (4).

Carcinogen-binding proteins from rodent liver and cell culture extracts have also recently been described that bind the unmetabolized carcinogens noncovalently and with high affinity (7–13). Novel intracellular transport systems for these very hydrophobic substances have been proposed to explain some of these findings (12, 13). An increasing amount of evidence also points to a receptor role for certain of these proteins where regulation of the cytochrome P-450 enzyme system and other enzymes crucial to the metabolism of xenobiotics is effected. The cytosolic TCDD[2]-binding protein, or *Ah* receptor, is probably the best understood of these receptor-like proteins. One function proposed for the *Ah* receptor is the induction of microsomal cytochrome P-450 and aryl hydrocarbon hydroxylase activity by a mechanism analogous to the steroid hormone receptor model. Evidence in support of this model derives from the following: (a) the presence of high-affinity TCDD binding in liver cytosol (7) and in nuclei (14, 15), (b) a time-dependent decrease

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[2] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *Ah*, aryl hydrocarbon; B[*a*]P, benzo[*a*]pyrene; B6, C57Bl/6J mice; BNF, β-naphthoflavone or 5,6-benzoflavone; BSA, bovine serum albumin; DB[*a,c*]A, dibenzo[*a,c*]anthracene or 1,2:3,4-dibenzanthracene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEDG buffer, buffer composed of 25 mM Hepes, 5 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol at pH 7.6; 3-MC, 3-methylcholanthrene; PCN, pregnenolone-16α-carbonitrile; SDS, sodium dodecyl sulfate.

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and reappearance of cytosolic *Ah* receptor levels in pretreated animals (14, 15), (c) a correspondence between the presence of nuclear *Ah* receptor and the induction of new cytochrome P-450 mRNA synthesis in a dose-dependent manner (16), and (d) a correlation of receptor affinity for TCDD and congeners and potency of P-450 induction (7, 17).

Noncovalent interactions also include the reported binding by rat and human glutathione *S*-transferases of an exceptionally diverse collection of drugs, metabolic products, and some carcinogens in the low micromolar range (18), at both substrate and nonsubstrate binding sites. Although this binding property has not been investigated in the mouse, these enzymes are present in mouse liver (19).

Our purpose in this study was to characterize the binding of the aromatic hydrocarbon B[*a*]P to cytosolic proteins of the B6 mouse liver. Certain properties of this binding resemble those of biological receptors. We describe these novel properties, and examine whether previously described binding proteins may be responsible for all or part of the observed carcinogen binding.

MATERIALS AND METHODS

Animals. Male B6 mice aged 6–8 weeks were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice, fed Charles River mouse chow and given distilled water ad libitum, were housed at 22°, 50% relative humidity, in a fluorescent light-controlled room (lights on from 0600 to 1800 hr). Before use in experiments, mice were acclimated for at least 3 days after arrival and then killed by decapitation at age 8–12 weeks.

Chemicals. B[*a*]P, BNF, glutathione (reduced), and dithiothreitol were obtained from Aldrich Chemical Company (Milwaukee, Wisc.). TCDD was purchased from KOR Isotopes (Cambridge, Mass.). Cortisol, corticosterone, dexamethasone, progesterone, 17β-estradiol, dihydrotestosterone, 3-MC, DB[*a*,*c*]A (all of highest purity available), dextran, Hepes, Coomassie Blue G250, and epoxy-activated Sepharose 6B were obtained from Sigma Chemical Company (St. Louis, Mo.). 1-Chloro-2,4-dinitrobenzene was from Eastman Organics (Rochester, N. Y.). Chrysene was a gift from Dr. Howard Liber, Massachusetts Institute of Technology (Cambridge, Mass.). PCN was a gift from Upjohn Company (Kalamazoo, Mich.). Norit-A charcoal was obtained from Fisher Scientific (Pittsburgh, Pa.). Glycerol was purchased from Mallinckrodt (St. Louis, Mo.). EDTA, disodium salt, was bought from Baker Chemical Company (Phillipsburg, N. J.). Bio-Sil A (325 mesh) and SDS-polyacrylamide gel molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, Calif.) (range 10,000–100,000). The following chemicals, of electrophoretic grade purity, were purchased from Bio-Rad Laboratories: acrylamide, *N*,*N*′-bisacrylamide, SDS, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, bromophenol blue, 2-mercaptoethanol, ammonium persulfate, dithiothreitol, and Coomassie Brilliant Blue R250. Tris, ultrapure glycine, and ultrapure sucrose were obtained from Bio-Rad Laboratories or BRL (Rockville, Md.).

The purity of B[*a*]P was determined by thin-layer chromatography in benzene as described for [^{3}H]B[*a*]P, and its concentration was determined by UV absorption at 296 nm. Cortisol and corticosterone were determined pure by thin-layer chromatography on Analtech silica gel GF plates (250 μm) developed in ethyl acetate:hexane:acetic acid:ethanol (144:27:20:9) and their concentrations based on weight. The following compounds were used without further purification: chrysene (>95%), TCDD (99.8%), BNF, DB[*a*,*c*]A, 3-MC, PCN, dexamethasone, progesterone, 17β-estradiol, and dihydrotestosterone. The UV spectra of DB[*a*,*c*]A, 3-MC, and BNF were obtained and their concentrations determined from extinction coefficients at two different wavelengths given by *CRC Handbook of Chemistry and Physics* (52nd ed.). Chrysene concentration was based on weight.

Radiochemicals. [1,3,6-^{3}H]B[*a*]P (specific activity 78.9 Ci/mmol) was obtained from New England Nuclear Corporation (Boston, Mass.) and was repurified in two steps before use in any experiment. First, an aliquot was applied to an Analtech silica gel G thin-layer plate (250 μm) and developed in benzene alongside authentic B[*a*]P. The major spot chromatographing as B[*a*]P was then scraped from the plate and the silica gel was applied to a 2- to 3-ml Bio-Sil column in benzene. Fractions (3-min) were collected, and those containing the peak [^{3}H]B[*a*]P (typically two fractions) were saved. Benzene was evaporated, and the [^{3}H]B[*a*]P was dissolved in *p*-dioxane to achieve the desired concentration for experiments as determined by liquid scintillation counting of 1-μl aliquots. The ligand was stored at −80° in benzene immediately after repurification, since it was found that storage of [^{3}H]B[*a*]P in *p*-dioxane led to some decomposition.

[1,6-^{3}H]TCDD was a generous gift from Dr. William Greenlee, Chemical Industry Institute of Toxicology (Research Triangle Park, N. C.). [*methyl*-^{14}C]Methylated ovalbumin (M_r = 46,000) and [*methyl*-^{14}C]methylated-BSA (M_r = 69,000) were obtained from New England Nuclear Corporation (Boston, Mass.).

Preparation of mouse liver cytosol. Male B6 mice were decapitated between 8 and 12 weeks of age. Livers were perfused with ice-cold 0.9% NaCl solution via the hepatic portal vein until blanched. Excised livers were minced finely, suspended in three volumes (w/v) of HEDG buffer (25 mM Hepes/5 mM EDTA/1 mM dithiothreitol/10% glycerol, pH 7.6) and homogenized with a glass-Teflon homogenizer on ice. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4°. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4°. This supernatant was removed without disturbing the top lipid layer or the microsomal pellet. This cytosol was used immediately or frozen under liquid N_2 and stored at that temperature; no changes were found in the binding parameters measured over a 6-month period. Protein concentrations were assayed by the method of Bradford (20) with crystalline BSA as the standard.

Standard binding assay. Our assay was based on the methods of Poland *et al.* (7) and Okey *et al.* (8). Radiolabeled or unlabeled ligands were prepared as described above or in the figure legends. Solvents used for compounds in the assay were *p*-dioxane (2% maximum), ethanol (0.8% maximum), or deionized and distilled water. The concentrations of these solvents were found not to disturb binding parameters relative to controls. All tubes and pipettes were siliconized before use, which reduced, but did not completely eliminate, ligand loss due to adsorption on the walls of the vessel.

In the assay, HEDG buffer (10–20 μl) and radiolabeled and/or unlabeled ligands were added to siliconized tubes (12 × 75 mm). Next, 0.5 ml of B6 liver cytosol in HEDG buffer was added to yield a final concentration of 1.0 mg/ml, which was determined to be optimal for this assay. Tubes were vortexed and placed at 20° for 40–60 min, protected from light; this time was found necessary to achieve equilibrium for binding. Before separation of bound from free ligand, a suspension of dextran-coated charcoal (0.5 ml; charcoal 30 mg/ml, dextran 3 mg/ml) in HEDG buffer was added to 1.5-ml Eppendorf tubes and centrifuged in a Fisher Microfuge at 13,000 × *g* for 2 min. The supernatant buffer was decanted and the pellets were kept at 4° until use. After the ligand-binding incubation, samples were placed on ice for 5 min, and duplicate or triplicate 25-μl aliquots were taken for determination of total free [^{3}H]B[*a*]P. Bound [^{3}H]B[*a*]P was separated from free by adding the remaining sample to the tubes with charcoal pellets, vortexing quickly to resuspend the charcoal, incubating at 4° for 5 min, and centrifuging at 13,000 × *g* for 2 min at 4°. Aliquots were taken for determination of total bound, or for layering onto sucrose density gradients.

Sucrose density gradients and fractionation. Samples of incubated cytosol (250 or 300 μl) were layered onto 5%–20% linear sucrose density gradients in HEDG buffer. [*methyl*-^{14}C]BSA or [*methyl*-^{14}C]ovalbumin was used as sedimentation-marker proteins. Gradients were run in either a Beckman SW 50.1 Ti rotor at 49,000 rpm for 16 hr or a

Beckman SW 56 Ti rotor at 49,000 rpm for 19 hr. Gradients were fractionated using an ISCO Model 185 gradient fractionator, and 150- or 200-μl fractions were collected for liquid scintillation counting. Gradient tube bottoms were cut off after fractionation and counted for any precipitable radioactivity. Samples were counted in a Beckman 3100 liquid scintillation counter with 36% counting efficiency for tritium.

Purification of glutathione S-transferases from B6 liver. Glutathione *S*-transferases were isolated from B6 mouse liver cytosol using a GSH affinity column by the procedure of Simons and Vander Jagt (21). The column (1 × 19 cm) was equilibrated with 22 mM potassium phosphate buffer (pH 7.0). B6 liver cytosol was freshly prepared, and an aliquot was saved for protein determination, GSH transferase activity, and SDS-gel electrophoresis. Cytosolic protein (145 mg) was applied to the column at a flow rate of 0.2 ml/min. Protein elution was monitored at 280 nm. The unbound effluent was collected until the A_{280} returned to baseline. The column was then developed with 50 mM Tris-HCl (pH 9.6) containing 5 mM glutathione, and a second small A_{280} absorbing peak was eluted. Glutathione *S*-transferase activity was measured according to the method of Habig *et al.* (22), using 1-chloro-2,4-dinitrobenzene as the substrate.

Electrophoresis. SDS-polyacrylamide slab gel electrophoresis was carried out using a modification of the method of Laemmli (23) where the Tris-glycine buffer system concentrations were doubled. Gels were stained and destained with Coomassie Blue by the method of Fairbanks *et al.* (24).

RESULTS

Demonstration of a saturable B[a]P binding site in mouse liver cytosol. Saturation binding curves of total, nonspecific, and specific binding of B[a]P are shown in Fig. 1a. Total binding continued to increase over the range of 0.1–65 nM [^{3}H]B[a]P. Nonspecific binding was linear. The difference between total and nonspecific binding, or specific binding, was saturable at about 3 pmol of B[a]P per milligram of protein. A typical experiment, plotted by the method of Scatchard (25), revealed a K_d of 7.7 nM and a B_{max} of 4.7 pmol/mg of protein (Fig. 1b). When the data were plotted according to the method of Klotz (26), a sigmoidal curve was obtained (Fig. 1c). These data indicate that all available binding sites are saturated under the conditions of our assay. Hill plot analysis (27) of these data indicated a Hill coefficient close to unity, $n = 0.92$ with best fit to a single class of sites. Experiments carried out with independently prepared cytosol samples yielded very similar results. Preliminary experiments have indicated that the binding species is labile with respect to protease and heat treatment and resistant to lipases and ribonuclease.[3]

Sucrose density gradient analysis of B[a]P binding components. When aliquots of B6 cytosol were preincubated with [^{3}H]B[a]P in either the absence or presence of unlabeled B[a]P and run on 5%–20% sucrose gradients, the binding species were found to consist of two components (Fig. 2). About 98% of the binding activity was present in a peak at approximately 4 S, whereas a very small percentage of the total binding was found in the region around 9 S on the gradient. Both peaks were reduced by the presence of excess unlabeled B[a]P in the incubation. Co-migration of the [^{3}H]B[a]P-labeled 9 S peak with [^{3}H]TCDD-labeled cytosol indicated that they sediment to identical positions (data not shown).

Competition assay with structurally related compounds. Some of the compounds used in this experiment bore close structural homology to B[a]P. Compounds that were demonstrated carcinogens, that were inducers of xenobiotic metabolizing systems, or that bound to other receptor-like proteins in liver were assayed for their ability to compete for the B[a]P-binding site(s). DB[a,c]A, BNF, chrysene, 3-MC, TCDD, cortisol, and PCN, in addition to B[a]P itself, were tested against [^{3}H]B[a]P in competition assays in which the unlabeled competitor concentration was varied. The results of these assays are plotted as B/B_0 (the fraction of bound ligand at each concentration of unlabeled competitor divided by bound ligand in absence of competitor) versus log unlabeled competitor concentration (Fig. 3).

Table 1 presents the IC_{50} values calculated from the competition curves. The IC_{50} for B[a]P is 14.1 nM. Those compounds most closely related, in structure to B[a]P

[3] S. Collins, J. D. Altman, and M. A. Marletta, unpublished results.

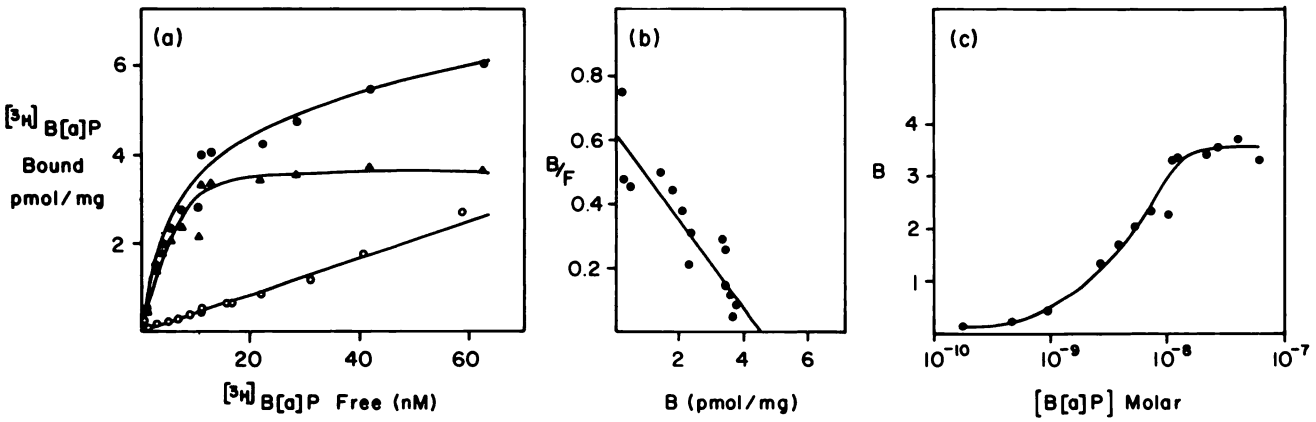

FIG. 1. *B[a]P binding plots*

a. Saturation binding curve of [^{3}H]B[a]P in B6 mouse liver cytosol. [^{3}H]B[a]P and unlabeled B[a]P were repurified as described under Materials and Methods, and stock solutions of [^{3}H]B[a]P were prepared in *p*-dioxane to complete the range of concentrations indicated. The binding assay and determination of free [^{3}H]B[a]P concentrations were carried out as described under Materials and Methods. Total binding (●) and nonspecific binding (○) were determined in the absence and presence of 1 μM unlabeled B[a]P, respectively. Specific binding (▲) is computed as the difference between total and nonspecific binding. b. Scatchard plot of specifically bound [^{3}H]B[a]P from a. c. Specifically bound [^{3}H]B[a]P from a, plotted by the method of Klotz (26), as bound (picomoles per milligram) versus log free [^{3}H]B[a]P.

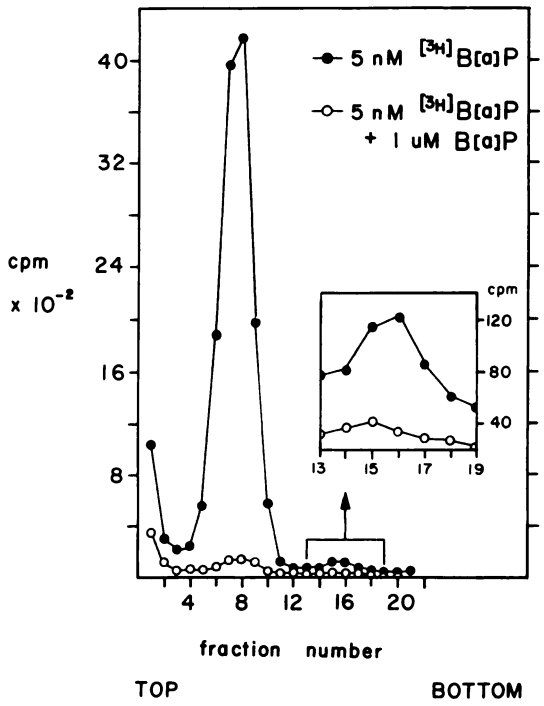

FIG. 2. *Sucrose density gradient analysis of [^{3}H]B[a]P-labeled B6 mouse liver cytosol*

Incubated cytosol (300 μl, 37,500 cpm) was layered onto 5%–20% linear sucrose density gradients prepared in HEDG buffer, and centrifuged in a Beckman SW 50.1 Ti rotor at 49,000 rpm for 16 hr at 4°. Fractions (200 μl) were collected into scintillation vials during fractionation with an ISCO Model 185 gradient fractionator. Aquasol (8 ml) was added to the vials and counted in a Beckman 3100 liquid scintillation counter.

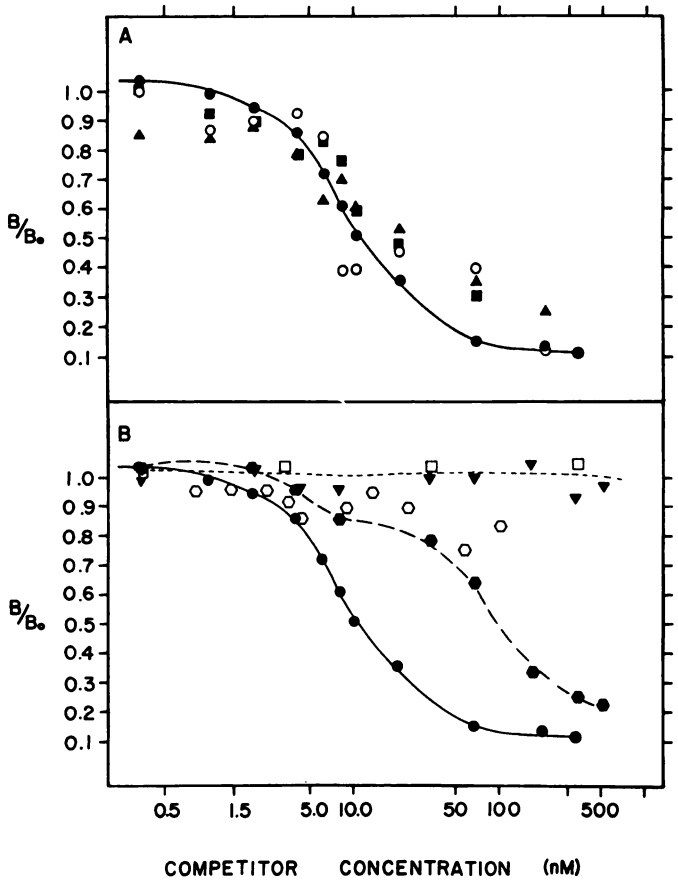

FIG. 3. *Competition studies for B[a]P binding sites*

Stock solutions of unlabeled ligands were prepared by dissolving the compounds in *p*-dioxane or ethanol to yield final concentrations of 0.25, 1.5, 5, 10, and 25 μM. All points were performed in duplicate, and a B[a]P control was run beside each test compound for comparison. [^{3}H]B[a]P (2–8 μM) in *p*-dioxane was added to all tubes before incubation so that a final concentration in the assay of 2–6 nM was obtained. Unlabeled competing ligands were added, and the binding assay was performed as described under Materials and Methods. B/B_0 is the fraction of bound ligand at each concentration of unlabeled competitor divided by bound ligand in the absence of competitor. *Solid lines* are best fit for the B[a]P curve. Unlabeled ligands were assumed to adsorb to the test tube surface to the same extent as that calculated for [^{3}H]B[a]P.

A. B[a]P, ●; BNF, ○; chrysene, ▲; DB[a,c]A, ■. B. B[a]P, ●; 3-MC, ◐; TCDD, ○; PCN, ▼; cortisol, □.

show competition binding which is similar to, if not identical with, B[a]P itself, indicating a similar affinity. The behavior of 3-MC is distinct from the other polycyclic aromatic hydrocarbons. The shift in the curve to a higher IC_{50} would indicate a weaker affinity for the B[a]P binding site. The 3-MC competition profile may also imply multiple binding sites and be related to this compound's reported ability to bind other sites in the liver (1, 3, 28). TCDD, while limited by its aqueous solubility, still appears not to be a competitor for this B[a]P site by this assay. The synthetic steroid PCN and the glucocorticoid cortisol also show no competition for this site.

In separate experiments, retinoids and representatives of the major steroid hormones were tested in competition binding assays at 10 μM (see Table 1). These ligands were not effective competitors at the concentrations tested. This same lack of competition by steroids was observed by investigators studying the *Ah* receptor (8, 15) and other carcinogen-binding proteins (10, 11).

Contribution of partially purified GSH-transferases to B[a]P binding. Glutathione *S*-transferases were isolated from B6 mouse liver cytosol using a GSH affinity column as described under Materials and Methods. The use of this affinity column to purify mouse GSH *S*-transferase activity has not been previously reported. The substrate used to follow transferase activity (1-chloro-2,4-dinitrobenzene) has been shown to have high activity with all of the isoenzymatic forms of this enzyme isolated from rats and humans (22, 29). Figure 4 shows that the transferases were isolated from the bulk of other cytosolic proteins by this method, and two major bands of M_r = 22,400 and 26,000 and a minor band at M_r = 29,000 on SDS-acrylamide gels were obtained. These values are in agreement with those reported previously for the subunits of rat and human enzymes (22, 29). On the basis of the GSH-transferase activity as measured by Habig *et al.* (22), a 10- to 15-fold purification was achieved. The B[a]P binding assay was performed on three samples from this procedure: cytosol, unbound effluent from the column, and eluted transferases. The results of the purification of GSH *S*-transferase activity and [^{3}H]B[a]P binding in these fractions is shown in Table 2. Although the GSH *S*-transferases were separated from the rest of

TABLE 1

IC_{50} values of competing ligands[a]

[^{3}H]B[a]P and competing ligands were incubated with mouse liver cytosol as described under Materials and Methods and in legend to Fig. 3. The final concentration of [^{3}H]B[a]P used was between 2 and 6 nM. IC_{50} values were determined for the individual competition curves in Fig. 3. TCDD, PCN, and cortisol are shown in Fig. 3B as representative ligands that do not compete at the concentrations tested.

Ligand	IC_{50}
● B[a]P[b]	14.1 ± 1.8 nM
■ DB[a,c]A	29.2 ± 5.5 nM
○ BNF	17.7 ± 8.3 nM
▲ Chrysene[c]	52.8 ± 13.9 nM
⬢ 3-MC	99.3 ± 12.6 nM
⭘ TCDD[d,e]	>100 nM
▼ PCN	>750 nM
□ Cortisol[f]	>200 μM
Corticosterone	>4 μM
Dexamethasone	>10 μM
Dihydrotestosterone	>10 μM
17β-Estradiol	>10 μM
Progesterone	>10 μM
Retinol	>10 μM
Retinoic acid	>10 μM

[a] Values, reported above with their standard error, were determined by Dr. M. B. Bolger, University of Southern California, using a curve-fitting program called FIGS (friendly interactive graphics and statistics program), which employs the Hartley modification of the Gauss-Newton method. IC_{50} values determined graphically were very similar to those calculated by the program.

[b] The average of three independent determinations.

[c] Data for a two-site model could also be fit with 90% confidence, giving IC_{50} No. 1 = 15.8 ± 9.0 nM and IC_{50} No. 2 = 1.35 ± 6.1 μM.

[d] Minimum IC_{50} values for TCDD, PCN, and cortisol were determined directly from Fig. 3B.

[e] TCDD not assayed beyond 100 nM.

[f] Steroids and retinoids were assayed in separate experiments at these higher concentrations; no competition was observed.

the cytosolic proteins, all of the B[a]P binding activity was retained in the unbound effluent and none was associated with the transferase preparation. This finding indicates that these affinity-purified enzymes are not responsible for the [^{3}H]B[a]P binding observed in the mouse liver cytosol.

DISCUSSION

Carcinogen-binding proteins, with relatively high affinity, have been recently described in rodent liver and cell culture extracts that bind certain unmetabolized carcinogens noncovalently (9–13).

We have shown that under equilibrium conditions a saturable high-affinity binding site for the polycyclic aromatic hydrocarbon carcinogen B[a]P exists in the B6 mouse liver cytosol. The charcoal-based separation of bound from free B[a]P is rapid and may provide more reliable values of K_d and B_{max} than other approaches that have been used. Our values are in the range of pharmacologically active receptor systems reported in the literature. The data suggest a single class of binding sites by the linearity of the Scatchard plot, a Hill coefficient of 0.92, and complete saturation of all sites by the Klotz plot. Despite this evidence, results from sucrose density gradient centrifugation indicate that the B[a]P binding

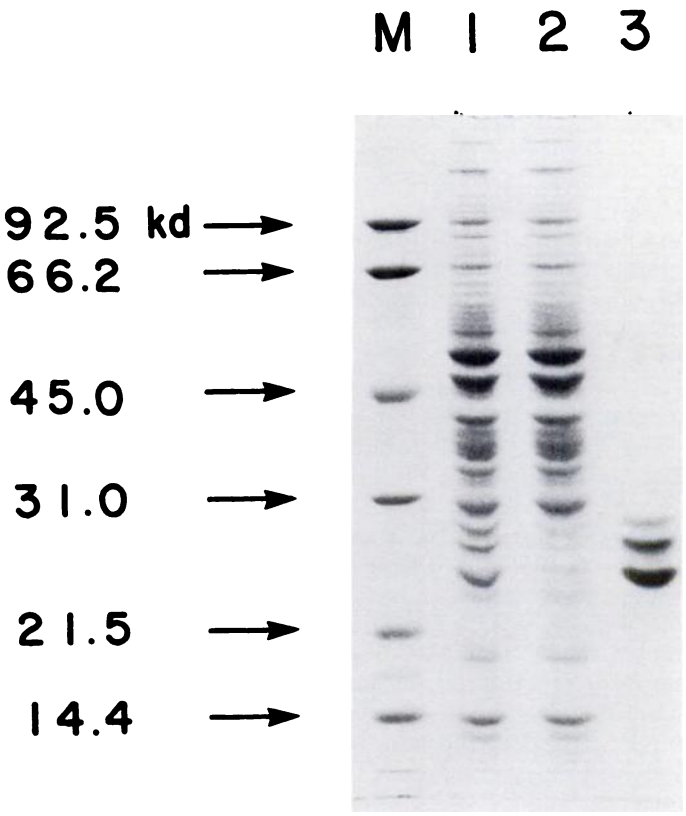

FIG. 4. *SDS polyacrylamide gel of samples from GSH-Sepharose 6B affinity chromatography*

Freshly prepared male B6 mouse liver cytosol in HEDG buffer (145 mg of protein) was applied to the column (1 × 19 cm) that had been equilibrated with 22 mM potassium phosphate buffer (pH 7.0) at 0.2 ml/min. When the A_{280} of the unbound effluent returned to baseline, the column was developed with 50 mM Tris-HCl (pH 9.6) containing 5 mM glutathione, and a second small protein peak was eluted.

Lane *M*: molecular weight standards included phosphorylase B (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Lane *1*: B6 mouse liver cytosol (55 μg). Lane *2*: Unbound effluent (75 μg). Lane *3*: GSH *S*-transferases eluted with 5 mM GSH (12 μg).

TABLE 2

Benzo[a]pyrene binding activity in GSH-affinity column fractions

Glutathione *S*-transferase were purified from mouse liver cytosol as described under Materials and Methods and in the legend to Fig. 4. Glutathione *S*-transferase activity was measured with 1-chloro-2,4-dinitrobenzene as substrate according to the method of Habig *et al.* (22). Samples from the column were diluted to a protein concentration of 1.0 mg/ml, and B[a]P binding was determined as described under Materials and Methods.

	GSH *S*-transferase specific activity	[^{3}H]B[a]*P-specific bound*
	μmol·mg^{-1} min^{-1}	*% control*
Cytosol	3.30 ± 0.20[a]	100
Unbound effluent	0.02 ± 0.001[b]	118.3
Eluted transferases	38.35 ± 3.88[a]	1.2[c]

[a] Mean ± standard deviation of three independent determinations.

[b] Mean ± standard deviation of two independent determinations.

[c] Binding was determined both before and after dialysis into HEDG buffer with no differences detected.

is composed of two discrete species. The major portion of this binding is the 4 S peak and is distinct from the TCDD-binding protein or *Ah* receptor at 9 S. The inability to detect two binding sites by the Scatchard or Hill analysis may be due to the relatively low abundance of the 9 S peak.

Holder *et al.* (9) identified two peaks on sucrose density gradients from [^{3}H]B[*a*]P-labeled rat liver cytosol that closely resemble our observations in mouse liver. The binding parameters obtained in rat liver using a charcoal adsorption assay (K_d = 2.54 nM, B_{max} = 530 fmol/mg) were similar to our results. Zytkovicz (11) recently described a cytosolic carcinogen-binding protein(s) in AKR mouse embryo cells using B[*a*]P as ligand. Although separation of bound from free ligand was performed on LH-20 columns, the binding parameters (K_d = 2.8 nM, B_{max} = 2 pmol/mg) were similar to those found in our studies. Whether this carcinogen-binding protein is identical with that in B6 liver is not known. In addition to the *Ah* receptor, which has been distinguished by its high affinity for TCDD, binding constants in the low nanomolar range have also been reported in support of a receptor function by investigators using these other aromatic hydrocarbon ligands (9–11). This protein-B[*a*]P binding we describe may be a receptor-ligand complex involved in some aspect of cellular regulation, but whose purpose remains speculative and requires further study. Other xenobiotic-metabolizing enzymes, inducible by aromatic hydrocarbons, have been shown not to segregate genetically with the *Ah* locus, and their mechanism of induction is presently unknown (30). Alternatively, transport proteins that may aid in the metabolism and removal of lipophilic substances have also been proposed (12, 13).

The *Ah* locus and receptor system have been the most thoroughly studied carcinogen-binding proteins to date, and it is the general consensus that the *Ah* receptor is necessary and sufficient for aryl hydrocarbon hydroxylase induction. However, when compounds other than TCDD have been used in studies of the *Ah* receptor, additional binding species are found, and it has been unclear what this binding represents. Okey *et al.* (8), Okey and Vella (17), and Hannah *et al.* (31) have examined [^{3}H]3-MC binding *in vitro* in B6 liver cytosol. Although the experiments we describe here have not used [^{3}H]3-MC as the ligand, our results with B[*a*]P bear a strong resemblance to their studies with 3-MC. These investigators observed a [^{3}H]MC-labeled peak, also in the 4 S region of sucrose density gradients, distinct from the *Ah* receptor. However, the 4 S [^{3}H]3-MC peak was not eliminated in the presence of a 100-fold molar excess of unlabeled 3-MC, and it was concluded that this major carcinogen-binding fraction represented binding of a nonspecific and nonsaturable nature. If, as our competition studies suggest, the affinity of the binding protein is weaker for 3-MC, then a greater than 100-fold molar excess of unlabeled 3-MC would be required to achieve competition at this 4 S site. Furthermore, the higher cytosolic protein concentrations used in those studies would significantly increase the total number of saturable binding sites, and this fact, coupled with a significant adsorption and net loss of ligand onto the vessel walls, may explain the incomplete competition observed with 3-MC. We have obtained similar incomplete competition with 10 nM [^{3}H]B[*a*]P and 1 μM B[*a*]P when cytosolic protein at 5 mg/ml was used. In another study, by Bresnick and co-workers (10), cytosolic 3-MC binding was also reported and evidence was presented that some of these binding proteins may be translocated to the nucleus, as in the *Ah* receptor model that has been proposed.

In competition binding assays, we selected test compounds that would provide information about the structural requirements for binding, that are demonstrated carcinogens and/or inducers of xenobiotic-metabolizing systems, or are known to bind to other characterized receptor-like binding proteins in liver. With the exception of 3-MC, compounds of related structure to B[*a*]P were equipotent in their ability to compete for the predominant 4 S species. Furthermore, there was no apparent correlation between binding to this site and induction at the *Ah* locus. Whether the values for chrysene and DB[*a,c*]A toward the extremes of the competition curve differ significantly from B[*a*]P is not known with certainty. Since, however, the IC_{50} values for these compounds were very similar in the most sensitive portion of the curve, these differences are probably small and inconsequential. The behavior of 3-MC as compared with other polycyclic aromatic hydrocarbons indicates a weaker affinity and suggests multiple cytosolic binding sites for this compound. However, to assess the shape of the 3-MC curve confidently, a wider concentration range would be necessary. [Computer fit of these data (see Table 1) indicates a good fit to a single-site model.] In addition, the synthetic steroid PCN, an inducer of a different set of P-450-mediated activities, does not compete for this site.

Steroid hormones do not reduce cytosolic B[*a*]P binding when incubated together in competition assays at saturating steroid levels. The glucocorticoids cortisol and corticosterone also display saturable high-affinity binding to a number of proteins in liver and kidney that are distinct from the native glucocorticoid receptor (32), but for which in most cases no known biological function has been defined. The fact that these glucocorticoids show no competition for the [^{3}H]B[*a*]P binding site additionally argues against the latter's being one of these corticosteroid binders. We have also examined B[*a*]P binding to serum albumin using BSA in concentrations ranging from 10 to 1000 μg/ml, and only nonspecific binding was detected. Furthermore, when serum prepared from these mice was examined at concentrations of 1 μg/ml–10 mg/ml, again only nonspecific binding was found.

We have found that an enhanced level of GSH *S*-transferase activity with 1-chloro-2,4-dinitrobenzene as a substrate is contained in the 4 S region of the sucrose density gradients. For this reason, together with the versatile binding characteristics reported for the rat and human liver transferases, we examined whether these enzymes were involved in the observed B[*a*]P binding in the B6 mouse. After affinity chromatography isolation of mouse liver GSH *S*-transferases, all of the B[*a*]P binding activity was recovered in the unbound cytosolic

proteins from the column, and none was found associated with the transferase preparation.

Our results demonstrate that the B[a]P binding, the major portion of which is a 4 S sedimenting species in sucrose density gradients, has properties similar to those of biological receptors in its affinity and number; yet it cannot be accounted for by several other previously described cytosolic binding proteins and receptors. The role of this binding protein(s) in liver, capable of specific interaction with B[a]P and related compounds, is unknown at present, and further biochemical characterization and study are necessary before any function can be assigned.

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