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Cytosolic Receptor for 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Evidence for a Homologous Nature among Various Mammalian Species

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SUMMARY

The presence and properties of the *Ah* receptor were examined in the guinea pig, rat, hamster, monkey, and three different strains of mice. These species and strains have demonstrated differences in sensitivity and variability of response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. All species examined, with the exception of DBA/2J mice, possess similar amounts of binding protein with high affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in hepatic tissue. Numerous dibenzo-*p*-dioxin congeners and polycyclic aromatic hydrocarbons demonstrated a similar rank order ability to bind to receptor molecules from these species. When analyzed by gel-exclusion high-performance liquid chromatography, hepatic cytosolic receptors from all species eluted at volumes corresponding to a similar molecular weight range. Association of the hepatic *Ah* receptor with the nuclear fraction was observed in all cases following the i.p. treatment of guinea pig, rat, C57BL/6J mouse, or hamster with [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin. In all species and tissues examined, with the exception of hamster duodenum and thymus, the highest concentrations of receptor were localized in the liver, lung, thymus, intestine, and kidney. Exceptionally high concentrations of receptors were also observed in guinea pig testes. These findings indicate that, despite species and tissue specific differences in the biochemical and toxicological responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds, a number of different mammalian species possess *Ah* receptors with similar properties. Thus, the correlative differences between certain strains of mice in terms of altered specific binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and sensitivity to this compound may be unique and not necessarily applicable to other species. Although all data indicate that the receptor mediates these responses, it appears that species- and tissue-specific differences may be determined by a number of additional factors. These results also suggest the conservation of some, as yet unknown, functional role of the receptor molecule.

INTRODUCTION

Polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, benzo[*a*]pyrene, and β-naphthoflavone, and certain halogenated heterocyclic aromatics such as TCDD[1] have been shown to be potent inducers of a number of enzymes in a variety of tissues and species (1). Some of these enzymes include two or more forms of cytochrome P-450 and associated monooxygenase activities (1). In certain strains of mice, the induction of these enzymes is regulated by a single genetic locus, the *Ah* locus (2), and mediated by the cytosolic *Ah* receptor (3, 4). Evidence to date suggests that the association of the inducer-receptor complex with the nucleus, and modu-

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[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; [^{3}H]TCDD, 2,3,7,8-[1,6-^{3}H]tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HAP, hydroxylapatite; HEDG, 25 mM Hepes/1.5 mM EDTA/1 mM dithiothreitol/10% glycerol (v/v), pH 7.6; PED, 200 mM sodium phosphate/1.5 mM EDTA/1 mM dithiothreitol, pH 7.4; HDM, 25 mM Hepes/1 mM dithiothreitol/3 mM $MgCl_2$, pH 7.5; HDMG, 25 mM Hepes/1 mM dithiothreitol/3 mM $MgCl_2$/10% glycerol (v/v), pH 7.5.

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lation of gene expression are essential events leading to the induction of these enzymes (5–7).

Research in this area has been facilitated by the availability of "responsive" and "nonresponsive" strains of mice. The "nonresponsive" strains (typified by DBA/2J mice) appear to have an altered receptor with lower affinity and subsequently decreased sensitivity to the inducers as compared with the "responsive" strains (typified by C57BL/6J mice) (3). In these and other species, as well as a number of cell culture systems, the induction of the enzyme systems noted above (particularly cytochrome P-450-associated aryl hydrocarbon hydroxylase) has been used to assess responsiveness to these compounds as well as to indicate the presence of the receptor. However, there are a number of other biochemical, biological, and toxicological responses to these compounds in a variety of tissues and species which are believed to be mediated by the cytosolic receptor (reviewed in ref. 8). The type and sensitivity of the response observed appear to be both species- and tissue-specific (8). Altered receptor content or properties, as in the case of the "responsive" or "nonresponsive" strains of mice, and/or major differences in the battery of enzymes expressed or repressed, may be responsible for this specificity in the response.

Results presented in this paper suggest that, despite absolute differences in the response to TCDD, and in contrast to different strains of mice, there is a notable conformity in the properties of these receptors among various mammalian species. These and other data suggest that, although all responses may be mediated by the receptor, many factors may contribute to the varied response observed. In addition, there may be a conservation of some, as yet unknown, functional role of this receptor molecule.

MATERIALS AND METHODS

Chemicals. [1,6-^{3}H]TCDD (50.5 Ci/mmole) was custom-synthesized and purified as previously described (9). This compound was >98% chemically pure as determined by HPLC (10). TCDF was a generous gift to Dr. J. A. Moore (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Unlabeled TCDD was obtained from KOR Isotopes (Cambridge, Mass.). Methylated [*methyl*-^{14}C]myosin, α-globulins, ovalbumin, bovine serum albumin, and cytochrome *c* were purchased from New England Nuclear Corporation (Boston, Mass.). Unlabeled blue dextran, aldolase, thyroglobulin, ferritin, bovine serum albumin, and cytochrome *c* were purchased from Pharmacia (Uppsala, Sweden). All HPLC-grade solvents were purchased from Fisher Scientific Company (Rochester, N. Y.). Phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, and leupeptin were purchased from Sigma Chemical Company (St. Louis, Mo.). The remainder of the chemicals were purchased from sources cited previously (11).

Animals. Male Sprague-Dawley rats (200–250 g), golden Syrian hamsters (110–130 g), and Hartley guinea pigs (300–350 g) were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). Male C57BL/6J, DBA/2J, and B6D2F$_1$/J mice (20–22 g) were obtained from Jackson Laboratories (Bar Harbor, Maine). These animals were housed under laboratory conditions for at least 3 days prior to use. They were fed commercial chow (Ralston Purina, Richmond, Ind.) and water ad libitum. Fresh liver samples from monkeys (*Macaca fascicularis*)[2] were provided by Dr. Paul Coleman (The University of Rochester).

Preparation of cytosol. With the exception of monkeys,[2] animals were killed by decapitation or cervical dislocation and tissues were excised and weighed. These tissues were homogenized in 3 volumes of HEDG buffer in a glass homogenizer fitted with a Teflon pestle. In some cases, specific tissues were pooled for a single preparation. The remainder of the cytosol preparation was carried out as previously described (11), and unless specified these and the remaining procedures were carried out at 0–4°. The cytosol preparations were used within 1 hr after preparation.

Treatment with [^{3}H]TCDD in vivo and preparation of nuclear extracts. Animals received i.p. injections of 30 μg (96 μCi) of [^{3}H]TCDD per kilogram of body weight 16 hr prior to sacrifice or 300 μg of unlabeled TCDD per kilogram 4 hr prior to the administration of [^{3}H]TCDD. Labeled and unlabeled TCDD was dissolved in 1,4-dioxane and added to olive oil. The 1,4-dioxane was removed by evaporation under nitrogen, and the olive oil (2.0/ ml/kg of body weight) was injected. Animals were killed 16 hr after injection for removal of tissues. Nuclear *Ah* receptor concentrations have been found to be near maximal in mice and rats at this time after treatment with [^{3}H]TCDD (5, 12). Procedures similar to that described by Okey *et al.* (13) and Legraverend *et al.* (14) were used to prepare nuclei. Livers were homogenized in HEDG or HDM buffer. The homogenate was centrifuged at 1,000 × *g* for 15 min and the supernatant fraction was removed. The crude nuclear pellet was resuspended in HEDG or HDM buffer and washed four times by repeated centrifugation at 1,000 × *g* for 10 min. The procedure described by Tierney *et al.* (15) utilizing dense sucrose was also used for the preparation of highly purified nuclei. As previously described (12, 13), the nuclear pellet was resuspended in HEDG or HDM buffer containing 0.5 M KCl, homogenized, and allowed to stand for 1 hr at 0° to extract the *Ah* receptor. The extract was centrifuged at 105,000 × *g* for 1 hr and the supernatant fraction was collected for use.

Protein analysis. The protein concentration of the cytosol and nuclear extracts was estimated by the absorbance at 215/225 nm (16), which was confirmed by the method of Lowry *et al.* (17).

Incubation conditions and assay of specific binding of [^{3}H]TCDD using hydroxylapaite. The incubation conditions used for the cytosol or nuclear extracts were similar to those previously described (11). Two milliliters of the 105,000 × *g* supernatant fraction (2–4 mg of protein per milliliter) were incubated with various concentrations (0.1–8.0 nM) of [^{3}H]TCDD and with [^{3}H]TCDD plus a 200-fold excess ot TCDF ([^{3}H]TCDD + TCDF) for 2 hr at 20° or 18–24 hr at 0°. These conditions were established to give equilibration of binding (11). The [^{3}H]TCDD and [^{3}H]TCDD + TCDF were added to the incubations in a volume of 5 μl of 1,4-dioxane/ml of cytosol. The determinations of the specific binding of [^{3}H]TCDD using the HAP assay system were carried out as previously described (11).

Competitive binding studies. The relative affinities of other compounds for the cytosolic receptor from various species were measured by their ability to compete with [^{3}H]TCDD for specific binding sites. This procedure was carried out as previously described (11).

Gel-exclusion HPLC. A Varian Model 5000 (Varian Instruments, Palo Alto, Calif.) liquid chromatograph was used to obtain an isocratic elution on a Micropak TSK 4000 SW exclusion column (7.5 × 300 mm) (Varian TSK pre-column 4000 SW; 7.5 × 100 mm). Each sample (250–500 μl) was applied with a syringe-loaded injector fitted with an interchangeable sample loop. Samples of the eluant were collected using an Isco Model 328 fraction collector (Lincoln, Neb.). In all cases, the column was kept at 2–4° by containment in a cold room or by the use of a water-jacket system. The column was eluted with PED buffer or PED buffer plus 0.4 M KCl at a flow rate of 0.7 ml/min. All buffers

[2] The animals were approximately 3.5 years old (4 kg) at the time of sacrifice. These animals had been treated for 78 days with a total dose of 45 mg of methylmercuric chloride. This treatment had ceased 190 days prior to sacrifice. The animals were killed by an overdose of pentobarbital.

and solvents were degassed and filtered prior to use. A more complete description of the system has been made (18).

Determination of radioactivity. Scintillation cocktail was added to vials containing HPLC column eluant or samples from the HAP assay. The radioactivity was determined using a Packard Tri-Carb Model 2450 scintillation spectrometer. Quenching was corrected by automatic external standardization. The counting efficiency for tritium ranged from 25% to 30%.

Data analysis. Specific binding was defined as the difference between total binding (samples containing [^{3}H]TCDD) and nonspecific binding (samples containing [^{3}H]TCDD + TCDF). When various concentrations of [^{3}H]TCDD were used, the number of binding sites (n) and apparent equilibrium dissociation constants (K_D) were determined by the method of Scatchard (19). Using n determined from Scatchard plots, the binding data was also analyzed by the method of Hill (20). In both cases, linear regression analysis was used to determine the best fit to the data. For the competitive binding studies, the total binding of [^{3}H]TCDD in the presence of the competitor was calculated and corrected for nonspecific binding. The ratio of association constants (expressed as percentage of that determined for TCDD) was estimated by the relationship described by Korenman (21). For the HPLC experiments, partition coefficients (K_d) were determined as defined by Ackers (22). Labeled and unlabeled proteins were used for column calibration. Relative molecular weights of the specific binding peaks for [^{3}H]TCDD were determined from a plot of K_d versus log molecular weight of the protein standards (data not shown). Representative results of multiple experiments are presented in the figures.

RESULTS

Presence of cytosolic receptors in hepatic tissue from various mammalian species. We compared the number of receptors, their respective binding affinities, and Hill coefficients for TCDD in hepatic cytosol prepared from male guinea pigs (Hartley), rats (Sprague-Dawley), hamster (golden Syrian), and a nonhuman primate (*Macaca fascicularis*), as well as C57BL/6J, DBA/2J, and B6D2F$_1$/J mice. These species and strains were selected because of the demonstrated differences in sensitivity and variability of their biochemical and toxicological responses to TCDD (8). Hepatic cytosolic receptor concentrations ranged from 23 to 74 fmoles/mg of protein (Table 1). As has been observed previously (3, 6, 11), little specific binding was found using cytosol from DBA/2J mice. In this case, the specific binding was 20–40% of the nonspecific binding and was considered too low to quantitate accurately. Receptors from all species (with the exception of DBA/2J mice) demonstrated high affinities for TCDD, with apparent equilibrium dissociation constants (K_D) ranging from 0.1 to 0.4 nM. Figure 1 shows representative Hill plots of the binding data obtained from individual species. Under the conditions used for the assay of specific binding (see Materials and Methods), the Hill coefficients for the receptors from all species examined were not significantly different from 1.0 (Table 1).

Competitive binding studies. The relative binding affinity of other compounds for the receptor in hepatic tissue from each species was measured by their capacity to compete with the specific binding of [^{3}H]TCDD. Figure 2 shows representative competitive binding curves for these compounds using guinea pig hepatic cytosol. The ratio of association constants was determined from the midpoint of each displacement curve and expressed as a percentage of the affinity for [^{3}H]TCDD. The rank order for the binding of all compounds tested was the same within each species tested (Table 2). TCDD and TCDF showed the highest affinities for the receptor, whereas dibenzo-*p*-dioxin demonstrated the lowest affinity. Phe-

TABLE 1

Concentration, apparent equilibrium dissociation constants, and Hill coefficients of hepatic cytosolic receptors for TCDD in various mammalian species

Specific binding was determined by the HAP assay system and with the use of various concentrations of [^{3}H]TCDD ± a 200-fold excess of TCDF. The number of specific binding sites (n) and apparent equilibrium dissociation constants (K_D) were determined by the method of Scatchard (19). Hill coefficients (n_H) were determined as the slope of a plot of log free [^{3}H]TCDD concentration versus log $\bar{Y}/(1-\bar{Y})$, where $\bar{Y}$ = the fraction of binding sites occupied at a given [^{3}H]TCDD concentration (see Fig. 1). Values are expressed as means ± standard deviation of number of determinations shown in parentheses.

Species	n^a	K_D	n_H
		nM	
Guinea pig (8)	59 ± 11	0.06 ± 0.02	0.99 ± 0.03
Rat (7)	61 ± 23	0.12 ± 0.03	1.01 ± 0.03
Monkey (3)	42 ± 8	0.26 ± 0.04	1.02 ± 0.03
Mouse			
C57BL/6J (4)	74 ± 10	0.29 ± 0.04	1.02 ± 0.04
B6D2F$_1$/J (3)	23 ± 2	0.42 ± 0.03	ND[b]
DBA/2J (3)	—[c]	—[c]	—[c]
Hamster (7)	67 ± 22	0.33 ± 0.07	1.03 ± 0.06

[a] Femtomoles of [^{3}H]TCDD specifically bound per milligram of cytosolic protein (assuming one binding site per molecule of receptor).
[b] ND, Not determined.
[c] —, Unable to be determined (see text).

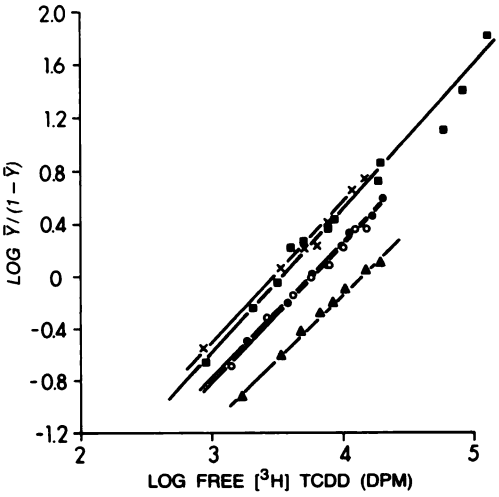

FIG. 1. *Hill plot analysis of [^{3}H]TCDD-specific binding in hepatic cytosol from various mammalian species*

Specific binding was determined by the HAP assay system and with the use of various concentrations (0.1–8.0 nM) of [^{3}H]TCDD ± a 200-fold excess of TCDF. The Hill coefficient (n_H) is the slope and $\bar{Y}$ is the fraction of [^{3}H]TCDD-binding sites occupied. "*Free*" [^{3}H]TCDD denotes total minus specifically bound. Representative data for the guinea pig (●), rat (■), monkey (○), C57BL/6J mouse (×), and hamster (▲) are plotted. n_H = 1.02 for guinea pig, 1.07 for rat, 1.03 for monkey, 1.05 for C57BL/6J mouse, and 1.01 for hamster.

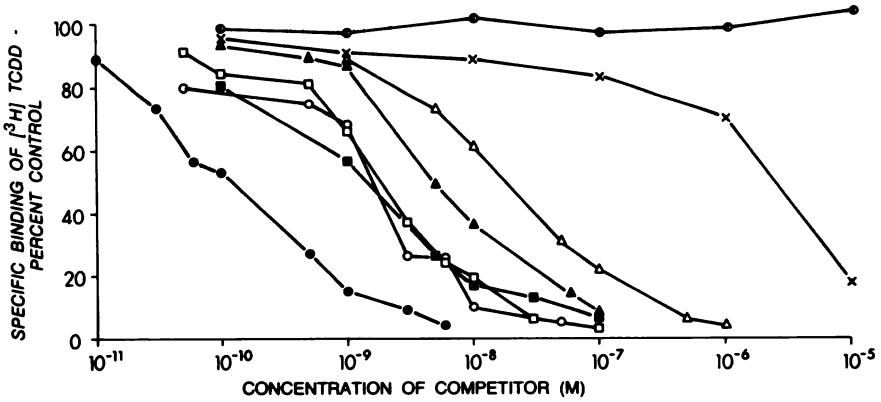

FIG. 2. *Competition by dibenzo-p-dioxin congeners and other compounds for the specific binding of [3H]TCDD to guinea pig hepatic cytosol*
The specific binding of [3H]TCDD to guinea pig hepatic cytosol (1.97 mg protein/ml) was determined by the HAP assay system. The concentration of [3H]TCDD was 0.3 nM. The concentration of other compounds is given on the abscissa. 2,3,7,8-Tetrachlorodibenzofuran, ●; 3-methylcholanthrene, ○; 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin, ■; β-naphthoflavone, □; benzo[*a*]pyrene, ▲; 1,2,4-trichlorodibenzo-*p*-dioxin, △; dibenzo-*p*-dioxin, ×; phenobarbital, ◐.

nobarbital showed no ability to compete for the specific binding with [3H]TCDD. In some cases, quantitative differences may exist between given species in an individual compound's ability to compete with [3H]TCDD. For example, dibenzo-*p*-dioxin and β-naphthoflavone appeared to have considerably less affinity for the receptor from hamster hepatic cytosol as compared with that from other species. However, with these exceptions and in consideration of the impurity of the receptor preparations, the relative affinities of individual compounds for the receptor in those species examined were quite similar.

Gel-exclusion HPLC analysis. To determine further whether the binding proteins for TCDD from each of these species were of a similar physicochemical nature, we determined their relative molecular weights by gel-exclusion HPLC. Figure 3 shows the profiles of total and nonspecific binding of [3H]TCDD to hepatic cytosol from these mammalian species. In all cases, specific binding of [3H]TCDD (total − nonspecific) was observed. The molecular weights of the specific binding peaks for [3H]TCDD from guinea pig, rat, and hamster ranged from 220,000 to 280,000. Those peaks from the C57BL/6J mouse and monkey hepatic cytosol eluted at a volume corresponding to a slightly higher molecular weight range, 300,000–330,000 and 280,000–300,000, respectively. Ranges are given in consideration of multiple determinations. Under these conditions, the recovery of specific binding of [3H]TCDD as determined by HPLC averaged between 77.9% and 94.7% of that determined by the HAP assay using the same cytosol preparations. When incubations of [3H]TCDD with hamster, guinea pig, or monkey hepatic cytosol were performed for longer time periods (up to 18 hr), much more (107–125%) specific binding was observed by the HPLC method than could be detected by the HAP assay. In these cases the additional "specific binding" occurred at fractions 42–48, corresponding to a molecular weight range of 100,000–140,000 (data not shown). As determined by HPLC, this binding was partially displaceable by TCDF. It is likely that the binding of [3H]TCDD at these fractions represents sites of much lower affinity, since they were not detected by the HAP assay system. The binding at these fractions, corresponding to a lower molecular weight and not associated with the *Ah* receptor, is likely

TABLE 2

Hepatic cytosolic binding affinity of dibenzo-p-dioxin congeners and other compounds relative to TCDD in various mammalian species

The relative affinity of other compounds for the receptor in hepatic tissue from each species was measured by their capacity to compete with the specific binding of [3H]TCDD. From the midpoint of each displacement curve (see Fig. 2), the ratio of the association constants, expressed as percentage of the affinity of TCDD for the receptor, was determined for each compound by the procedures described by Korenman (21). Thus, TCDD is assigned a relative binding affinity of 100.

Compound	Guinea pig	Rat	C57BL/6J mouse	B6D2F$_1$/J mouse	Hamster	Monkey
TCDD	100	100	100	100	100	100
TCDF	46	49	39	42	35	54
1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin	6.6	1.6	0.82	2.5	0.88	2.3
1,2,4-trichlorodibenzo-*p*-dioxin	0.31	0.066	0.076	0.15	0.67	0.44
Dibenzo-*p*-dioxin	0.035	0.0018	0.0012	0.0014	0.00048	0.003
β-Naphthoflavone	2.2	2.6	5.9	5.9	1.7	4.7
3-Methylcholanthrene	3.7	4.2	3.8	3.2	4.2	5.4
Benzo[*a*]pyrene	1.4	0.86	0.44	0.43	0.46	2.3
Phenobarbital			Inactive (1 × 10^{-5})[a]			

[a] The highest concentration (M) tested.

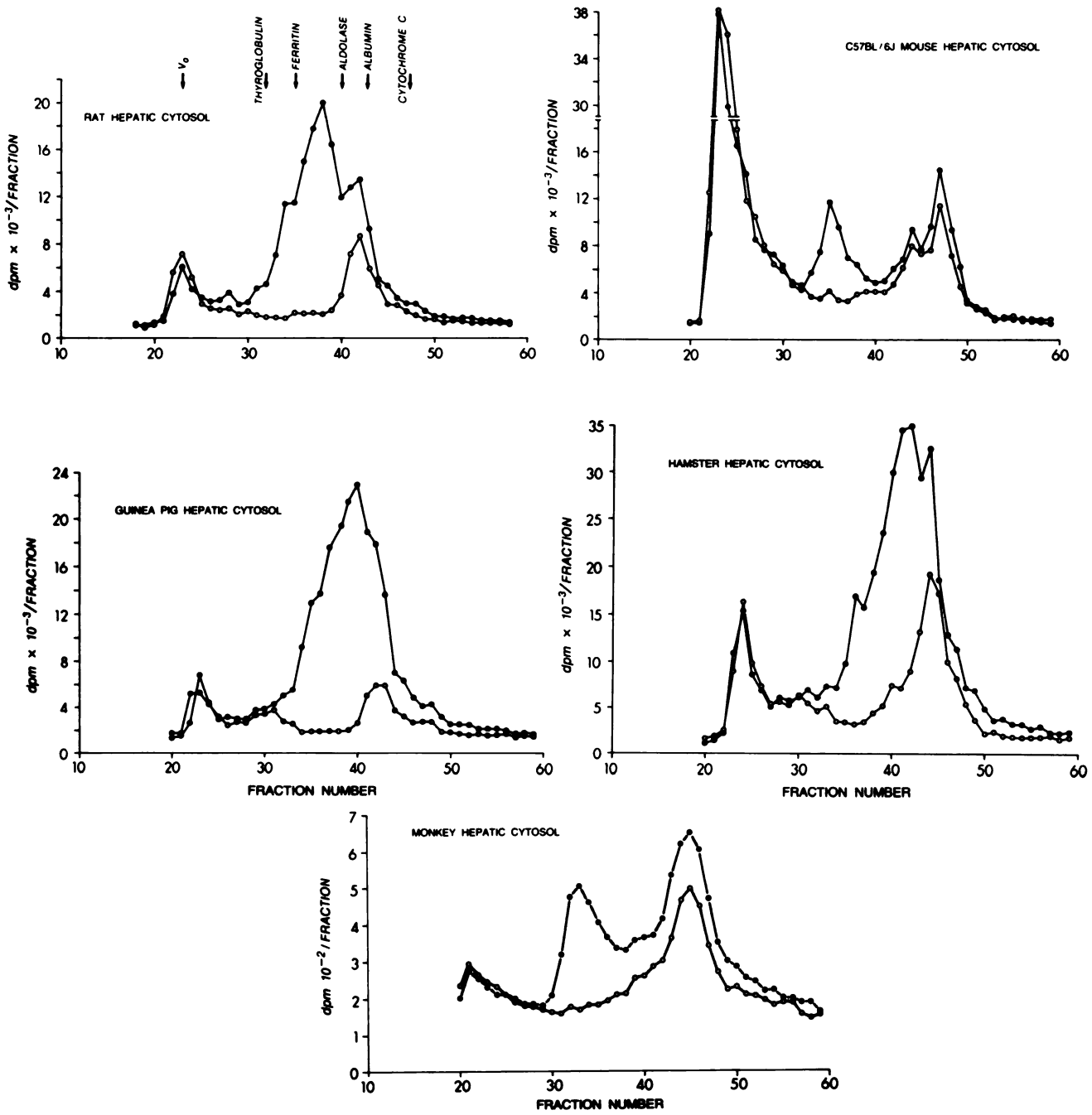

FIG. 3. *Binding profile of [^{3}H]TCDD to hepatic cytosol from various mammalian species as determined by gel-exclusion HPLC*

Hepatic cytosol from each species was incubated for 2 hr at 20° with [^{3}H]TCDD (2 nM) in the presence (○) or absence (●) of a 200-fold excess of TCDF. The cytosol was chromatographed on a TSK 4000 SW HPLC column. The column was eluted with PED buffer at a flow rate of 0.7 ml/min (0.4 min/fraction). Protein concentrations used for rat, mouse, guinea pig, hamster, and monkey were 2.85, 2.03, 3.11, 2.85 and 1.60 mg of protein per milliliter of cytosol, respectively. Further details are described under Materials and Methods.

similar to that previously described by Hannah *et al.* (23). As noted previously (18), no change in the elution profile of [^{3}H]TCDD binding to hepatic cytosol was observed when the elution buffer contained 0.5 M KCl.

Nuclear association of receptors in vivo. In this study we examined the ability of specifically bound [^{3}H]TCDD to be associated with hepatic nuclei from guinea pigs, rats, mice, and hamsters which had been treated by i.p. injection of [^{3}H]TCDD or [^{3}H]TCDD plus unlabeled TCDD (see Materials and Methods). When nuclei from untreated hamsters or mice were prepared by the method of Legraverend *et al.* (14) using HDM buffer, considerably less (50–100%) specific binding of [^{3}H]TCDD to hepatic cytosol or nuclear extracts was observed as compared with that using HEDG buffer (data not shown). This loss of specific binding was not apparent using livers from untreated guinea pigs or rats. The reason for this species difference with the use of HDM buffer is unknown. Subsequent to this finding, all nuclei were

prepared by the method of Okey *et al.* (13) using HEDG buffer or by the method described by Tierney *et al.* (15).

As previously described, high levels of specific binding were observed in the cytosol from all species treated with olive oil only (Table 3). Small, but significant, amounts of specific binding with [^{3}H]TCDD were also found in the nuclear extracts from olive oil treated *or* untreated animals. Similar results were obtained when highly purified hepatic nuclei were prepared using dense sucrose (15). However, and as noted above, no or much less specific binding in these nuclear extracts was observed when HDM buffer was used to prepare nuclei. As compared with olive oil-treated animals, decreased cytosolic and increased nuclear specific binding of [^{3}H]TCDD was observed in all treated animals. Under these conditions *in vivo*, the amount of specific binding of [^{3}H]TCDD remaining in the cytosol of rats, guinea pigs, mice, and hamsters previously treated with [^{3}H]TCDD represented approximately 9.3, 3.3, 25.0, and 5.2%, respectively, of that in cytosol of untreated animals. The specific binding associated with nuclei from these respective species treated with [^{3}H]TCDD represented approximately 4.8-, 87.4-, 21.4- and 5.7-fold increases over olive oil-treated animals.

Relative tissue distribution of receptors. For this study, the guinea pig and hamster were chosen because of their great difference in sensitivity to TCDD (8). Table 4 shows the relative concentrations of receptors in a variety of tissues from untreated guinea pig and hamster, as well as a few selected tissues from the rat and mouse. In all species examined, the liver and lung contained the highest or nearly the highest concentration of receptors per milligram of cytosolic protein. Although not examined in this study, in a previous investigation high concentrations of the *Ah* receptor were observed in the mouse lung (12). Of all tissues examined, rat thymus contained the highest concentration of receptors. The thymus of the guinea pig and mouse also contained very high levels of the receptor, whereas the hamster thymus contained low or in some cases undetectable levels. Lower concentrations of receptor were observed in the duodenum, kidney, heart, and spleen from the guinea pig. Detectable levels of receptor have also been observed in rat and mouse kidney and intestine (12). Among these tissues in the hamster, only the kidney and spleen showed detectable receptor levels. Of the brain sections examined in the guinea pig and hamster, only the cerebrum and cerebellum showed measurable receptor levels. The testes from the guinea pig showed exceptionally high levels of the receptor. This is in contrast to the hamster, rat, and mouse (12) testes, where no measurable concentration of the *Ah* receptor has been detected.

For these studies, all concentrations of receptor in tissue cytosol were determined by extrapolation of the Scatchard plot of data obtained using multiple concentration of [^{3}H]TCDD ± a 200-fold excess of TCDF. In all instances where measurable levels of receptor were observed, K_D values ranged from 0.1 to 0.5 nM. In tissues such as the guinea pig testes and rat thymus, where exceptionally high levels of the receptor were observed using the HAP assay, the presence and level of the receptor were confirmed by the use of the HPLC method. In these cases, the specific binding peak for [^{3}H]TCDD eluted at a volume similar to that observed in hepatic cytosol (Fig. 3).

In tissues, for example hamster thymic cytosol, where low or undetectable levels of receptor were observed, it is possible that some factor or proteolytic activity was

TABLE 3

Analysis of cytosol and nuclear extracts from animals treated with TCDD

Guinea pigs, rats, mice (C57BL/6J), and hamsters received i.p. injections of [^{3}H]TCDD [30 μg (96 μCi)/kg of body weight] 16 hr prior to sacrifice or unlabeled TCDD (300 μg/kg) 4 hr prior to the administration of [^{3}H]TCDD. Binding of [^{3}H]TCDD to the cytosol and nuclear extracts from untreated or treated animals was determined by the HAP assay system (11). For the animals treated with TCDD, specific binding represents the difference of total binding ([^{3}H]TCDD) and nonspecific binding ([^{3}H]TCDD + TCDD). Value are results of representative experiments.

Species	Treatment	Cytosol			Nuclear extract		
		Total binding	Nonspecific binding	Specific binding	Total binding	Nonspecific binding	Specific binding
		dpm/mg protein			*dpm/mg protein*		
Rat	Olive oil	8,718	1,059	7,659	1,955	1,177	778
	[^{3}H]TCDD	1,453	—	713	4,556	—	3,781
	[^{3}H]TCDD + TCDD	—	740		—	775	
Guinea pig	Olive oil	4,629	663	3,966	976	855	121
	[^{3}H]TCDD	743	—	134	11,364	—	10,578
	[^{3}H]TCDD + TCDD	—	609		—	786	
Mouse (C57BL/6J)	Olive oil	3,916	803	3,113	1,879	1,425	454
	[^{3}H]TCDD	1,289	—	779	10,823	—	9,752
	[^{3}H]TCDD + TCDD	—	510		—	1,071	
Hamster	Olive oil	4,579	721	3,858	1,867	1,140	727
	[^{3}H]TCDD	727	—	203	4,993	—	4,165
	[^{3}H]TCDD + TCDD	—	524		—	828	

TABLE 4

Relative tissue concentrations of receptors for TCDD

Specific binding was determined by the HAP assay system and with the use of various concentrations of [^{3}H]TCDD ± a 200-fold excess of TCDF. The number of specific binding sites was determined by Scatchard analysis (19). In all cases where detectable levels of receptor were observed, K_D ranged from 0.1 to 0.5 nM. Values are expressed as means ± standard deviation of number of determinations shown in parentheses.

Tissue	Guinea pig	Hamster	Rat	Mouse (C57BL/6J)
	fmoles/mg cytosolic protein			
Liver	59 ± 11 (8)	67 ± 22 (7)	61 ± 23 (7)	74 ± 10 (4)
Lung	86 ± 28 (4)	35 ± 20 (4)	76 (2)	—
Thymus	47 ± 7 (8)	5 ± 6 (4)	121 ± 30 (4)	24 ± 2 (3)
Duodenum	18 (2)	ND[a](2)	—	—
Kidney	24 ± 19 (4)	13 (2)	—	—
Spleen	17 (2)	6 ± 5 (3)	—	—
Testes	50 ± 7 (4)	ND (2)	—	—
Heart	16 (2)	ND (2)	—	—
Pancreas	ND (2)	ND (2)	—	—
Muscle	ND (2)	ND (2)	—	—
Adrenal	3 ± 3 (3)	ND (2)	—	—
Brain				
Midbrain	ND (3)	ND (2)	—	—
Cerebrum	11 (2)	14 (2)	—	—
Medulla	ND (2)	ND (2)	—	—
Cerebellum	12 (2)	ND (2)	—	—
Hypothalamus	ND (3)	ND (3)	—	—

[a] ND, Not detectable.

present in the tissue which prevented us from accurately quantitating the receptor. To determine this, rat and hamster thymic cytosol were prepared in the presence of the protease inhibitors phenylmethylsulfonyl fluoride (1.0 mM), soybean trypsin inhibitor (2500 BAEE U), and leupeptin (0.5 mM). The presence of these inhibitors had no effect upon the level of receptor observed in these tissues. We also examined the effect of tissue mixing prior to homogenization on the expected specific binding of [^{3}H]TCDD to the resulting cytosolic fraction. If, for example, some factor or proteolytic enzyme was present in the hamster thymus, the same factor should also alter the level of receptor present in a tissue such as rat thymus mixed with the hamster thymus. With the exception of the pancreas, no tissue examined appeared to contain any factor which may alter the specific binding of [^{3}H]TCDD in another tissue (data not shown). In the case of the pancreas, the observed specific binding ranged from 65% to 78% of that expected.

DISCUSSION

The induction of a limited number of enzymes, primarily aryl hydrocarbon hydroxylase, has been used to indicate a response to TCDD and related polycyclic aromatic hydrocarbons via the *Ah* receptor molecule. However, interspecies variability in the sensitivity and responses observed following treatment with TCDD or related compounds limits the use of these assays in examining a general mechanism of action of these compounds. In consideration of this variability and the proposed role of the receptor molecule for the action of these compounds in specific strains of mice (3), a major goal of the present study was to compare the biochemical properties of the receptor molecules from a variety of mammalian species.

Despite varied individual responses to TCDD, the rat, C57BL/6J mouse, guinea pig, hamster, and monkey possess binding proteins for TCDD in hepatic cytosol in similar numbers and which have high affinity for this compound. Only in the different strains of mice did there appear to be any relationship between the relative binding affinity of the receptor for TCDD and the sensitivity of these strains to the action of this compound. Thus, the correlative differences between certain strains of mice in terms of altered specific binding of TCDD and sensitivity to this compound may be unique and not necessarily applicable to most other species. However, data showing the correlation of rank-order structure-activity for the binding of compounds and their ability to elicit various toxic responses suggest that the receptor does mediate these responses in all species yet examined (8). Thus, species- and tissue-specific toxicological effects of TCDD, for example hepatotoxicity, may, in part, be due to a variance in the battery of enzymes expressed or repressed as a result binding and not necessarily a function of the properties of the receptor molecule. Following exposure to TCDD or 3-methylcholanthrene, aryl hydrocarbon hydroxylase activity is induced in hepatic tissue from mice, rats, and hamsters (1, 8, 24). Although hepatic benzo[*a*]pyrene hydroxylase and DT-diaphorase activities are induced to a minimal extent or not at all in the guinea pig (25), 4-biphenyl hydroxylase is increased to a moderate degree (26). Thus, although altered gene expression appears to take place in the liver of all these species following TCDD exposure, it is only in the liver from mice and rats that specific systems are expressed (or repressed), resulting in significant hepatotoxicity. Also, the specific induction of various forms of cytochrome P-450 by TCDD has been found to be tissue-dependent in the rabbit (27). These may be only two of many cases where biochemical systems controlled by the *Ah* locus may be species- and tissue-specific (8).

The binding of TCDD to the receptor in all species examined appears to follow the principles of mass action by virtue of the linearity of Scatchard plots (11) and Hill coefficients which are not significantly different from 1.0 (Fig. 1; Table 1). However, this conclusion may be valid only under the specific conditions in which these incubations were performed. The concentrations (0.08–0.2 nM) of receptor used in the incubations are likely far below that present in intact cells. Notides *et al.* (28) have observed that the concentration of estrogen receptors in cell-free systems profoundly affects the ability to detect the positive cooperative binding of estradiol to this receptor molecule. In this case, low concentrations of estrogen receptor (0.3 nM) resulted in linear Scatchard plots and Hill coefficients near 1.0. At higher concentrations (1.5 nM), convex Scatchard curves characteristic of positive cooperative effects and Hill coefficients of 1.4 and 1.6 were observed. Further studies are needed to determine whether this may be the case with the *Ah* receptor.

In addition to having a high affinity for TCDD, the receptor molecules from the mammalian species examined possess the same rank order ability to bind a number of dibenzo-*p*-dioxin congeners and other polycyclic com-

pounds (Table 3). This is strong evidence that the receptor molecules are of a similar nature. Similarly, when analyzed by HPLC, hepatic cytosolic and nuclear receptors from different species elute at volumes corresponding to similar molecular weight ranges. Small differences in these determined parameters may exist because of the impure nature of the cytosol preparation and influences of, for example, other hydrophobic molecules present in the cytosol. Purification of the receptor molecules is necessary to confirm the homology.

Although the presently reported molecular weight range from the receptor is similar to that described by Hannah *et al.* (23), it is 2–3 times as large as that reported by Poellinger *et al.* (29). At present, the reasons for this disparity are unknown. Some possible explanations include aggregation, effects of different buffer systems, the presence or absence of proteolytic enzymes causing degradation of the receptor, an asymmetrical nature of the protein (29), and/or different methodologies of analysis. Notably, the HPLC method used in the present investigation facilitates analysis of these receptors in a short period of time (20 min), thus minimizing possible effects of proteolytic enzymes or dissociation of the [^{3}H]TCDD-receptor complex. Furthermore, a molecular weight similar to that reported by Hannah *et al.* (23) was observed (a) when the buffer used for the incubation of the cytosol and elution of the HPLC column contained 0.4 M KCl (18), and (b) following the HPLC analysis of receptor extracted (using 0.4 M KCl) from hepatic nuclei of animals treated with TCDD *in vivo* (18).

Association of the hepatic *Ah* receptor with the nuclear fraction was observed in all cases following the i.p. treatment of guinea pig, rat, mouse, or hamster with [^{3}H] TCDD. Although not specifically examined in this study, this nuclear association of the receptor occurs in all rat and mouse tissues of measurable cytosolic receptor content following treatment wth TCDD (12). Our study also did not investigate the possibility that the receptor which binds TCDD (like the steroid hormone receptors) must undergo modification before the ligand-receptor complex is capable of being associated with the nucleus. All data suggest that the binding of TCDD to the receptor results in a biochemical and/or conformational change since (a) the ligand-receptor complex appears to be more thermostable than the unbound form (3, 11), and (b) the nuclear association of the receptor *in vivo* and binding to DNA-cellulose *in vitro* is dependent upon ligand binding (5, 12, 30). The actual kinetic and molecular mechanisms by which these events occur and their relationships to the control of modulated gene expression induced by TCDD remain to be established clearly.

In all species and tissues examined, with the exception of hamster duodenum, the highest concentrations of receptors are localized in the liver, lung, intestine, and kidney. In general, these tissues also exhibit the highest levels of induced aryl hydrocarbon hydoxylase activity (24). An important exception to this is the guinea pig, where little or no induction of the enzyme or the associated cytochrome P-450 has been observed following TCDD (26) or 3-methylcholanthrene administration (25). It seems likely that, if the receptor molecule does serve some function in this species, it may not be primarily for the regulation of the cytochrome P-450-associated monooxygenases. Yet from a toxicological standpoint, the guinea pig is extremely sensitive to TCDD. It remains to be determined whether this toxicity is due to an interference with the normal function(s) of this receptor molecule.

Rat, guinea pig, and mouse thymus also contained very high levels of the *Ah* receptor. In the rat, this tissue contained the highest receptor level of all tissues and species examined. Although TCDD-induced aryl hydrocarbon hydroxylase activity in the mouse thymus is less than 1/1000 that in the liver, in terms of percentage increase the thymus activity is approximately twice as inducible as in the liver (31). Involution of the thymus and impairment of thymus-dependent immune function are consistent features of TCDD exposure in most mammalian species (32). The cellular or biochemical mechanisms by which these events occur have not been determined. It is of interest that the rat and guinea pig, which have been found to be most sensitive to TCDD-induced thymic atrophy (8), also have the highest number of receptors per milligram of protein, whereas the hamster thymus, which showed the lowest or undetectable levels of receptor, appears to be the least sensitive to thymic involution (or any other toxic effects) induced by TCDD (33). These data may suggest that TCDD-induced thymic involution is directly associated with the presence of receptor in this tissue. However, it is clear that more direct evidence is needed to establish a causal relationship between these events.

Exceptionally high concentrations of the receptor were observed in the guinea pig testes. Our studies and those of Mason and Okey (12) detected no receptor in this organ from the hamster, rat, or mouse. In contrast to the elevation of cytochrome P-450-associated enzymes in liver and other tissues, TCDD has been shown to produce a depression of microsomal cytochrome P-450 content in the testes from both the guinea pig and rat (34). This appears to be related to a TCDD-induced depression in testicular heme synthesis (34).

Despite species and tissue specific differences in the responses to TCDD and related compounds, all those mammalian species examined in this study possess the *Ah* receptor. Among these species the receptors have similar biochemical properties, translocate to the nucleus following TCDD exposure, and are distributed within the animal in a similar manner. Similar age-related differences in the concentration of the hepatic cytosolic *Ah* receptor in various mammalian species have also been observed (35). All of these results suggest the conservation of some, as yet unknown, functional role of this receptor molecule. Although previous studies have mainly focused on the role of the receptor in the regulation of cytochrome P-450-associated monooxygenases, it is clear that this is only one of many responses controlled by the *Ah* locus (8). The observed tissue and species specificity of these responses creates an obvious difficulty in associating a function of this receptor molecule. A unifying factor may be the observation that the main

effects of TCDD appear to be on epithelial tissues or tissues with epithelial cell subpopulations and that all of these effects involve cell division, involution, or differention (8).

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