

## Regulatory Gene Product of the *Ah* Complex

COMPARISON OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN AND 3-METHYLCHOLANTHRENE BINDING TO SEVERAL MOIETIES IN MOUSE LIVER CYTOSOL\*

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The major regulatory gene product of the murine *Ah* complex appears to be a cytosolic receptor. 2,3,7,8-[1,6-<sup>3</sup>H]Tetrachlorodibenzo-*p*-dioxin or [<sup>3</sup>H]3-methylcholanthrene binding to the *Ah* receptor and other moieties in hepatic cytosol was examined by gel permeation chromatography, velocity sedimentation (sucrose density gradient centrifugation), dextran-charcoal adsorption, and anion exchange chromatography. In the liver of *Ah*-responsive C57BL/6N and the *Ah*<sup>b</sup>/*Ah*<sup>d</sup> heterozygote, both radioligands bind to three major components: peak I, a large aggregate which is eluted in the void volume of Sephacryl S-300 columns and which sediments as a residue to the bottom of sucrose density gradients; peak II, an asymmetric protein (*M<sub>r</sub>* ≈ 245,000) with a Stokes radius of about 75 Å; and peak III, a globular protein (*M<sub>r</sub>* ≈ 87,000) with an estimated Stokes radius of 40 Å. The peak I aggregate is not adsorbed by dextran-coated charcoal and therefore represents the large proportion of nonsaturable radioligand binding measured by dextran-charcoal adsorption. The peak II protein has a size of about 9.0 S in low ionic strength and 7.5 S in high ionic strength, high affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and saturability at TCDD concentrations greater than about 1.0 nM. The peak II protein is not detectable in the liver of *Ah*-nonresponsive DBA/2N and the *Ah*<sup>d</sup>/*Ah*<sup>d</sup> homozygote and therefore represents the *Ah* receptor. The peak III protein has an estimated size of 5.0 S, is not saturable with either TCDD or 3-methylcholanthrene under the conditions of these experiments, and is not associated with the *Ah*<sup>b</sup> allele. 3-Methylcholanthrene binds to the peak III protein to a greater extent than TCDD.

These data explain the discrepancies between the dextran-charcoal adsorption and sucrose density gradient assays. Any further studies of the function of the *Ah* receptor and these other ligand-binding moieties (e.g. nuclear translocation) should include gel permeation chromatography in order to distinguish among the various binding components.

The murine *Ah* locus controls the induction (by polycyclic aromatic compounds such as 3-methylcholanthrene, benzo[*a*]pyrene, *β*-naphthoflavone, and TCDD<sup>1</sup>) of many drug-me-

tabolizing enzyme activities in virtually all tissues examined (reviewed in Ref. 1). The *Ah* complex is believed to comprise regulatory, structural, and probably temporal genes which may or may not be linked (2). Multiple forms of cytochrome P-450 are believed to be among the many structural gene products "turned on" during the sequence of events following exposure of the animal to the polycyclic aromatic inducer (3, 4).

A cytosolic receptor for these inducers in genetically "responsive" mice was postulated (5, 6) and is now believed to be the major product of the *Ah* regulatory genes. Experimental evidence in support of this hypothesis has been reported, with the use of a dextran-coated charcoal adsorption assay (7-9), isoelectric focusing following trypsin treatment (10, 11), sucrose density gradient following dextran-charcoal adsorption (12, 13), and a detergent-washing procedure with purified nuclei (14). There is no detectable receptor in "*Ah*-nonresponsive" strains of mice (8, 12). The cytosolic receptor with specifically bound inducer appears to translocate into the nucleus during cytochrome P<sub>1</sub>-450 induction by polycyclic aromatic compounds in the intact liver of *Ah*-responsive mice (12), in cytosolic and nuclear preparations *in vitro* (14), and in cell cultures (13). The cytosolic location of the *Ah* receptor (7-14) and the temperature-dependent translocation of the inducer-receptor complex (13) are very similar to the properties of the steroid hormone receptors.

Estimates of TCDD-specific binding sites (8, 12, 13) have ranged between about 10 and 90 fmol/mg of cytosolic protein (~900 to 8,100 sites/cell), with an apparent *K<sub>d</sub>* for [<sup>3</sup>H]TCDD of approximately 0.7 nM (8, 10, 12). The cytosolic location of a 3-methylcholanthrene-specific binding protein and apparent nuclear uptake of this complex were recently reported; estimates of 3-methylcholanthrene-specific binding sites and apparent *K<sub>d</sub>* were 770 fmol/mg of cytosolic protein and 2.8 nM, respectively (15). In the current study we use differences at the *Ah* locus and four independent separatory methods to demonstrate that mouse liver cytosol contains at least two distinct proteins that bind TCDD and 3-methylcholanthrene. Only one, however, is shown to be the *Ah* receptor, because this peak is absent in *Ah*-nonresponsive (*Ah*<sup>d</sup>/*Ah*<sup>d</sup>) mice.

### EXPERIMENTAL PROCEDURES

**Chemicals**—[<sup>3</sup>H]TCDD (55 Ci/mmol) was purchased from KOR Isotopes (Cambridge, MA); gas chromatographic analysis by the manufacturer indicated that the product might contain as much as 20% tri-, penta-, and hexachlorodibenzo-*p*-dioxins. Nonlabeled TCDD was a generous gift of Dow Chemical Company (Midlands, MI). [<sup>3</sup>H]3-Methylcholanthrene (generally labeled, 35 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Nonlabeled 3-methylcholanthrene, Hepes, trypsin, dextran 200C, and Triton X-100 were purchased from Sigma; Norit A was from Fischer Scientific Company (Pittsburgh, PA); Sephacryl S-300 (superfine) was from

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<sup>1</sup> The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; [<sup>3</sup>H]TCDD, 2,3,7,8-[1,6-<sup>3</sup>H]tetrachlorodibenzo-*p*-dioxin; B6, the inbred C57BL/6N mouse strain; D2, the inbred DBA/2N mouse strain; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Pharmacia (Uppsala, Sweden); DEAE-52 (preswollen, microgranular) was from Whatman (Irvine, CA); Aquasol was from New England Nuclear; sodium deoxycholate was from Mann Research Laboratory (New York, NY); blue dextran, thyroglobulin, ferritin, bovine serum albumin, and cytochrome c were from Bio-Rad Laboratory (Richmond, CA); and [ $^{14}\text{C}$ ]formaldehyde-labeled bovine serum albumin (20  $\mu\text{Ci}/\text{mg}$ ) was from New England Nuclear. NP-40 (polyoxyethylene-9-*p*-isooctylphenyl ether) was a generous gift from Dr. Leonard M. Hjelmeland, Developmental Pharmacology Branch, National Institute of Child Health and Human Development, Bethesda, MD. The remainder of the chemicals were purchased from the sources cited previously (13).

**Animals**—*Ah*-responsive B6 ( $Ah^b/Ah^b$ ) and *Ah*-nonresponsive D2 ( $Ah^d/Ah^d$ ) mice were obtained from the Veterinary Resources Branch, National Institutes of Health. Breeding to obtain responsive heterozygotes ( $Ah^b/Ah^d$ ) and nonresponsive homozygotes ( $Ah^d/Ah^d$ ) from the B6D2F<sub>1</sub>  $\times$  D2 backcross was carried out in the Developmental Pharmacology Branch mouse colony, National Institute of Child Health and Human Development. Phenotyping progeny of the B6D2F<sub>1</sub>  $\times$  D2 backcross, with respect to the *Ah* locus, was carried out by the zoxazolamine paralysis test (16) on weanlings (either sex) between 3 and 4 weeks of age. The rigid environment and free access to food and water were described previously (13).

**Buffers and Solutions**—HEDG consisted of: 25 mM Hepes, 1.5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 10% glycerol (v/v), pH 7.6; dextran charcoal consisted of 5 mg of charcoal (Norit A) and 0.5 mg of dextran/ml of HEDG buffer, pH 7.6.

**Preparation of Cytosol**—Mice were killed by cervical fracture. The portal vein was cut and the livers were perfused *in situ* with HEDG buffer containing 0.1 M NaCl. All subsequent operations were carried out at 4 °C. The livers were removed, minced, and then homogenized in 2 volumes of HEDG buffer (with 0.1 M NaCl) using a Teflon-glass homogenizer. The homogenate was centrifuged at 10,000  $\times g$  for 15 min and the supernatant fraction was centrifuged at 100,000  $\times g$  for 1 h. The supernatant (cytosolic) fractions were quickly frozen in 2-ml aliquots and stored in liquid nitrogen. No losses of [ $^3\text{H}$ ]TCDD or [ $^3\text{H}$ ]3-methylcholanthrene binding capacity were noted during storage in liquid nitrogen for 3 months.

**Treatment of Cytosol with Radioligand In Vitro**—Usually 1 ml of cytosol was treated with the desired concentration of [ $^3\text{H}$ ]TCDD or [ $^3\text{H}$ ]3-methylcholanthrene for 1 h at 0–4 °C. Unless otherwise specified, 15 mg of cytosolic protein/ml were used in all experiments, except sucrose density gradient centrifugation, in which case 5 mg of protein/ml was used. In some samples, nonlabeled TCDD or 3-methylcholanthrene (in 100-fold excess) was added to the radioligand before treating the cytosol. As noted before (13), *p*-dioxane was the solvent for TCDD. A minimal amount of acetone was the solvent for 3-methylcholanthrene. After treatment at 4 °C for 1 h, the cytosol was examined by one of four independent techniques.

**Gel Permeation Chromatography**—Sephacryl S-300 gel was equilibrated in HEDG buffer containing 0.5 M NaCl, and columns with dimensions of 35  $\times$  0.9 cm were prepared. The chromatography of 1 ml of the radioligand-treated cytosol was performed by gravity flow (approximately 5 ml/h). Proteins and blue dextran were used as markers to calibrate the columns.

**Dextran-coated Charcoal Adsorption**—The radioligand-treated cytosol was added to a dextran-charcoal pellet (10 mg of charcoal/mg of dextran, pelleted from HEDG buffer). Dextran-charcoal was resuspended on a Vortex mixer, and the sample was incubated at 4 °C for 15 min before the dextran-charcoal was removed by centrifugation at 4000  $\times g$  for 15 min. Aliquots of cytosol were taken both before and after dextran-charcoal treatment for determination of “total” and “bound” radioactivity (8).

**Velocity Sedimentation**—Following dextran-charcoal adsorption, 300  $\mu\text{l}$  of cytosol was layered onto linear (5 to 20%) sucrose density gradients prepared in HEDG buffer containing 0.1 M NaCl. Gradients were centrifuged at 2 °C for 16 h at 235,000  $\times g$  in a Beckman SW 60 Ti rotor. The gradients were then separated in 0.2-ml fractions with an ISCO model 640 gradient fractionator. [ $^{14}\text{C}$ ]Formaldehyde-labeled bovine serum albumin and other protein standards were sometimes used as internal sedimentation markers (12).

**Anion-exchange Chromatography**—DEAE-52 gel was equilibrated in HEDG buffer containing 0.05 M NaCl, and columns with dimensions of 10  $\times$  1.5 cm were prepared. Chromatography of 1 ml of the radioligand-treated cytosol was performed by gravity flow (approximately 10 ml/h), with a linear NaCl elution.

**Determination of Radioactivity**—Samples of 0.2 ml or less were dissolved in Aquasol. Tritium was measured at 40 to 50% efficiency,

and  $^{14}\text{C}$  at 90% efficiency, with the use of a Mark III Tracor spectrometer.

## RESULTS

**Comparison of Gel Permeation Chromatography with Velocity Sedimentation: [ $^3\text{H}$ ]TCDD Binding to B6 and D2 Cytosol**—The sucrose density gradient assay is designed to resolve macromolecules with sedimentation coefficients of about 15 S or less. In order to detect larger components, we therefore used gel permeation chromatography (Figs. 1 and 2). [ $^3\text{H}$ ]TCDD binding in the *Ah*-responsive B6 mouse was observed in three regions (Fig. 1A): peak I eluting in the void fractions; peak II eluting in fractions 23 to 26 (Stokes radius  $\approx 75$  Å); and peak III eluting in fractions 30 to 33 (Stokes radius  $\approx 40$  Å). Peak I appears to represent a very large complex or aggregation. The saturability of components in these peaks was examined by incubating B6 cytosol with the radioligand plus a 100-fold excess of nonlabeled TCDD, and only peak II exhibited saturability (Fig. 1A).

In order to relate these peaks to those observed on sucrose density gradients, we applied samples from the peak I, peak II, and peak III regions of the Sephacryl S-300 eluate directly to sucrose gradients (Figs. 1B and 2B). Peak I resulted in a “trailing” of [ $^3\text{H}$ ]TCDD in the upper fractions of the gradient and a large amount on the bottom of the centrifuge tube. Peak II resulted in a peak with a maximum at gradient fractions 10 to 12 (approximately 9 S). Peak III resulted in a peak at gradient fractions 6 and 7 (about 5 S).

Fig. 2 illustrates that the *Ah*-nonresponsive D2 mouse does not have the saturable component in peak II but does possess peaks I and III. These data indicate that peak II represents the *Ah* receptor and that the B6 liver cytosol has at least two other TCDD-binding moieties that are nonsaturable, not associated with the *Ah* locus, and present also in D2 liver cytosol.

**Comparison of Gel Permeation Chromatography with Dextran-Charcoal Adsorption**—Sephacryl S-300 and other chromatographic matrices quantitatively adsorb free [ $^3\text{H}$ ]TCDD which can be released only by organic solvents such as *p*-dioxane (12). The [ $^3\text{H}$ ]TCDD eluted from the Sephacryl S-300 column (Figs. 1A and 2A) therefore represents radiolabel bound to macromolecules. More than 45% of the total [ $^3\text{H}$ ]TCDD eluted from the column was associated with the nonsaturable peak I (Table I). [ $^3\text{H}$ ]TCDD associated with peak I was not adsorbed to dextran-coated charcoal (data not illustrated). Comparing the two methods (Table I), we found approximately the same amount of bound radioligand in the dextran-charcoal adsorption assay as by gel permeation chromatography (the sum of peaks I, II, and III).

**Comparison of Velocity Sedimentation with Dextran-Charcoal Adsorption**—The dextran-coated charcoal adsorption assay (7–9) provides a rapid method for separating free from macromolecular-bound [ $^3\text{H}$ ]TCDD; however, all three components of binding are included in this measurement. The addition of excess nonlabeled TCDD as a competitor has variable effects on the nonsaturable components. As shown in Figs. 1 and 2, [ $^3\text{H}$ ]TCDD binding to peak I is often decreased; however, at higher protein and radioligand concentrations, [ $^3\text{H}$ ]TCDD binding to peak I is increased by addition of competitor (data not shown). These effects make it extremely difficult to use dextran-coated charcoal adsorption alone as the basis for measuring [ $^3\text{H}$ ]TCDD binding to the peak II moiety. In sucrose density gradients, peak I material sediments to the bottom of the centrifuge tube (Figs. 1B and 2B); hence, peak I does not interfere with the detection of peak II.

**Effect of Protein Concentration**—There are certain situations (e.g. estimation of “free” TCDD concentrations when

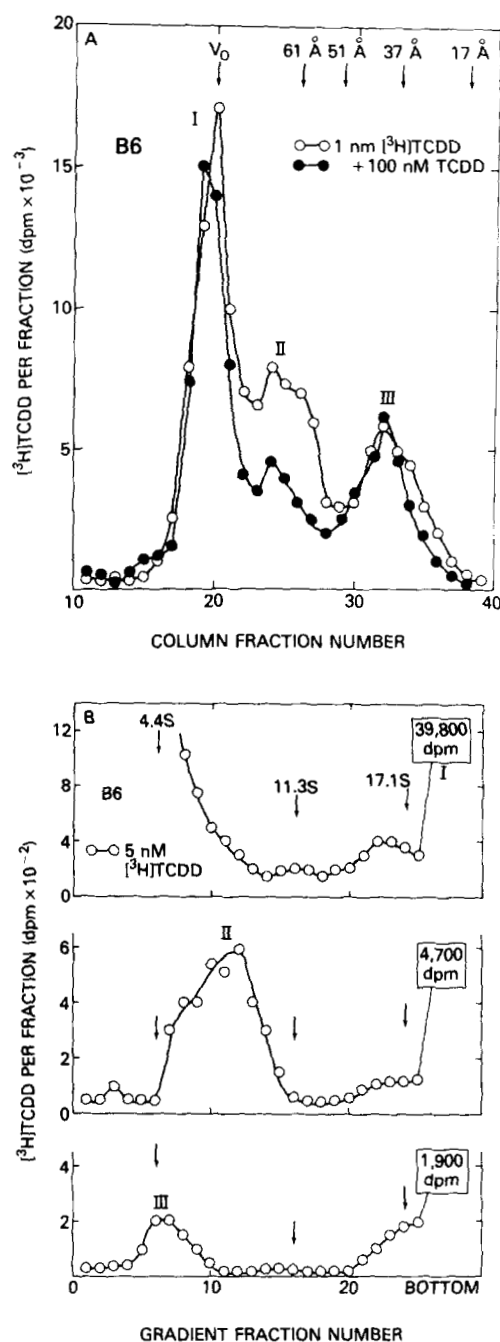


FIG. 1.  $[^3\text{H}]\text{TCDD}$  binding to B6 cytosol. A, gel permeation chromatography of B6 hepatic cytosol treated with  $[^3\text{H}]\text{TCDD}$ .  $V_0$ , void volume determined with blue dextran, thyroglobulin, ferritin, bovine serum albumin, and cytochrome c, with each of their Stokes radii indicated in Å, were used to calibrate the columns. B, analysis of peaks I, II, and III by velocity sedimentation. Cytosol (15 mg protein/ml) was treated with 5 nM  $[^3\text{H}]\text{TCDD}$  for 1 h at 4°C and then chromatographed on a Sephacryl S-300 column. Samples from the peak I (fractions 16 to 22), peak II (fractions 23 to 27), and peak III (fractions 28 to 35) regions were then added to sucrose density gradients (5% to 20%). Because of the low concentration of protein in these samples, the material was not treated with dextran-coated charcoal before application to the sucrose gradients. In addition to the usual gradient fractions, the bottoms of the centrifuge tubes were cut off, soaked in Aquasol, and counted for radioactivity (disintegrations per min shown in boxes). Approximate sedimentation values are shown for bovine serum albumin (4.4 S), bovine liver catalase (11.3 S), and ferritin (17.1 S); these standards were centrifuged in a separate gradient.  $[^{14}\text{C}]\text{Albumin}$  (2,000 dpm) was also included as an internal standard. Further details are described under "Experimental Procedures."

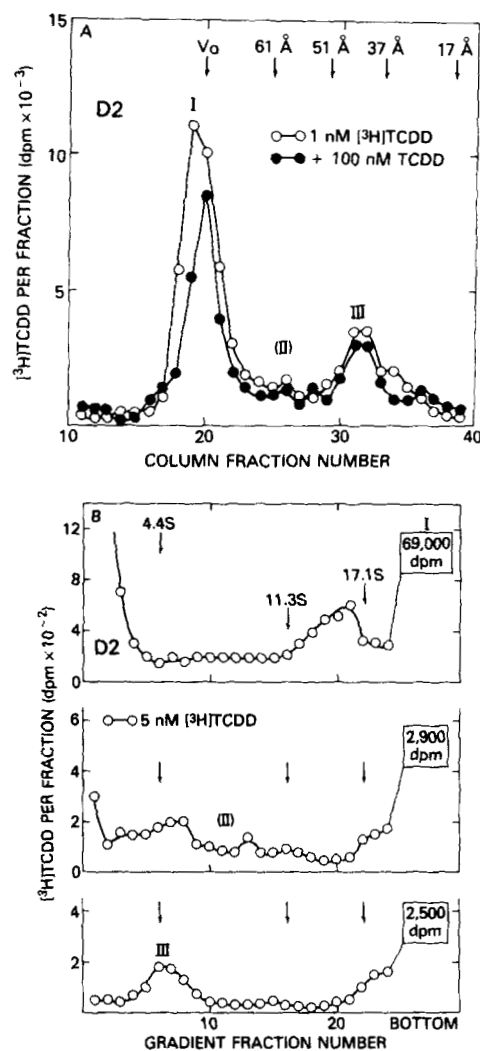


FIG. 2.  $[^3\text{H}]\text{TCDD}$  binding to D2 cytosol. A, gel permeation chromatography of D2 hepatic cytosol treated with  $[^3\text{H}]\text{TCDD}$ . B, analysis of peaks I, II, and III by velocity sedimentation. These experiments with D2 mice were identical to those with B6 mice illustrated in Fig. 1.

TABLE I

Comparison of gel permeation chromatography with dextran-charcoal adsorption

Following treatment of B6 liver cytosol with [ $^3\text{H}$ ]TCDD alone or in the presence of a 100-fold excess of nonlabeled TCDD, aliquots were examined by dextran-coated charcoal adsorption. The remainder of the sample was examined by Sephacryl S-300 chromatography and represents the data illustrated in Fig. 1A. The chromatogram was divided into three regions: fractions 16 to 22 (peak I); fractions 23 to 27 (peak II); and fractions 28 to 35 (peak III). The values shown reflect the total radioactivity in these regions, and no corrections are made for possible overlap of components. Peak I material is not totally separable from peak II material (Fig. 1A), so that quantitation of peak II by gel permeation chromatography should require estimation of the degree of contribution by peak I radioactivity.

In vitro treatment	Total [ $^3\text{H}$ ]TCDD bound				Dextran-charcoal adsorption assay
	Gel permeation chromatography				
	Peak I	Peak II	Peak III	Total	
	dpm $\times 10^{-3}$				
1.0 nM [ $^3\text{H}$ ]TCDD	53	35	26	114	129
1.0 nM [ $^3\text{H}$ ]TCDD plus 100 nM TCDD	42	15	24	81	110

attempting to perform Scatchard analysis) in which it becomes essential to know the amount of [ $^3\text{H}$ ]TCDD associated with peak I. Peak I represents approximately one-fourth of the total [ $^3\text{H}$ ]TCDD added *in vitro* to cytosol concentrations of 5 and 10 mg of protein/ml (Fig. 3A). At 15 mg of protein/ml, however, peak I comprised almost 70% of the total [ $^3\text{H}$ ]TCDD added *in vitro*. Although peak II increased in proportion to protein concentration, peak III was markedly diminished at 15 mg/ml. Under conditions when peak I is greatest (i.e. high protein concentration) and therefore "free" [ $^3\text{H}$ ]TCDD would be lowest, relatively little binding to the peak III moiety occurred. It is thus concluded that peak III material has lower affinity for TCDD than peak II material. These data illustrate why there has been so much difficulty with Scatchard analysis by either the dextran-charcoal adsorption method (8, 9) or the sucrose density gradient method (12).

[ $^3\text{H}$ ]TCDD binding in the 5.0 S region of sucrose density gradients (peak III material) was quite low at both high and

low cytosolic protein concentrations (Fig. 3B), in contrast to what was seen for peak III with gel permeation chromatography. Both the gel permeation chromatography and velocity sedimentation methods measure ligand binding under nonequilibrium conditions. The time required for analysis on sucrose density gradients (about 18 h) is considerably longer than that for gel permeation chromatography (about 6 h). The quantitative differences in peak III in Fig. 3 between A and B are therefore probably related to dissociation of ligand ("TCDD off-rate") from the peak III macromolecule(s) as a function of time after the *in vitro* [ $^3\text{H}$ ]TCDD treatment. The large proportion of radioligand associated with peak I has made it impossible for us to study accurately the kinetics of [ $^3\text{H}$ ]TCDD binding to the peak II and peak III moieties. The partial saturability of peak III may reflect heterogeneity, as well as a large number of binding sites.

**Estimation of Size**—The approximate molecular weights of the peak II and peak III moieties were calculated from their respective Stokes radii and sedimentation coefficients (Table II). The peak II component, believed to represent the Ah receptor, appears to be an asymmetric molecule ( $M_r \approx 245,000$ ;  $f/f_0 \approx 1.6$ ). The peak III material is presumably more symmetric ( $M_r \approx 87,000$ ;  $f/f_0 \approx 1.2$ ).

**Comparison of Gel Permeation Chromatography with Velocity Sedimentation: [ $^3\text{H}$ ]3-Methylcholanthrene Binding to B6 and D2 Cytosol**—Various properties of the peak III material in mouse liver caused us to wonder if this were analogous to the 3-methylcholanthrene-binding protein in rat liver recently reported by Fuller *et al.* (18) and Tierney *et al.* (15). Three major peaks were detected in B6 cytosol by gel permeation chromatography (Fig. 4A), and the positions of elution of the [ $^3\text{H}$ ]3-methylcholanthrene-binding peaks were essentially the same as those of the [ $^3\text{H}$ ]TCDD-binding peaks. Peak III was proportionately larger with [ $^3\text{H}$ ]3-methylcholanthrene than with [ $^3\text{H}$ ]TCDD. Peak II [ $^3\text{H}$ ]3-methylcholanthrene binding was not detectable in the Ah-nonresponsive D2 cytosol (Fig. 4B).

With sucrose density gradient analysis (Fig. 5) [ $^3\text{H}$ ]3-methylcholanthrene eluted in the positions of the peak II and peak III material, as had been seen for [ $^3\text{H}$ ]TCDD (Fig. 1B). At either 1 or 10 nM [ $^3\text{H}$ ]3-methylcholanthrene, 100-fold excess concentrations of nonlabeled 3-methylcholanthrene decreased peak II almost completely while decreasing peak III less than 50%. These data suggest that the saturable binding for [ $^3\text{H}$ ]3-methylcholanthrene is principally in peak II but that some saturable binding sites may exist in the peak III material.

**Effect of Trypsin and Detergents**—Peak II was entirely destroyed by trypsin treatment under conditions that did not affect peak I or peak III to any substantial degree (Fig. 6).

The detergents sodium deoxycholate and NP-40 abolished binding to peak II, but the [ $^3\text{H}$ ]TCDD was then eluted in the void fractions of the Sephacryl S-300 column after detergent treatment, i.e. as an increase in peak I (data not illustrated). TCDD and other polycyclic aromatic inducers are extremely hydrophobic and insoluble, and it seems plausible that cyto-

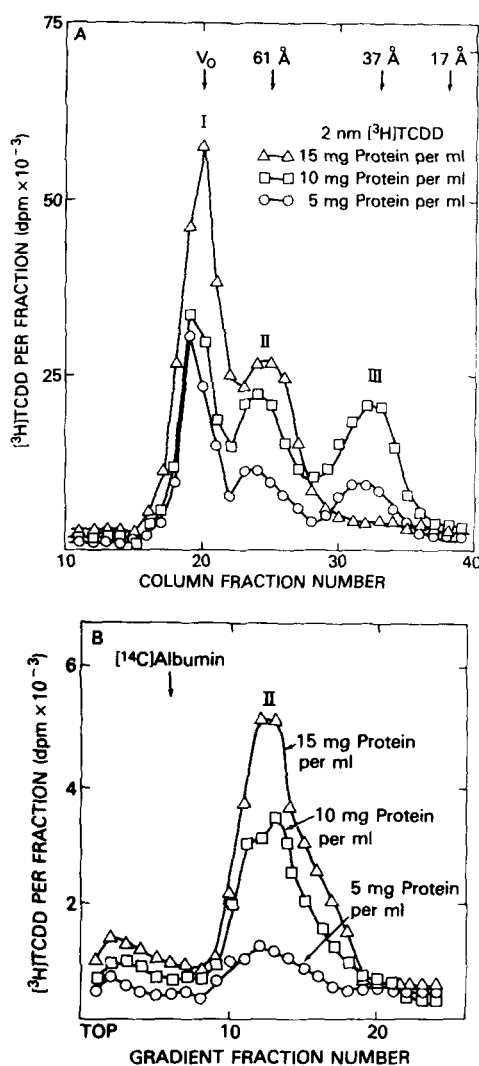


FIG. 3. Distribution of [ $^3\text{H}$ ]TCDD binding to peaks I, II, and III as a function of protein concentration. A, gel permeation chromatography. B6 liver cytosolic preparations (1 ml of three different protein concentrations) were treated with 2 nM [ $^3\text{H}$ ]TCDD for 1 h at 4°C and then chromatographed on Sephacryl S-300 columns. B, velocity sedimentation analysis. Following treatment with 2 nM [ $^3\text{H}$ ]TCDD for 1 h at 4°C and then dextran-coated charcoal adsorption, B6 cytosolic preparations were examined with the use of 5 to 20% sucrose density gradients, as detailed under "Experimental Procedures."

TABLE II

## Physical parameters of the peak II and peak III moieties

The sedimentation characteristics were determined in sucrose density gradients containing 0.5 M NaCl. The  $M_r$  and  $f/f_0$  values were calculated as described by Sherman *et al.* (17); assumed values included  $\bar{v} = 0.74$  and  $\delta = 0.2$ .

Parameter	Peak II	Peak III
Stokes radius (nm)	7.5	4.0
Sedimentation coefficient ( $s_{20,w}$ )	7.5	5.0
$M_r$ estimation	245,000	87,000
$f/f_0$ estimation	1.6	1.2

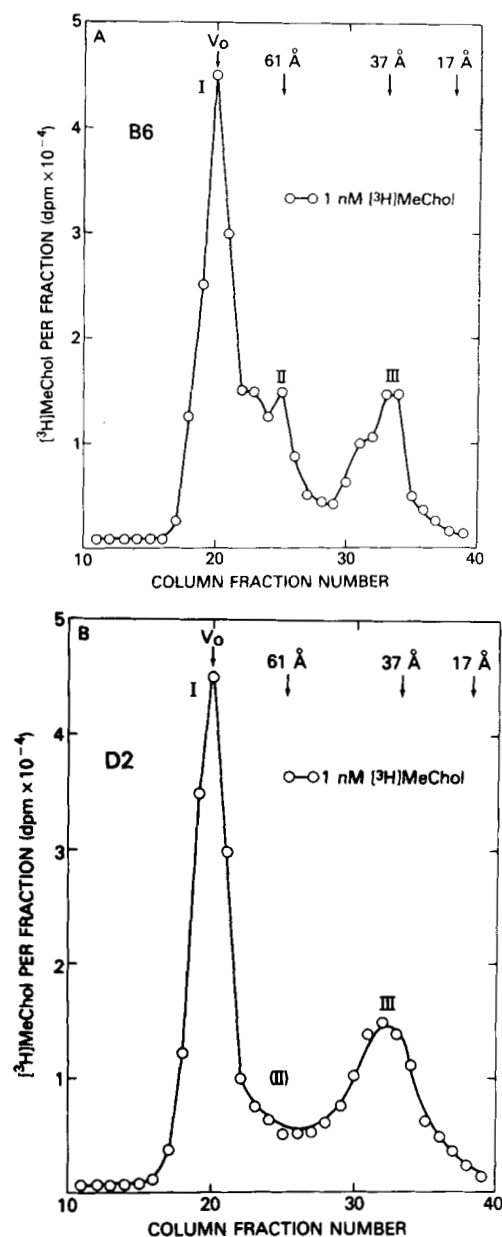


FIG. 4. Gel permeation chromatography of B6 liver cytosol (A) and D2 liver cytosol (B) treated with  $[^3\text{H}]$ 3-methylcholanthrene ( $[^3\text{H}]\text{MeChol}$ ). Following treatment of 1 ml of cytosol (15 mg of protein) with 10 nM  $[^3\text{H}]$ 3-methylcholanthrene for 1 h at 4 °C, the samples were chromatographed on Sephacryl S-300 columns, as described under "Experimental Procedures."

solic components such as proteins or lipids could form large micellar structures that might include these ligands.

**Genetic Analysis of  $[^3\text{H}]$ 3-Methylcholanthrene and  $[^3\text{H}]$ -TCDD Binding to Liver Cytosol**—*Ah*-responsive B6 ( $Ah^b/Ah^b$ ) and the heterozygote ( $Ah^b/Ah^d$ ) possess levels of receptor that are sufficient for the induction of cytochrome P<sub>1</sub>-450 and its associated aryl hydrocarbon hydroxylase activity, whereas *Ah*-nonresponsive D2 ( $Ah^d/Ah^d$ ) and the  $Ah^d/Ah^d$  homozygotes as children of the B6D2F<sub>1</sub> × D2 backcross do not exhibit detectable levels of cytosolic receptor (1, 12). Because B6 and D2 mice represent very different inbred strains, we chose to study the relationship of peak II and peak III  $[^3\text{H}]$ 3-methylcholanthrene binding as a function of the *Ah*<sup>b</sup> allele.

Individual offspring from the B6D2F<sub>1</sub> × D2 backcross were phenotyped 10 days earlier by the xoxazolamine paralysis test

(16) and therefore classified as *Ah*-responsive  $Ah^b/Ah^d$  or *Ah*-nonresponsive  $Ah^d/Ah^d$  individuals (Fig. 7). The responsive heterozygote exhibited both peak II and peak III  $[^3\text{H}]$ 3-methylcholanthrene binding, whereas the nonresponsive homozygote demonstrated large peak III  $[^3\text{H}]$ 3-methylcholanthrene binding only. With  $[^3\text{H}]\text{TCDD}$ , peak II was prominent in the  $Ah^b/Ah^d$  mouse and absent in the  $Ah^d/Ah^d$  mouse, and peak III was negligible in both; this result is consistent with that previously reported (12). We therefore conclude that this  $[^3\text{H}]$ 3-methylcholanthrene binding moiety of peak III is not associated with the *Ah* receptor, whereas the peak II binding correlates well.

**Analysis of Peak II and Peak III  $[^3\text{H}]$ 3-Methylcholanthrene Binding by Anion-Exchange Chromatography**—In all the above experiments, peak II and peak III have been separated on the basis of their hydrodynamic properties. Anion-exchange chromatography also can resolve  $[^3\text{H}]\text{TCDD}$  and

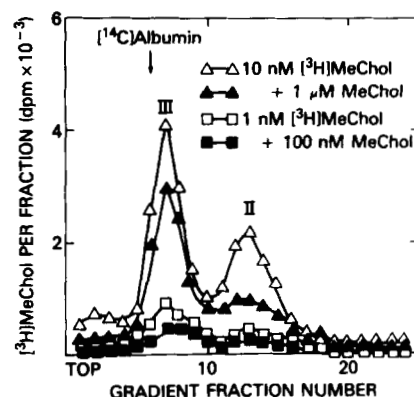


FIG. 5. Velocity sedimentation analysis of B6 hepatic cytosol treated with  $[^3\text{H}]$ 3-methylcholanthrene ( $[^3\text{H}]\text{MeChol}$ ). Treatment for 1 h at 4 °C included 10 nM  $[^3\text{H}]$ 3-methylcholanthrene alone ( $\Delta$ — $\Delta$ ) and with 1.0 μM nonlabeled 3-methylcholanthrene ( $\blacktriangle$ — $\blacktriangle$ ), or 1 nM  $[^3\text{H}]$ 3-methylcholanthrene ( $\square$ — $\square$ ) and with 100 nM nonlabeled 3-methylcholanthrene ( $\blacksquare$ — $\blacksquare$ ). Following treatment and then dextran-charcoal adsorption, the samples were examined with the use of 5 to 20% sucrose density gradients, as detailed under "Experimental Procedures."

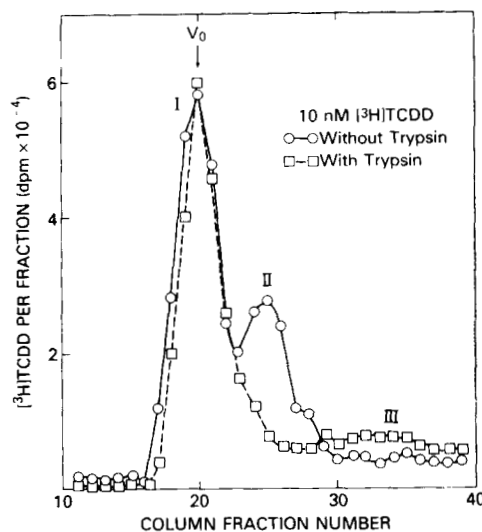


FIG. 6. Effect of trypsin on the  $[^3\text{H}]\text{TCDD}$ -binding moieties in B6 liver cytosol, examined by gel permeation chromatography. Following incubation of the cytosol (15 mg of protein in 1 ml) at 22 °C for 1 h in the presence or absence of 100 μg of trypsin, the cytosolic samples were cooled to 4 °C and treated for 1 h with 10 nM  $[^3\text{H}]\text{TCDD}$ . The material was then applied to Sephacryl S-300 columns, as described under "Experimental Procedures."

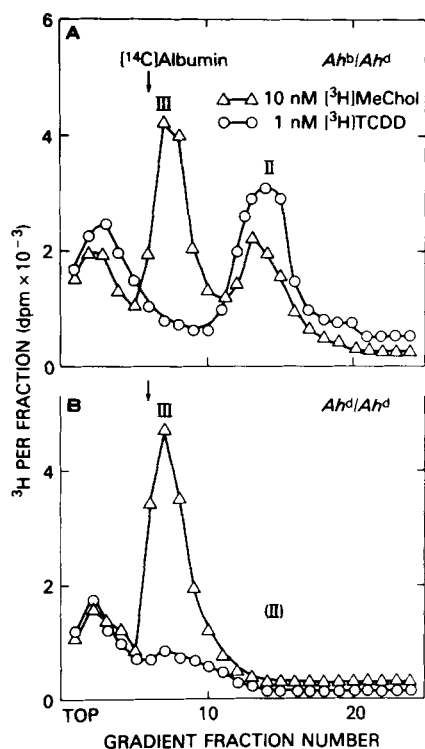


FIG. 7. Velocity sedimentation analysis of [ $^3\text{H}$ ]TCDD- and [ $^3\text{H}$ ]3-methylcholanthrene ([ $^3\text{H}$ ]MeChol)-binding moieties in hepatic cytosol of progeny from the B6D2F $_1$   $\times$  D2 backcross. Mice were from the same litter: A, responsive  $Ah^b/Ah^d$  mouse; B, nonresponsive  $Ah^d/Ah^d$  mouse. The backcross mice had been phenotyped by the xoxazolamine paralysis test (16) 10 days earlier. One ml of cytosol (10 mg of protein) from individual mice was treated with 1 nM [ $^3\text{H}$ ]TCDD or 10 nM [ $^3\text{H}$ ]3-methylcholanthrene for 1 h at 4  $^\circ\text{C}$ ; following dextran-coated charcoal adsorption, the samples were then examined on sucrose density gradients, as detailed under "Experimental Procedures."

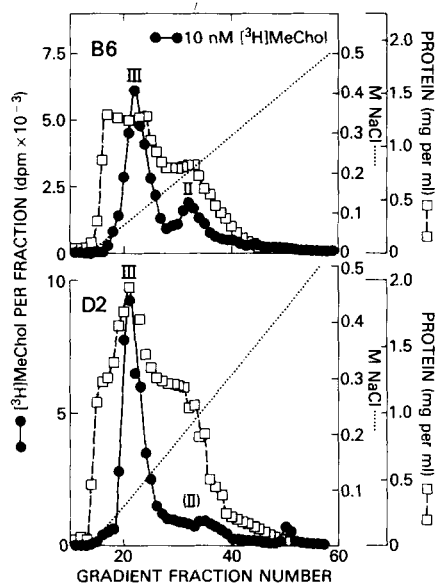


FIG. 8. Separation of [ $^3\text{H}$ ]3-methylcholanthrene ([ $^3\text{H}$ ]MeChol)-binding peaks II and III by anion-exchange chromatography. A, B6 liver. B, D2 liver. Five ml of cytosol (75 mg of protein) was treated with 10 nM [ $^3\text{H}$ ]3-methylcholanthrene for 1 h at 4  $^\circ\text{C}$  and then applied to a DEAE-cellulose column (bed volume of 20 ml). After the column was washed with 100 ml of HEDG buffer containing 50 mM NaCl, the proteins were eluted with a linear NaCl gradient and 1-ml fractions were analyzed for radioactivity, protein content ( $A_{280}$ ), and conductivity. Further details are described under "Experimental Procedures."

[ $^3\text{H}$ ]3-methylcholanthrene binding into distinct components. Okey *et al.* (12) previously showed that anion-exchange chromatography resolved [ $^3\text{H}$ ]TCDD binding into a single major component that eluted at concentrations between 0.16 and 0.20 M NaCl; those experiments were carried out under conditions (high cytosolic protein concentrations), however, that would lead to minimal [ $^3\text{H}$ ]TCDD binding to peak III material. With changes in the experimental conditions (Fig. 8), we were able to separate clearly peak II and peak III [ $^3\text{H}$ ]3-methylcholanthrene binding: peak II was eluted at concentrations of 0.16 to 0.20 M NaCl and was found only in the  $Ah$ -responsive B6 cytosol; peak III was eluted at lower concentrations of NaCl (about 0.10 M NaCl) and was found to be equally large in both the  $Ah$ -responsive B6 and the  $Ah$ -nonresponsive D2 cytosol.

#### DISCUSSION

With the use of gel permeation chromatography, we have resolved the apparent discrepancies between the dextran-charcoal adsorption assay (7-9) and the sucrose density gradient assay (12, 13) for the  $Ah$  receptor. If saturating concentrations of [ $^3\text{H}$ ]TCDD are used, the sucrose density gradient assay provides an accurate measure of [ $^3\text{H}$ ]TCDD binding to the peak II moiety. Using this assay, we have shown that binding is saturable and proportional to protein concentration over a wide range of experimental conditions. The aggregated material (peak I) does not correlate with the  $Ah$  locus. With the sucrose density gradient assay, this material sediments to the bottom of the tube and therefore does not interfere with determination of  $Ah$  receptor levels (peak II). We have also shown in this report that the major 3-methylcholanthrene binding moiety (peak III in this report) described in rat liver cytosol (15, 18) is not associated with the  $Ah$  receptor and the cytochrome P $_1$ -450 induction process.

Gel permeation chromatography and velocity sedimentation techniques both involve significant departures from equilibrium binding conditions; these methods can therefore be used only for estimates of parameters such as dissociation constants and numbers of binding sites. Both these techniques, however, are able to separate distinctly the major TCDD- and 3-methylcholanthrene-binding components. Peak II appears to represent the  $Ah$  receptor, with a size of about 9.0 S in low ionic strength, high affinity for TCDD, and saturability at TCDD concentrations greater than 1 nM. Peak III is much more prominent when 3-methylcholanthrene rather than TCDD is used as the ligand, is not associated with the  $Ah$  receptor, is not saturable, and represents at least 100 times more binding sites than peak II. Although peak III is not destroyed by mild trypsin treatment that destroys peak II (Fig. 6), Bresnick and co-workers (15) have demonstrated that this moiety is destroyed by prolonged Pronase treatment. The binding reflected in both peak II and III must therefore represent protein. Peak I is some sort of large aggregate. At low cytosolic protein concentrations, the proportion of peak I binding is considerably less (Fig. 3) explaining the success with dextran-charcoal adsorption under certain experimental conditions (7-9).

TCDD, as the most potent inducer, exhibits a large peak II and small peak III, whereas 3-methylcholanthrene binding is much more prominent in peak III than in peak II. Benzo[*a*]pyrene, an inducer less potent than TCDD and perhaps equivalent in potency to 3-methylcholanthrene, showed binding characteristics similar to those of 3-methylcholanthrene on sucrose density gradients (12): a small but detectable peak in the  $Ah$  receptor range (peak II) and a very large binding component of lower molecular weight (peak III).

Polycyclic aromatic inducers are able to enhance aminopyrine *N*-demethylase (19), aniline hydroxylase, *d*-benzphet-



amine *N*-demethylase, chlorocycline *N*-demethylase, ethylmorphine *N*-demethylase, and pentobarbital hydroxylase (20) activities in *Ah*-nonresponsive mice (usually 40% to 2-fold enhancement). Small increases in electrophoretic bands are detectable by sodium dodecyl sulfate-polyacrylamide gels of liver microsomes from *Ah*-nonresponsive mice.<sup>2</sup> Hepatic glutathione transferase induction (with 1-chloro-2,4-dinitrobenzene dinitrobenzene as substrate) by 3-methylcholanthrene is also not associated with the *Ah* complex and occurs in some nonresponsive mice having no detectable *Ah* receptor (21). It is therefore possible that we are technically unable to measure the presence of some *Ah* receptor in these nonresponsive mice. Alternatively, some other receptor or a "nonreceptor" mechanism<sup>3</sup> might be responsible for this induction process by polycyclic aromatic compounds in *Ah*-nonresponsive mice. Also, peak III could be heterogeneous and might contain a component responsible for this process in *Ah* nonresponsive mice.

Although *Ah*-nonresponsive mice have no detectable *Ah* receptor, there is significant aryl hydrocarbon hydroxylase induction in mouse fetal cell cultures exposed to benzo[*a*]anthracene (23) and in numerous tissues *in vivo* when nonresponsive mice receive sufficient doses of TCDD (5). This difference in sensitivity (*Ah*-nonresponsive mice requiring 12 to 18 times the dose of inducer given to *Ah*-responsive mice in order to attain the same response (6)) implies that the *Ah* structural genes are intact and that some defect lies in the *Ah* regulatory genes, presumably the *Ah* receptor. This 12- to 18-fold difference in sensitivity cannot be readily explained by the binding data in this report, however, which show a complete absence of peak II in liver cytosol from *Ah*-nonresponsive mice. It is possible that a 12- to 18-fold decrease in affinity would result in dissociation of the inducer-receptor complex during gel permeation chromatography (which takes about 6 h) or other lengthy methods of analysis. It would be useful to be able to measure ligand binding under equilibrium conditions, but this does not appear to be possible in crude cytosol because of the large proportion of nonsaturable binding of inducer to the peak I aggregate.

Several recent studies have dealt with the interesting possibility that the TCDD- or 3-methylcholanthrene-binding moieties undergo translocation to the cell nucleus during the process of induction. Okey *et al.* (12) have shown that the 7.5 S moiety moves to the nucleus after *in vivo* injection of [<sup>3</sup>H]TCDD. Okey *et al.* (13) more recently have demonstrated an apparent temperature-dependent step during the nuclear translocation of the [<sup>3</sup>H]TCDD-receptor complex with the use of cells in culture. Using a detergent-washing procedure with isolated purified liver cell nuclei (14), Greenlee and Poland showed that the [<sup>3</sup>H]TCDD-receptor complex moved presumably from the cytosol to the nucleus *in vitro*. Okey *et al.* (12),

with the use of sucrose density gradients, have been unable to confirm, however, the *in vitro* nuclear translocation demonstrated by Greenlee and Poland (14), who used the dextran-charcoal adsorption method. Tierney *et al.* (15) demonstrated that the "peak B" 3-methylcholanthrene-binding component (equivalent to peak III in this report) undergoes temperature-dependent translocation to the nucleus.

We believe the best approach to studying nuclear translocation and all other aspects of any polycyclic aromatic binding moiety is to separate and characterize the various components contributing to the overall binding. In this report we have demonstrated the usefulness of gel permeation chromatography and anion-exchange chromatography, combined with an appreciation of the best protein and salt concentrations to obtain maximal resolution.

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<sup>2</sup> James S. Felton and Daniel W. Nebert, unpublished data.

<sup>3</sup> Unknown nonreceptor mechanisms leading to increases in enzyme activity might include: (i) direct binding of chemicals to cytochromes P-450 or other membrane moieties; (ii) perturbation of various membrane components (e.g. changes in membrane fluidity); (iii) interaction of nonmetabolized parent drug, or its metabolites, directly with nucleic acids or proteins in the nucleus without the need of a cytosolic receptor; (iv) activation of pre-existing inactive enzyme protein; and (v) inhibition of degradation. In cultured hepatocytes from fetal rat liver (22), for example, aryl hydrocarbon hydroxylase activity can be stabilized, and even enhanced, in the presence of the inducer benz[*a*]anthracene or phenobarbital and in the almost complete absence of RNA and/or protein synthesis.