ANALYTICAL BIOCHEMISTRY 142, 28–36 (1984)

Protamine Sulfate Precipitation: A New Assay for the *Ah* Receptor

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Received March 19, 1984

The ability of protamine sulfate to effect the quantitative precipitation of 2,3,7,8-[^{3}H]tetrachlorodibenzo-*p*-dioxin (TCDD):*Ah* receptor complexes from rat liver cytosol has been developed into a new assay for the identification, quantitation, and characterization of the *Ah* receptor. The method is reliable, uncomplicated, and rapid, and can be applied to large numbers of samples. The major advantage of the assay is that protamine sulfate appears to selectively precipitate the *Ah* receptor protein and does not precipitate a number of other proteins that bind [^{3}H]TCDD nonspecifically.

KEY WORDS: *Ah;* 2,3,7,8-TCDD; receptor; protamine.

The induction of hepatic microsomal cytochrome P_1-450 (*P*-448) and associated aryl hydrocarbon hydroxylase (AHH)[3] activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and several polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (MC) and benzo[*a*]pyrene (BP) is well documented in several species (1,2,30). Induction appears to be initiated by binding of the inducer to a cytosolic receptor protein termed the *Ah* receptor (3–5). The inducer–receptor complex subsequently translocates into the nucleus (4,6,7), interacts with DNA (8), and initiates the transcription of mRNAs encoding information for the synthesis of cytochrome P_1-450 (9–11). Since chemicals that interact with the *Ah* receptor are typically highly toxic, mutagenic, teratogenic, and/or carcinogenic, considerable effort recently has been devoted to characterizing the receptor.

Methodology employed to detect, quantitate, and characterize the *Ah* receptor relies primarily on measurement of specific binding of appropriate radioligands (e.g., TCDD, MC) to either crude cytosol or ammonium sulfate fractions of cytosol. Several techniques have been used, including dextran-coated charcoal (DCC), to remove free ligand (3,12), DEAE-cellulose chromatography (4,13), sucrose density centrifugation of radioligand-containing cytosol following DCC treatment (4), and isoelectric focusing (8). These techniques present a number of practical problems. All yield relatively high levels of nonspecific binding of the radioligand. Since the latter techniques typically are quite time consuming the possible dissociation of ligand from the receptor, denaturation, and/or alteration of the receptor during the assay cannot be discounted. A vertical tube rotor assay (14) significantly reduces the centrifugation time but is of limited value, because of the few

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[3] Abbreviations used: AHH, aryl hydrocarbon hydroxylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; MC, 3-methylcholanthrene; BP, benzo[*a*]pyrene; DCC, dextran-coated charcoal; HAP, hydroxylapatite; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; BSA, bovine serum albumin; DTT, dithiothreitol; Mops, 4-morpholine propanesulfonic acid; MDEG, 25 mM Mops (pH 7.4), 1 mM EDTA, 1mM DTT and 10% (v/v) glycerol; BNF, β-naphthoflavone; Tween 80, polyethylene sorbitan monooleate; SDC, sucrose-density centrifugation; PS, protamine sulfate; TBC, total binding capacity.

0003-2697/84 $3.00

samples that can be run at one time. A recently described assay utilizing hydroxylapatite (HAP) offers several advantages over previous procedures since it can be conducted relatively rapidly and provides a much lower level of nonspecific binding (15).

During recent attempts to partially purify and quantitate the rat hepatic cytosolic *Ah* receptor, we observed that treatment of cytosol with protamine sulfate caused the quantitative precipitation of the *Ah* receptor protein while apparently not precipitating other proteins that bound TCDD nonspecifically. This report describes the use of protamine sulfate as a new and potentially valuable reagent for use in the identification and quantitation of hepatic cytosolic *Ah* receptors.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley-derived rats (200 g) were purchased from Blue Spruce Farms (Altamont, N. Y.); they were housed in plastic cages on corncob bedding, provided food and water *ad libitum,* and maintained on a 12-h diurnal light cycle.

Chemicals. 2,3,7,8-[1,6-^{3}H]Tetrachlorodibenzo-*p*-dioxine (TCDD) (36.1 Ci/mmol), unlabeled TCDD, and 2,3,7,8-tetrachlorodibenzofuran (TCDBF) were kindly provided by Dr. A. Poland (McArdle Laboratory for Cancer Research, Madison, Wisc.). [^{3}H]-Methylcholanthrene (MC) (26 Ci/mmol) and ACS counting scintillant were purchased from Amersham Corporation (Arlington Heights, Ill.), and [*methyl*-^{14}C]bovine serum albumin (BSA) (95 mCi/mmol) was from New England Nuclear (Boston, Mass.). BSA, dithiothreitol (DTT), NADPH, MC, phenobarbital, estradiol-17β,dexamethasone, protamine sulfate, 4-morpholinepropanesulfonic acid (Mops), and dextran ($M_{r_{av}}$ = 177,000) were obtained from Sigma Chemical Company (St. Louis, Mo.), and benzo[*a*]pyrene (BP), dioxane, and β-naphthoflavone (BNF) were obtained from Aldrich Chemical Company (Milwaukee, Wisc.). Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad Inc. (Rockville Heights, N. Y.). Tween 80 (polyethylene sorbitan monooleate) was from Fisher Scientific (Rochester, N. Y.), and ultrapure sucrose was from Schwarz/Mann Inc. (Song Valley, N. Y.). Charcoal (active carbon PX-21) was kindly provided by Amoco Research Corporation (Chicago, Ill.) and pregnenolone 16α-carbonitrile was a gift from Dr. N. Elshourbagy (Medical College of Virginia, Richmond, Va.). All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Cytosol preparation. The animals were killed by decapitation and the liver was perfused, *in situ,* with ice-cold MDEG buffer; the liver was gently massaged during perfusion to ensure a complete and even removal of blood. The liver was removed and finely minced in ice-cold MDEG buffer and after thorough rinsing the tissue was homogenized in 2 vol of MDEG buffer (w/v) in a Potter–Elvehjem glass homogenizer. The homogenate was centrifuged at 13,500*g* for 20 min and, after decantation through glass wool, the supernatant was recentrifuged at 105,000*g* for 1 h. The resulting supernatant was stored at −80°C in 1.5-ml aliquots until required. No significant loss of specific [^{3}H]TCDD-binding capacity was detected on storage for up to 4 months. Protein concentration was measured by the method of Lowry *et al.* (16).

The sucrose-density centrifugation (SDC) assay was carried out as described by Okey *et al.* (4). Cytosol (8 mg protein/ml) was incubated for 1 h at 4°C with 10 nM [^{3}H]TCDD in the absence or presence of 1 μM unlabeled TCDD. Aliquots (300 μl) were layered onto linear 5–20% sucrose gradients and centrifuged for 15 h at 225,000*g*. Following centrifugation each tube was pierced with a 22-gauge needle and 2-drop fractions were collected in 7-ml polyethylene scintillation vials containing 5 ml of ACS counting scintillant. [^{14}C]BSA was included in each gradient as an internal sedimentation marker (4.4 S) and sedimentation coefficients were calculated by the method of Martin and Ames (17).

The protamine sulfate (PS) assay was carried out using a modification of the technique described by Chamness *et al.* (18) for measuring estrogen receptor. A stock solution of PS (1 mg/ml) was prepared by slowly dissolving PS in MDEG buffer at room temperature; the stock solution was stored at 4°C and was prepared frèshly every 2 weeks.

Cytosol (1 ml; 2 mg protein/ml) was incubated with radioligand (TCDD or MC, 0.5 to 5.0 nM) in the presence or absence of a 200-fold molar excess of unlabeled competitor (TCDBF) for 2 h at 20°C; radioligands and competitors were each added to the incubation mixture in 5 μl of *p*-dioxane/ml of cytosol. At the termination of the incubation period 0.25-ml aliquots of the incubation mixture were transferred to 12 × 75-mm disposable tubes containing 0.25 ml of PS stock solution (0.25 mg of PS). The tubes were incubated at 4°C for 20–30 min, followed by the addition of 1.0 ml of cold MDEG buffer containing 0.5% Tween 80 (v/v). The samples were vortexed and, following centrifugation at 500*g* for 10 min, the supernatants were carefully decanted and discarded. The resulting pellets were each washed with two additional 1.0-ml aliquots of MDEG/Tween 80 buffer before being vortexed with 1.0 ml of 95% ethanol and transferred by Pasteur pipet to 20-ml scintillation vials containing 10 ml of ACS counting scintillant. The tubes and pipets were washed with an additional 1.0 ml of ethanol and the washes added to the appropriate scintillation vials prior to counting.

Cytosol (10 mg/ml) for SDC analysis of receptor precipitated by PS was incubated with 10 nM of [^{3}H]TCDD or [^{3}H]MC for 2 h at 4°C. The unbound or loosely bound radioligand was removed by addition of the samples to a dextran/charcoal pellet (10 mg charcoal and 1 mg dextran, pelleted from MDEG buffer). The charcoal pellet was resuspended on a vortex mixer and the samples were incubated for 15 min at 4°C prior to centrifugation at 500*g* for 10 min. Samples of supernatant (0.6 ml) were carefully transferred to 12 × 75-mm disposable tubes containing 0.3 ml of PS (5 mg/ml MDEG) and, following incubation for 5 min at 4°C, the mixtures were centrifuged at 500*g* for 10 min. Aliquots (300 μl) of the supernatant were layered on linear 5–20% (w/w) sucrose gradients prepared in MDEG buffer. [^{14}C]BSA (1,000 dpm) was included in each gradient as an internal sedimentation marker (4.4 S). Gradients were centrifuged and analyzed as described.

The hydroxylapatite assay was carried out as described by Gasiewicz and Neal (15). The incubation and washing conditions were similar to those detailed in the PS assay. In studies comparing the HAP and PS assays, 0.25 ml of the incubation mixture was added to each HAP pellet rather than the 0.20 ml generally used.

Competitive binding studies. The ability of other ligands to compete with TCDD for hepatic cytosol was determined by their ability to inhibit specific [^{3}H]TCDD-specific binding. In competition studies, [^{3}H]TCDD (1 nM) and the desired test compounds (200 nM) were added to the incubation mixtures in 5 μl of *p*-dioxane. The total cytosolic binding of [^{3}H]TCDD in the presence and absence of various competitors was determined and the specific binding measured in the presence of the competitor was divided by the total specific binding. The resulting values were expressed as the percentage inhibition of [^{3}H]TCDD specific binding.

Radioisotopes and liquid scintillation counting. [^{3}H]TCDD was stored at 2–4°C in toluene/ethanol (v/v) and [^{3}H]MC was stored in toluene at −20°C. Aliquots of the labeled solutions were evaporated to dryness under a stream of nitrogen and redissolved at the desired concentration in *p*-dioxane; the solutions were allowed to stand overnight at room temperature prior to use.

Radioactivity was determined with a Packard liquid scintillation spectrometer (Model 2425). Sample quench was corrected by automatic external standardization and counting error was maintained at a maximum of 5%.

The efficiencies of counting for ^3H and ^{14}C were 35 and 85%, respectively.

RESULTS

Receptor Measurement

As indicated by the data in Table 1, the protamine sulfate (PS) and HAP assays provided comparable estimates of [^{3}H]TCDD specific binding to rat liver cytosol when Tween 80 was included in the wash buffer. In agreement with previous reports with the HAP assay (15) measurement of specific binding by the PS assay was not possible in the absence of Tween 80, which in each case effectively removes [^{3}H]TCDD absorbed to HAP. Direct measurement of PS or the amount removed in sequential washes was not possible since, in the absence of protein, PS remains in solution with the free ligand. Although values of [^{3}H]TCDD specific binding were similar when measured by either the PS or the HAP assay (Tables 1 and 2) the HAP assay yielded values approximately 2.5-fold greater than the PS assay when [^{3}H]MC was used as the radioligand (Table 2).

Estimates of the apparent dissociation constants (K_d) and total binding capacities (TBC) of [^{3}H]TCDD with rat liver cytosol were obtained from Scatchard plot analysis (19) of data from each assay (Table 3). The K_d values estimated by the PS assay were slightly lower than those obtained by the HAP assay although the difference was not significant. The TBC values determined using the PS assay were not significantly different from those obtained using established assays (HAP and SDC). The TBC values observed are significantly higher than published values and may reflect differences resulting from the particular strain of animal used.

Specific binding of [^{3}H]TCDD to rat liver cytosol was maximal following a 1-h incubation at 20°C. Values remained relatively constant for up to about 4 h but decreased somewhat (33% by 8 h), possibly due to receptor inactivation or degradation (Fig. 1).

TABLE 1

EFFECT OF TWEEN 80 ON [^{3}H]TCDD BINDING: COMPARISON OF PROTAMINE SULFATE AND HYDROXYLAPATITE ASSAYS

Buffer	Binding[b]	Binding assay[a] PS	HAP
With Tween 80	TB	1918 ± 59	1960 ± 106
	NSB	511 ± 21	589 ± 30
	SB	1401 ± 59	1371 ± 59
Without Tween 80	TB	4204 ± 363	4608 ± 186
	NSB	4002 ± 140	3834 ± 344
	SB	369 ± 427	168 ± 222

[a] Values are expressed as the mean ± SD of the number of dpms of [^{3}H]TCDD bound/0.25 ml of incubation mixture from duplicates of each of three determinations.

[b] Binding is expressed as the total binding (TB), nonspecific binding (NSB), and specific binding (SB) of [^{3}H]TCDD to cytosol as described under Materials and Methods.

TABLE 2

SPECIFIC BINDING OF [^{3}H]TCDD AND [^{3}H]MC TO RAT HEPATIC CYTOSOL MEASURED BY THE PROTAMINE SULFATE AND HYDROXYLAPATITE ASSAYS[a]

Binding assay	[^{3}H]TCDD	[^{3}H]MC
Protamine sulfate	4234 ± 145	676 ± 255
Hydroxylapatite	4201 ± 176	1674 ± 104

[a] Values are expressed as the mean ± SD of the number of dpms of each radioligand bound/mg of cytosolic protein from duplicates of three determinations.

TABLE 3

TOTAL SPECIFIC BINDING CAPACITY (TBC) AND APPARENT DISSOCIATION CONSTANTS (K_d) ESTIMATED FROM SCATCHARD PLOT ANALYSIS OF [^{3}H]TCDD SPECIFIC BINDING TO HEPATIC CYTOSOL USING THE PROTAMINE SULFATE AND HYDROXYLAPATITE ASSAYS[a]

Binding assay	TBC (fmol/mg protein)	K_d (nM)
Protamine sulfate	134 ± 5	1.07 ± 0.06
Hydroxylapatite	138 ± 4	0.85 ± 0.06

[a] Values are expressed as the mean ± SD of duplicate samples of each of three determinations. In each experiment, portions of the same cytosol were used for each assay method.

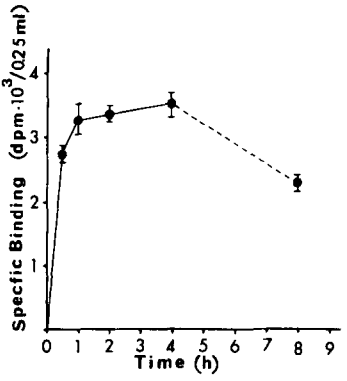

FIG. 1. Effect of time of incubation on the specific binding of [^{3}H]TCDD to rat hepatic cytosol as determined by the PS assay. Each point represents the mean ± SD of duplicates of two determinations.

The specific binding of [^{3}H]TCDD increased linearly with increasing protein concentration up to approximately 3 mg cytosolic protein/ml (Fig. 2).

Maximal precipitation of [^{3}H]TCDD specific binding occurred at a PS concentration of 0.25 mg/assay (Fig. 3) and was not enhanced by further increases to 0.75 mg/assay.

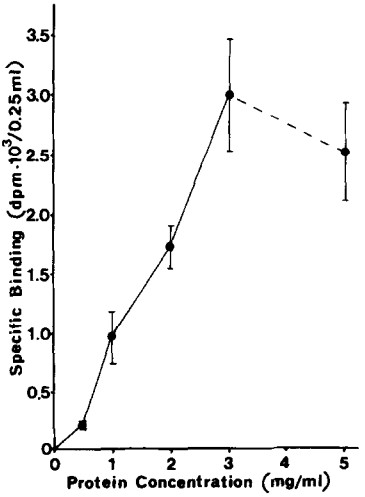

FIG. 2. Effect of cytosolic protein concentration of the specific binding of [^{3}H]TCDD as measured by the PS assay. Cytosol, of various protein concentrations, was incubated with [^{3}H]TCDD (1 nM) in the presence or absence of TCDBF (200 nM). Each point represents the mean ± SD of duplicates of two determinations.

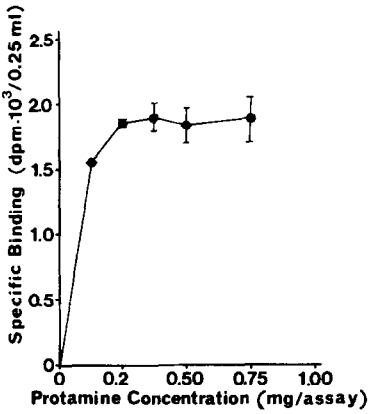

FIG. 3. Effect of protamine sulfate concentration on the specific binding of [^{3}H]TCDD to rat hepatic cytosol. Cytosol was incubated with [^{3}H]TCDD (1 nM) in the presence or absence of TCDBF (200 nM) and aliquots (0.25 ml) were added to 0.25 ml of protamine sulfate solution of increasing concentration and assayed as described under Materials and Methods. Each point represents the mean ± SD of duplicates of two determinations.

Receptor-Binding Competition

The abilities of several compounds to interact with the *Ah* receptor were evaluated for their abilities to compete with [^{3}H]TCDD for specific binding sites as measured by the PS and HAP assays. The data (Table 4) clearly show that PS-precipitable [^{3}H]TCDD binding was effectively inhibited by compounds known to be agonists for P_1-450 induction (TCDBF, MC, benzo[*a*]pyrene, BNF) and was unaffected by those compounds (e.g., phenobarbital, pregnenolone 16α-carbonitrile, 17β-estradiol, dexamethasone) known not to be specific *Ah* receptor ligands. In all cases, the results obtained by the PS method were comparable to those observed with the HAP technique.

Sucrose Density Centrifugation (SDC) Analysis

Radiolabeled complexes formed as a result of incubating rat liver cytosol with [^{3}H]TCDD or [^{3}H]MC sedimented into two distinct regions (3–4 S and 7–8 S) following centrifu-

TABLE 4

INHIBITION OF [^{3}H]TCDD SPECIFIC BINDING BY VARIOUS COMPOUNDS AS MEASURED BY THE PROTAMINE SULFATE (PS) AND HYDROXYLAPATITE (HAP) ASSAYS[a]

Competitor	Inhibition of specific binding (percentage of control)	
	PS	HAP
No competitor	0	0
2,3,7,8-Tetrachlorodibenzofuran	100	100
3-Methylcholanthrene	100	98
Benzo[*a*]pyrene	93	100
β-Naphthoflavone	100	100
Phenobarbital	0	2
Pregnenolone 16α-carbonitrile	2	5
17β-Estradiol	0	0
Dexamethasone	0	0

[a] Specific binding was measured as described under Materials and Methods using 1.0 nM [^{3}H]TCDD in the presence or absence of a 200-fold molar excess of competitor indicated. Values represent the mean percentage inhibition of specific binding of [^{3}H]TCDD estimated from duplicates of at least two determinations.

gation on linear sucrose gradients prepared in low-ionic-strength buffer.

The binding of [^{3}H]TCDD and [^{3}H]MC to the 7–8 S region was essentially eliminated in the presence of a 100-fold molar excess of unlabeled TCDD or MC, respectively (Figs. 4A, B); [^{3}H]TCDD binding to the 3–4 S peak was not affected by the presence of excess cold TCDD (Fig. 4A). Although the binding of [^{3}H]MC to the 3–4 S peak was substantially decreased in the presence of unlabeled MC (Fig. 4B), it was not affected by unlabeled TCDD (data not shown). TCDBF in a 100-fold molar excess displaced both [^{3}H]TCDD and [^{3}H]MC from the 7–8 S peak (Fig. 5) but displaced only [^{3}H]MC from the 3–4 S peak; approximately 4–5 times more [^{3}H]-TCDD than [^{3}H]MC was bound to the 7–8 S region (Table 5).

Treatment with PS of cytosol incubated with either [^{3}H]TCDD or [^{3}H]MC followed by SDC analysis of the resulting supernatants indicated the effective removal of ligand binding to the 7–8 S region and limited removal of binding to the 3–4 S region (Fig. 6).

DISCUSSION

The data provided clearly indicate that PS quantitatively precipitates [^{3}H]TCDD:*Ah* re-

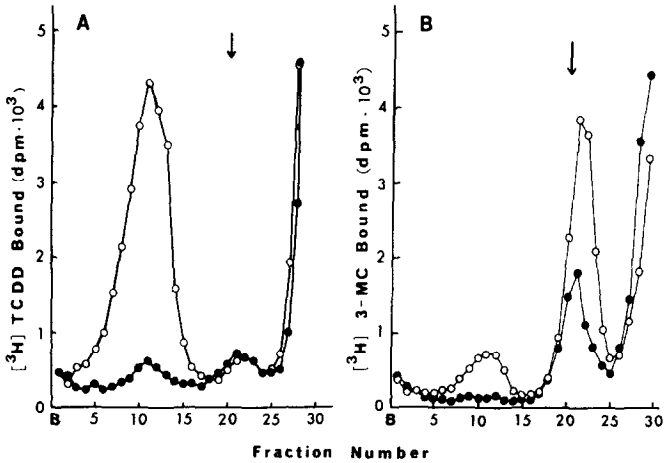

FIG. 4. Determination of the binding of radioligands in rat hepatic cytosol. Cytosol was incubated with (A) 10 nM [^{3}H]TCDD in the presence (●) or absence (○) of 1 μM of unlabeled TCDD or (B) 10 nM [^{3}H]MC in the presence (●) or absence (○) of 1 μM of unlabeled MC and each was analyzed by sucrose density centrifugation as described under Materials and Methods. The arrow indicates the peak sedimentation of [^{14}C]BSA (4.4 S) and "B" indicates the bottom of the tube.

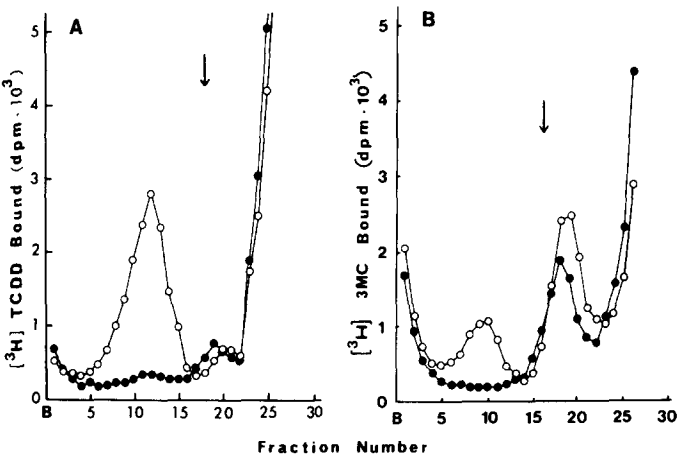

FIG. 5. Determination of the binding of radioligand to rat hepatic cytosol. Cytosol was incubated with (A) 10 nM [^{3}H]TCDD or (B) [^{3}H]MC in the presence (●) or absence (○) of 1 μM unlabeled TCDBF and was analyzed by sucrose density centrifugation as described under Materials and Methods. The arrow indicates the peak sedimentation of [^{14}C]BSA (4.4 S).

ceptor complexes from rat hepatic cytosol and consequently constitutes a potentially useful reagent for the assay and characterization of the *Ah* receptor. Like the HAP assay, the PS assay is simple, rapid, and reproducible and has several advantages over most existing assay procedures. It does not suffer from the high nonspecific binding background of the DCC assay (12), obviates the need for the potentially damaging effects of trypsinization required by the isoelectric focusing technique (20), and is much less time consuming than the SDC assay (4).

Recent studies (13,21) have indicated that the *Ah* receptor in rat hepatic cytosol sediments at approximately 8–10 S following centrifugation in low-ionic-strength sucrose gradients; a 4–5 S component binding [^{3}H]-TCDD nonspecifically also was identified but subsequently has been shown not to be involved in the mechanism of AHH induction (21) based on ligand specificity and genetic evidence. The 7–8 S and 3–4 S components observed in the present study are undoubtedly the same as 8–10 S and 4–5 S components reported by (21). The identity of the 3–4 S (4–5 S) component(s) is presently unknown but may comprise one or more of a variety of cytosolic proteins such as ligandin and the "h-protein," both of which are known to bind numerous polycylic aromatic hydrocarbons (22–25).

TABLE 5

ESTIMATION OF THE SPECIFIC BINDING OF [^{3}H]TCDD AND [^{3}H]MC TO THE RAT HEPATIC CYTOSOLIC *Ah* RECEPTOR BY THE SUCROSE DENSITY CENTRIFUGATION TECHNIQUE

Radioligand	Specific binding[a] (fmol/mg cytosolic protein)
[^{3}H]TCDD	115 ± 21
[^{3}H]MC	24 ± 9

[a] Values are expressed as the mean ± SD of four determinations.

PS treatment apparently causes the quantitative precipitation of a variety of cytosolic proteins, including the 7–8 S (8–10s) component(s) that specifically binds [^{3}H]TCDD and the estrogen receptor. The ability of PS to only partially precipitate the 3–4 S fraction that binds [^{3}H]TCDD nonspecifically is similar to its failure to precipitate several other cytosolic and serum proteins that bind estrogens nonspecifically (26,27,18).

It has been suggested that the abilities of various proteins to form insoluble complexes

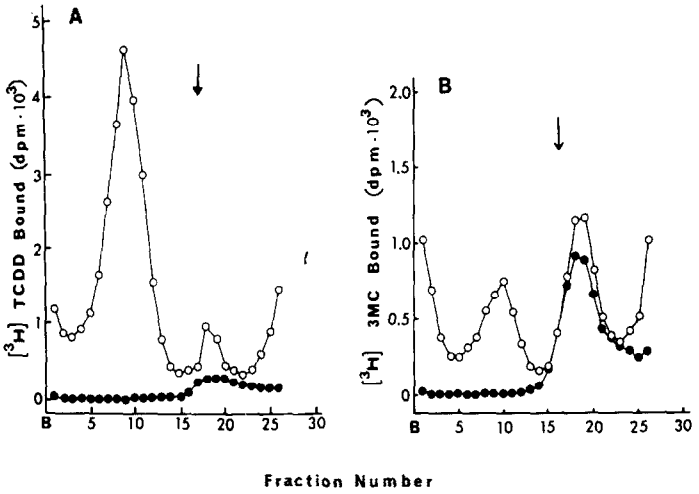

FIG. 6. Effect of protamine sulfate on the binding of radioligand to rat hepatic cytosol. Cytosol was incubated with (A) 10 nM [^{3}H]TCDD or (B) 10 nM [^{3}H]MC for 2 h at 4°C. Aliquots (0.3 ml) of the incubation mixture, after dextran-coated charcoal treatment, were either directly analyzed by sucrose density centrifugation (○) or incubated with protamine sulfate and centrifuged at 500*g* for 10 min and then 0.3 ml of the resulting supernatant was analyzed by sucrose density centrifugation (●) as described under Materials and Methods. The arrow indicates the peak sedimentation of [^{14}C]BSA (4.4 S).

with polycations, such as protamine, are due to their "acidic natures" (26) and/or the presence of an acidic region on the proteins. Although selective precipitation of acidic proteins by polycations has been reported (28,29) the failure of PS to form insoluble complexes with some acidic proteins, such as BSA (26), strongly suggests that additional factors must be involved in determining specificity. The incomplete precipitation of radioligand associated with the 3–4 S region may indicate the presence of a variety of different proteins, some of which may bind radioligand specifically or nonspecifically, yet vary in their reactions with protamine.

Accurate estimation of *Ah* receptor levels by both the PS and HAP assays can be made only if [^{3}H]TCDD is used as the radioligand because [^{3}H]TCDD specifically binds only to the *Ah* receptor (7–8 S component). The assays are not adequate with [^{3}H]MC as the radioligand and unlabeled MC as the competitor since under these conditions the specific binding that is measured results from the combined displacement of [^{3}H]MC from both the 7–8 S and the 3–4 S components. Quantitation of the specific binding of [^{3}H]MC to the *Ah* receptor can be made using unlabeled TCDD as the competitor since TCDD displaces [^{3}H]MC binding only from the 8–10 S component (21). Measurement of the specific binding of [^{3}H]MC to the *Ah* receptor would probably be feasible using unlabeled TCDD as the competitor. However, due to the extremely low solubility of TCDD in dilute protein solutions (3), TCDBF, a congener of TCDD that binds to the *Ah* receptor and has a somewhat greater aqueous solubility, is usually used in the PS and HAP assays. TCDBF allows accurate quantitation of [^{3}H]TCDD specific binding, since it displaces [^{3}H]TCDD only from the *Ah* receptor; TCDBF is inadequate for measurement of the specific binding of [^{3}H]MC since it also displaces this radioligand from the 3–4 S component. Consequently, only the SDC technique, in which binding to and displacement from each component can be independently quantitated, provides an accurate estimation of the specific binding of [^{3}H]MC to the *Ah* receptor.

Since [^{3}H]TCDD undoubtedly binds non-

specifically to a number of different cytosolic proteins, studies using the PS, HAP, and DCC assays to measure the capacity of various compounds to compete with [^{3}H]TCDD specific binding should always be validated by SDC analysis. Since displacement of radioligand from the 3–4 S component will result in an overestimation of the competitor's apparent binding affinity, it should be clearly established whether a particular competitor is displacing [^{3}H]TCDD from the 7–8 S component, the 3–4 S component, or from both.

In contrast to recent reports (21) that [^{3}H]TCDD and [^{3}H]MC exhibit similar K_d and TBC values with the *Ah* receptor, the results in the present study (using the SDC assay) show that significantly more [^{3}H]-TCDD than [^{3}H]MC is bound to the *Ah* receptor. Since identical concentrations of each radioligand were used in the experiments described here it is possible that the apparent difference in binding of the two ligands results from differences in their TBC and/or binding affinities. Several earlier studies have indicated that the *Ah* receptor exhibits a considerably higher affinity for TCDD than for MC (3,15). The reason for these differences remains to be determined.

ACKNOWLEDGMENTS

This work was supported by a grant (ES 01902) from the National Institute of Health; M. S. Denison gratefully acknowledges support from a predoctoral NRSA (ES 07052) fellowship.

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