0026-895X/88/050682-07$02.00/0

MOLECULAR PHARMACOLOGY, 34:682-688

A Competitive Binding Assay for 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin and Related Ligands of the *Ah* Receptor

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Received May 16, 1988; Accepted August 4, 1988

SUMMARY

A sensitive competitive binding assay for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other ligands of the *Ah* receptor was developed using a stable preparation of the *Ah* receptor, the 40–55% ammonium sulfate fraction of liver cytosol from C57BL/6J mice, and the radioligand [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin (specific radioactivity, 2176 Ci/mmol, and binding affinity, K_D = 6.5 pM). Conditions are described which maximize assay precision and sensitivity, while minimizing sample counting time, ensuring ligand solubility, and permitting attainment of binding equilibrium for competing ligands. Assay conditions were developed to allow calculation of the binding affinity for competing ligands and to ensure that an unknown competitor could be quantified in terms of "TCDD binding equivalents." Standard assay conditions consisted of incubation of 8 pM radioligand and 18–20 pM *Ah* receptor with 5–1000 pM TCDD, in a 1-ml volume, for 16 hr at 4°. Statistical analysis of the standard curve of bound radioligand *versus* the log of the concentration of competing TCDD indicated the minimal detectable concentration of TCDD to be 10 pM (3.2 pg in a 1-ml assay $\alpha \leq 0.01$). The simplicity, sensitivity, and reproducibility of this competitive binding assay should prove useful as a screen to detect planar halogenated aromatic hydrocarbons and other ligands of the *Ah* receptor. The availability of this ^{125}I-labeled dioxin congener also permitted the characterization of *Ah* receptor-ligand binding over a range of ligand and receptor concentrations not possible with currently available ^{3}H-ligands.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin is one of the most potent small molecule toxins known (1–3) and serves as the prototype for a large number of planar halogenated aromatic hydrocarbons which elicit their biological effects by a common mechanism (e.g., certain isomers of halogenated dibenzo-*p*-dioxins, dibenzofurans, azo[*xy*]benzenes, and biphenyls). The risk presented by the widespread dispersion of these compounds into the environment is a function of 1) their toxic potency, 2) their resistance to chemical and biological degradation (4), and 3) their lipophilicity and hence potential for accumulation in the food chain (5).

Analysis of trace concentrations of these compounds in environmental and biological samples has been made possible through recent advances in chromatographic and mass spectral technologies. Current methodologies involve varying degrees of sample preparation, separation of isomers by liquid or gas chromatography, and identification and quantification of congeners by mass spectrometry. Extremely low concentrations of TCDD in environmental samples, picogram per gram levels (i.e., parts per trillion), are now routinely quantified using this technology (6). Despite the sensitivity of mass spectrometer-based methods, their use is limited by cost and availability of instrumentation.

Bioassays have played a prominent early role in the identification and monitoring of chlorinated dibenzo-*p*-dioxins: e.g., the formation of pericardial edema in the newborn chick (7), and the production of chloracne in the rabbit pinna (8). More recently, cell culture bioassays [e.g., the induction of aryl hydrocarbon hydroxylase activity in rat hepatoma cells (9), and keratinization in XB/3T3 cell cultures (10)] have achieved remarkable sensitivity, 10 pg of TCDD, but have not gained widespread use. Radioimmunoassays have been developed to detect TCDD, 2,3,7,8-tetrachlorodibenzofuran, and chlorinated biphenyls with detection limits approaching 25 pg (11–13). The limited use of these radioimmunoassays may be attributable to 1) the need to characterize each antisera for its reactivity toward a large number of isomers and cross-reacting com-

This work was supported in part by the National Institute of Environmental Health Sciences Grant ES-01884, National Cancer Institute Core Grant-07175, and National Cancer Institute Postdoctoral Training Grant T32-CA09020.

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, (ethylenedinitrilo)-tetraacetic acid); K_D, equilibrium dissociation constant; *L*, ligand (labeled or unlabeled); *R*, receptor; *RL*, receptor-radioligand complex; *RL'*, transformed receptor-radioligand complex; $[L]_T$, total radioligand concentration; $[R]_T$, total receptor concentration; B_{max}, concentration of receptor determined by Scatchard analysis; *B*, bound radioligand at equilibrium; B_0, receptor-bound radioligand in the presence of no competing ligand; B_x, *receptor-bound radioligand in the presence of competing ligand*; *C*, competing ligand; $[C]_F$ concentration of "free" competing ligand at equilibrium; k_{-1}, dissociation rate constant; NSB, nonspecific binding (radioligand binding in the presence of a 200–1000-fold excess of 2,3,7,8-tetrachlorodibenzofuran); EC_{20}, EC_{50}, EC_{80}, competing ligand concentration which produces 20%, 50%, or 80% reduction in specific binding, respectively; $t_{1/2}$ half-life of dissocation.

pounds, 2) difficulties in the solubility of radioligand (or antigen), and 3) the limited availability of the radioligands.

TCDD and related halogenated aromatic hydrocarbons are all approximate isostereomers and produce a characteristic pattern of morphological and biochemical changes. The biological effects elicited by these compounds appear to result from their stereospecific binding to a soluble protein, the *Ah* receptor, and the resultant gene expression which is initiated by this ligand-receptor complex (14–17). In support of this model: for chlorinated dibenzo-*p*-dioxins, the apparent binding affinities (K_D) of congeners for the *Ah* receptor correspond to their rank-ordered potencies to produce biological responses (e.g., induction of monooxygenase activity, lethality, chloracne, and tumor promotion). Secondly, in mice there is a genetic polymorphism in the *Ah* locus that determines the receptor. Strains which express a high or low affinity receptor are more or less sensitive to the effects of agonists, respectively, and a variety of toxic responses (e.g., thymic involution, porphyria, epidermal hyperkeratosis, and teratogenicity) have been shown to segregate with the *Ah* allele which determines the high affinity receptor (for a review see Ref. 16).

Since these halogenated aromatic hydrocarbons produce their biological effects by virtue of their binding to the *Ah* receptor, a competitive binding assay of sample and radiolabeled ligand for *Ah* receptor occupancy would appear to offer a direct and simple assay system. As an analytical method, a competitive radioligand-*Ah* receptor binding assay has a number of advantages: 1) theoretical foundation based on the law of mass action, 2) elimination of the biological variability inherent in the use of whole animals or cells, and 3) rapid screening not requiring expensive instrumentation.

The sensitivity, and hence utility, of *Ah* receptor-based competitive binding assays has been limited to date by the low specific activity of available radioligands (e.g., [^{3}H]TCDD, 58 Ci/mmol). We have previously reported the synthesis and binding kinetics of a new radioligand of the *Ah* receptor, [^{125}I] 2-iodo-7,8-dibromodibenzo-*p*-dioxin (18). This radioligand possesses a high affinity for the *Ah* receptor (K_D = 6.5 pM) and a high specific activity (2176 Ci/mmol). We now report the use of this radioligand in the development of a highly sensitive competitive binding assay for ligands of the *Ah* receptor.

Rationale

Our approach to the development of this competitive binding assay consisted of: 1) synthesis of a radioligand of high specific activity and high receptor affinity; 2) characterization of a stable and reproducible preparation of the *Ah* receptor; 3) use of competitive binding theory to approximate optimal conditions; and 4) refinement of the conditions by experiment.

Radioligand

The sensitivity of a competitive binding assay is proportional to the [specific activity of the radioligand × $1K_D$]$^{1/2}$ (19). Thus, use of [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin (2176 Ci/mmol, counting efficiency ~70%, K_D = 1.7 K_D for TCDD) should increase assay sensitivity approximately 6-fold compared to use of [1,6-^{3}H]TCDD (58 Ci/mmol, counting efficiency ~40%).

Receptor Preparation

The 40–55% ammonium sulfate precipitate fraction of hepatic cytosol from C57BL/6J mice was chosen because 1) we have recently characterized the kinetics of radioligand binding to this preparation in detail (18), and 2) the receptor concentration in this preparation is slightly enriched compared to cytosol, the lipid contamination is reduced, and binding characteristics are unchanged for over 1 year when stored at −80°.

Mathematical and Statistical Theory Used to Optimize Assay Sensitivity

Equilibrium binding. The binding of a radioligand, L, to its receptor, R, under conditions of equilibrium, can be described by the law of mass action, where the equilibrium dissociation constant, K_D, is equal to the ratio of the constituents: $[R]$ = concentration of free receptor; $[L]$ = concentration of free radioligand; and $[RL]$ = concentration of radioligand-receptor complex. Classically, this interaction is represented as:

$$R + L \rightleftarrows RL \tag{1}$$

$$K_D = \frac{[R][L]}{[RL]} \tag{2}$$

We have recently examined the binding of the *Ah* receptor from C57BL/6J mouse liver and [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin (18) and found it to be best characterized by the following model:

$$R + L \rightleftarrows RL \rightleftarrows RL' \rightleftarrows R' + L \tag{3}$$

where RL' is a distinct form of the ligand-receptor complex, which has a much slower ligand dissociation rate than does RL.

Under the binding conditions employed (*Ah* receptor from C57BL/6J mice, incubation at 4°, for 16 hr), the transformed receptor-ligand complex, RL', is less than 25% of the total bound ligand; and equations 1 and 2 provide a rough approximation of equilibrium binding.[1]

Optimization of a competitive binding assay. The quantity of radioligand bound to receptor, $[RL]$ or B, at a fixed concentration of radioligand and receptor, is progressively decreased by the addition of increasing concentrations of unlabeled competitor, C, which competes for receptor occupation. The concentration of radioligand bound, as a function of unlabeled ligand added, describes a competitive binding curve and can be derived from the following relationship:

$$\left(\frac{[RL]}{[L]}\right)^2 + \left(\frac{[RL]}{[L]}\right)\left(1 + \frac{[L]_T}{K_D} - \frac{[R]_T}{K_D}\right) - \frac{[R]_T}{K_D} = 0 \tag{4}$$

where $[L]_T$ = concentration of total ligand, and $[R]_T$ = concentration of total receptor.

A major goal in developing a competitive binding assay is to optimize the precision and sensitivity of the assay. This is accomplished by adjusting the concentrations of radioligand $[L]_T$ and receptor $[R]_T$. Berson and Yalow (20) and Ekins and co-workers (19) independently developed theoretical solutions to calculate optimum reagent concentrations. Although these solutions differ in their definitions of sensitivity and considerations of error, they predict similar optimal conditions. Berson and Yalow define conditions which yield maximal sensitivity to those which give an initial ratio of bound to total radioligand ($B_0/[L]_T$) = 0.33 to 0.5, $[R]_T = 0.5\ K_D$, and $[L]_T$ that approaches zero. Ekins and co-workers define optimal conditions as those which yield $B_0/[L]_T = 0.5$; $[R]_T \leq 1.25\ K_D$ and $[L]_T \leq 2.25\ K_D$, when considering only counting error, or which approach $[R]_T = 0.5\ K_D$ and $[L]_T = 0$, with increasing experimental error. These derivations assume simple mass action and equilibrium conditions which only approximate ligand binding to the *Ah* receptor (i.e., equations 1, 2, and 4). Additionally, they are dependent upon experimental as well as counting errors. Therefore, we used these theoretical values to determine initial ranges of receptor and radioligand concentrations and further optimized assay sensitivity empirically.

[1] This assumption was necessary since the optimization theory used was derived from Eqs. 1, 2, and 4. The assumption was supported by the fact that optimal sensitivity was achieved in accordance with the theoretically determined optimal reagent concentration ranges. (see "Results" and "Discussion").

Quantification of competing ligands with differing receptor affinity. A host of halogenated aromatic hydrocarbon isomers are ligands for the *Ah* receptor, but differ widely in their binding affinities. We foresee this competitive binding assay as being useful in the quantification of the sum of these compounds present in environmental or biological samples, in terms of "TCDD binding equivalents." Ekins *et al.* (19) and Rodbard and Lewald (21) have examined the complex relationship between the relative binding potency of an unlabeled competitor, C, with an equilibrium dissociation constant K_C, and radioligand, L (with affinity K_D). When the affinity of the radioligand is equal to or greater than that of the competing ligand (and the ratio of bound radioligand/free radioligand is less than 0.5), the estimate of binding potency is nearly linear (i.e., $EC_{50C}/EC_{50L} = K_C/K_D$). Since we have chosen a radioligand with a receptor affinity approximately one-half that of TCDD, it is very likely the estimate of binding equivalency of unknown ligands will always be nearly linear. We have chosen to optimize the assay sensitivity for detection of TCDD; one may enhance the sensitivity of detecting a known ligand of lower affinity by adjusting the concentration of receptor $[R]_T$ and radioligand $[L]_T$ (19).

Time for equilibrium. To accurately estimate the binding equivalency of a competing unlabeled ligand, it is important that binding equilibrium be achieved for both the unlabeled compound and the radioligand. The time required to achieve equilibrium binding is a function of the dissociation rate constant of the slower dissociating ligand. Since the radioligand has a very high affinity for the *Ah* receptor, comparable to or greater than that of any known competing ligand, we assumed that 5 times the half-life of its dissociation [$t_{1/2} = 1.9$ hr (18)] or $3.5/k_{-1}$ is a good approximation of equilibrium conditions for all potential ligands (22).

Calculation of dissociation constants. The equilibrium dissociation constant of a competing ligand (K_C) is most commonly calculated by the Cheng-Prusoff equation (23), which is applicable to situations where $[L] \approx [L]_T$. These conditions are not met in competitive binding assays which have been optimized for sensitivity (i.e., $B_0/[L]_T = 0.3$–0.5). We estimated the K_C of competing ligands from EC_{50} values by the equations of Linden (24) which place no constraints on the fraction of radioligand or competitor bound. First the concentration of "free" competing ligand is calculated:

$$[C]_F = EC_{50} - [R]_T + \frac{[R]_T}{2}\left[\left(\frac{[L]_T}{K_D + [L]_T}\right) + \left(\frac{K_D}{K_D + [L]_T + [R]_T/2}\right)\right] \quad (5)$$

then $[C_F]$ is substituted into the following equation to determine K_C:

$$K_C = \frac{[C]_F}{1 + \frac{[L]_T}{K_D} + \frac{[R]_T}{K_D}\left(\frac{K_D + [L]_T/2}{K_D + [L]_T}\right)} \quad (6)$$

Materials and Methods

Reagents. [^{125}I]2-Iodo-7,8-dibromodibenzo-*p*-dioxin (2176Ci/mmol) was synthesized and purified as described previously (18). TCDD was a generous gift of Dow Chemical Co. (Midland, MI), 2,3,7,8-Tetrachlorodibenzofuran and 2,3-dichlorodibenzo-*p*-dioxin were a gift from Drs. David Firestone and Albert Pohland (Food and Drug Administration, Washington, DC). [^{14}C]TCDD (specific activity 114 mCi/mmol), and 2,3-dibromodibenzo-*p*-dioxin were synthesized as described (25, 26). 3,4,3′,4′-Tetrachlorobiphenylether was a gift from Dr. Andrew Kende (University of Rochester). Estradiol was purchased from Calbiochem (San Diego, CA). Pregenolone-16α-carbonitrile was purchased from Upjohn Diagnostics (Kalamazoo, MI). Sodium phenobarbital was purchased from Merck Chemical Division (Rahway, NJ). Active charcoal, grade PX-21, was a gift from Amoco Research Corp. (Chicago, IL). Bacto-Gelatin was purchased from Difco Laboratories (Detroit, MI). Glycerol was purchased from J. T. Baker (Phillipsburg, NJ). EDTA was purchased from EM Scientific (Cherry Hill, NJ). Testosterone, cortisol, Na-L-thyroxine, dithiothreitol, β-mercaptoethanol, sodium azide, and MOPS (free acid and sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). *p*-Dioxane (anhydrous 99+% pure) and dimethyl sulfoxide (anhydrous, 99% pure, stored under N_2 gas) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Buffers. MN represents the stock buffer which contains 25 mM MOPS and 0.02% sodium azide, pH 7.5 (at 4°). MEN is the stock buffer plus 1 mM EDTA. MβENG and MDENG represent MEN with the addition of 10% (w/v) glycerol, plus the addition of either 10 mM β-mercaptoethanol or 1 mM dithiothreitol (respectively).

Receptor preparation. The 40–55% ammonium sulfate precipitate of C57BL/6J mouse hepatic cytosol was prepared as described previously (18).

Standard assay protocol. To a series of 12- × 75-mm borosilicate assay tubes was added 5 μl of dimethyl sulfoxide containing TCDD standards (0, 5, 10, 20, 40, 60, 100, 200, 500, and 1000 fmol) or unknowns. The frozen 40–55% ammonium sulfate precipitate (15 mg of protein/tube) was dissolved in ice-cold MDENG buffer and diluted to a concentration of 150 μg of protein/ml (equivalent to an *Ah* receptor concentration of 18–20 fmol/ml). The radioligand in dimethyl sulfoxide was added to the solution of receptor to a concentration of 8.0 fmol/ml (~40,000 dpm/ml, added as a 1 μl stock solution/ml of buffer) before dispensing 1 ml of the solution to each assay tube (without vortexing). The tubes were then incubated at 4° for 16 hr. The assay was terminated by the addition of a 0.5 ml of suspension of charcoal/gelatin (3%/0.3%, w/v) in MN buffer with vigorous stirring on a vortex mixer for 3 sec, followed by incubation for 10 min at 4°C. The tubes were then centrifuged at 2000 × *g* for 10 min at 4°. A 1-ml aliquot of the supernatant fraction of each tube was transferred to 12- × 75-mm polypropylene tubes (VWR Scientific, San Francisco, CA), and the bound radioligand was quantified in a MINAXI Series-5000 gamma scintillation counter (United Technologies/Packard Instrument Co., Downers Grove, IL). Sample counting times were adjusted to obtain a counting error of 3% (range of 0.1–2.0 min, counting efficiency of 75%). Each sample and standard were analyzed in quadruplicate. Total radioligand, $[L]_T$, was determined by the transfer of 0.67 ml of assay solution directly to the polypropylene tubes for counting (no charcoal/gelatin is added to these tubes).

Data analysis. The γ-scintillation counter was connected by a serial communication port to an IBM-XT personal computer equipped with software for analysis of radioimmunoassay data and quality control (SECURIA PLUS, Packard Instrument Co., Downers Grove, IL).

Total radioligand, $[L]_T$, was defined as the concentration of radioligand in solution after the 16-hr incubation. (This value is generally 10% lower than the concentration of radioligand originally added to the tubes, i.e., 8 pM.) NSB was defined as the amount of radioligand bound in the presence of 8 nM 2,3,7,8-tetrachlorodibenzofuran (1000-fold molar excess). The total radioligand bound at each concentration of competing ligand (i.e., 5–1000 fmol of TCDD/ml) minus the NSB was defined as the specific binding: B_0 = specific binding in absence of competing ligand and B_x = specific binding at "x" concentration of competing ligand.

To generate a standard curve, the response parameter, B_x/B_0, i.e., the ratio of specific binding at each concentration of TCDD relative to the specific binding in the absence of competing ligand, was plotted *versus* the log of the concentration of TCDD. For data analysis we chose to fit these results, via an iterative curve-fitting technique, to the four-parameter logistic model described by DeLean *et al.* (27).[2] The concentration of ligand in unkown samples is quantified as "equivalents" of TCDD (in picomolar concentration or femtomoles per milliliter), by interpolation of the fractional bound response metameter on

[2] $B_x/B_0 = \frac{B_0 - NSB}{1 - ([C]/EC_{50})^S} + NSB$
where S is slope of log logit plot.

the standard curve. Outliers were defined as values varying more than 5 standard deviations from the mean ($n = 4$ at each point).

The variation between assays was monitored by following a variety of quality control parameters for the standard curve, which included: 1) B_0, (specifically bound ligand in the absence of competing ligand; 2) B_0/L_T, (specifically bound ligand as a fraction of total radioligand per milliliter); 3) NSB; 4) estimates of EC_{20}, EC_{50}, and EC_{80} (competing ligand concentration which produces a 20%, 50% and 80% reduction in specific binding, respectively;), 5) the calculated slope of the four-parameter logistic model (i.e., slope of the logit-log plot), and 6) a control standard yielding a TCDD concentration of 45 pM.

Results

A representative saturation binding isotherm of [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin binding to the *Ah* receptor in the 40–55% ammonium sulfate precipitate fraction of liver cytosol is shown in Fig. 1A. The data in Fig. IA were transformed by the method of Scatchard (28) to generate the plot described in Fig. 1B. From an average of three experiments the concentration of binding sites, B_{max} = 120 ± 7 fmol/mg of protein, the apparent K_D = 16 ± 3 pM, and the correlation coefficient = 0.99 ± 0.00. The Hill coefficient of these plots is 0.97 ± 0.02 with a correlation coefficient = 0.99 ± 0.00 (29).

As outlined under "Rationale," the approximate conditions for optimal precision and sensitivity of a competitive binding assay are: 1) $B_0/[L]_T = 0.33–0.5$; 2) $[R]_T \leq 1.25\ K_D$ and $[L]_T \leq 2.25\ K_D$. In Fig. 2, are displayed a series of competitive binding curves generated at three radioligand concentrations ($[L]_T$ = 2.4, 7.7, and 23 pM) and three receptor concentration ($[R]_T$ = 7, 28, and 112 pM), with TCDD as the competing unlabeled ligand. The initial $B_0/[L]_T$ ratios (in the absence of unlabeled ligand) varied from 0.2 to 0.7. For a given radioligand concentration, decreasing the concentration of the receptor decreased the initial $B_0/[L]_T$ and increased the initial slope of the competitive binding assay.

As a test of the relative assay sensitivity obtained at differing reagent concentrations, we compared the statistical power[3] of these assay conditions to detect a 5 pM concentration of TCDD. As shown in Table 1, the power of the assay increased with a reduction of receptor concentration, at a receptor concentration of 7 pM, the power increased as radioligand concentration decreased.

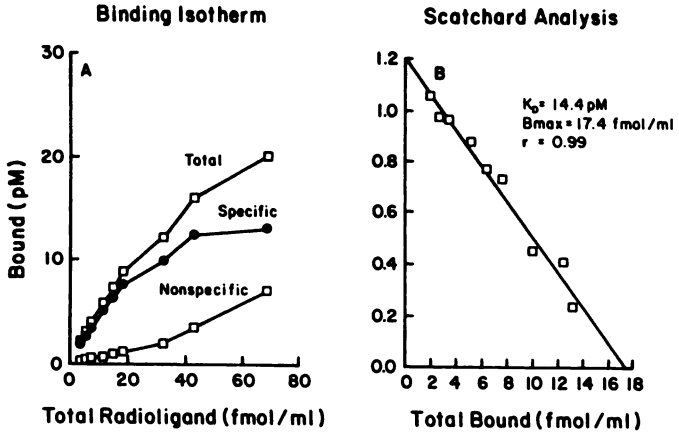

Fig. 1. A. Equilibrium binding of [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin to the 40–55% ammonium sulfate precipitate fraction of hepatic cytosol. The receptor preparation (145 μg of protein/ml) was incubated at 4° for 16 hr, with increasing concentrations of radioligand (3–70 pM). Nonspecific binding was estimated in the presence of a 200-fold excess of 2,3,7,8-tetrachlorodibenzofuran. Each point represents the average of two determinations. B. Scatchard analysis of equilibrium binding data from A. The binding parameters were calculated by a linear least squares estimate of specifically bound/free radioligand versus specifically bound radioligand. The K_D and B_{max} results (inset) are derived from the experiment shown, the mean and standard deviations from three experiments are K_D = 16 ± 3 pM and B_{max} = 18 ± 3 fmol/ml (120 ± 17 fmol/mg of protein).

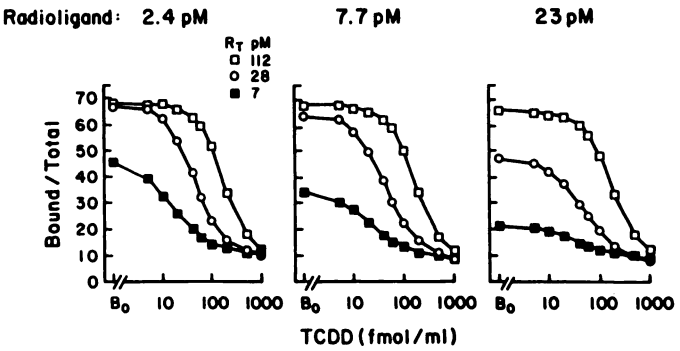

Fig. 2. Effect of radioligand and receptor concentration on binding curves generated with TCDD as competing ligand. Assays were incubated at 4° for a period of 16 hr (see text). All data points are the result of quadruplicate determinations. Ordinate: total radioligand bound divided by total radioligand in solution. Abscissa: concentration of unlabeled competitor (TCDD, picomolar concentration). Receptor concentration: 112 pM (□); 28 pM (○); and 7 pM (■). Left, total radioligand concentration, $[L]_T$, is 2.4 pM (11,600 dpm/ml); center, total radioligand concentration is 7.7 pM (37,200 dpm/ml); right, total radioligand concentration is 23 pM (111,000 dpm/ml).

TABLE 1

Statistical power of the competitive binding assay

Statistical power (see footnote 3) of various assay conditions to detect the decrease in radioligand bound in the presence of 5 pM TCDD, as compared to radioligand bound in the absence of TCDD (i.e., B_0). Ekins *et al.* (19) have shown that the sensitivity of a competitive binding assay is equivalent to the error in B_0 divided by the initial slope of the assay curve, Δ bound/Δ competing ligand added. The statistical power ($1 - \beta$) was calculated using the Student's *t* value with $\alpha \leq 0.01$ (30, 31).

Radioligand Concentration	Statistical Power at Receptor Concentration		
	7 pM	28 pM	112 pM
pM			
2.4	0.99	0.10	0.00
7.7	0.95	0.30	0.00
23	0.60	0.10	0.10

TCDD and related halogenated aromatic hydrocarbon have very limited solubilities in aqueous solution. We examined the concentration of radioligand that remained in solution, after a 16-hr incubation at 4°, as a function of the concentration of the receptor preparation used. As seen in Fig. 3, only at protein concentrations of 150 μg/ml ($[R]_T$ = 18 pM) or greater, did at least 95% of the added radioligand remain in solution. While maximal sensitivity is achieved at receptor concentrations of 7 pM, we chose to use the higher concentration (18 pM, 150 μg/ml) to increase the solubility of competing ligands.

Based on the observations presented above, the standard conditions for the competitive binding assays were chosen to be: radioligand concentration, $[L]_T$, of 7.2–7.7 pM (~4 × 10^4 dpm/ml added initially), a receptor concentration of 18–20 pM

[3] The statistical power of an assay (30) is the probability that the decrease in bound radioligand, due to the presence of 5 pM TCDD, will be detected when compared to B_0 (Student's *t* value, $\alpha \leq 0.01$). Thus, assay conditions which yield the greatest statistical power are the conditions which yield the greatest assay sensitivity. Statistical power is equal to $1 - \beta$ (where β is the probability of making a type II statistical error) (31).

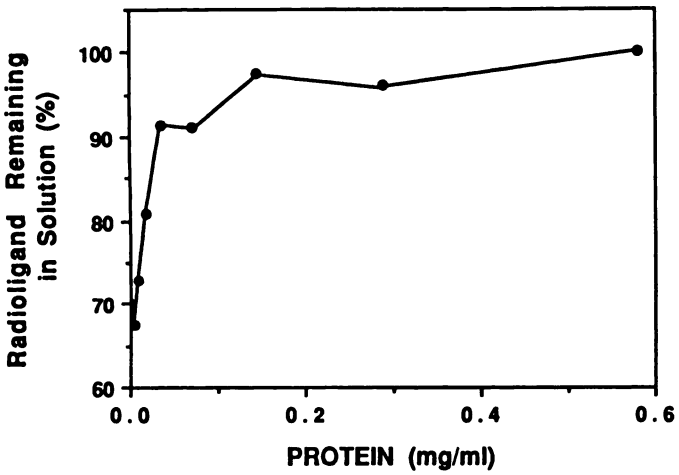

Fig. 3. Effect of protein concentration on radioligand solubility. The radioligand (8.3 pM) was incubated at 4° with varying concentrations of the receptor preparation (0, 5, 10, 19, 38, 75, 150, 300, 600, and 1200 μg of protein/ml. After incubation for 16 hr, total radioligand in solution, $[L]_T$, was determined as described in the text. Each point represents the average of two determinations.

(approximately 150 μg of protein/ml), and an incubation time of 16 hr at 4°. A typical standard curve for the competitive binding of TCDD determined under these conditions is shown in Fig. 4. The initial total bound ligand is 17,176, NSB is 1996 dpm, initial specifically bound ligand, B_0, is 15,180 dpm and $B_0/[L]_T = 0.40$. The concentration of TCDD which produces a reduction in specifically bound radioligand equal to one-half the initial value (EC_{50}) is 41 ± 3 pM (Table 2). Under the standard assay conditions the minimal detectable concentration of TCDD which reproducibly produced a statistically significant reduction in bound radioligand is 10 pM (10 fmol/ml = 3.2 pg/ml, $\alpha \leq 0.05$). Thus, to ensure ligand solubility with an increase in $[R_T]$, we have reduced sensitivity about 2-fold. By reducing the assay volume to 0.25 ml, one can decrease the minimal detectable concentration to 0.8 pg of TCDD (data not shown). The advantage of the 1.0-ml incubation volume is greater bound radioactivity, and thus reduced counting time per sample to achieve the same precision.

We have previously found that [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin has a biphasic dissociation from the *Ah* receptor, with the major component (75%) having a dissociation rate constant, $k_{-1} = 0.36$/hr at 4° ($T_{½} = 1.9$ hr^{-1}) (18). Thus, binding equilibrium for competing ligands with dissociation rate constants equal to or greater than that of the radioligand should be achieved by $5 \times t_{½}$, or 10 hr (22) (see Rationale). Competing ligands with slower dissociation rates than the radioligand [presumably TCDD, since its K_D is approximately one-half that of the radioligand (Table 3)] may require more than 10 hr to reach binding equilibrium. To confirm that binding equilibrium was obtained for TCDD by 16 hr, we examined the competitive binding curves at 4° as a function of incubation time (Fig. 5). The initial bound radioligand B_0 and slope of the competitive binding curved reached a maximum by 16 hr, remained virtually unchanged between 16 and 48 hr, and then progressively declined. The decrease in B_0 at longer incubation times is probably attributable to degradation of receptor with time.

The accuracy of the assay is dependent on the standard, i.e., the accuracy in weighing and dilution of small amounts of TCDD. We checked this by determining the competitive bind-

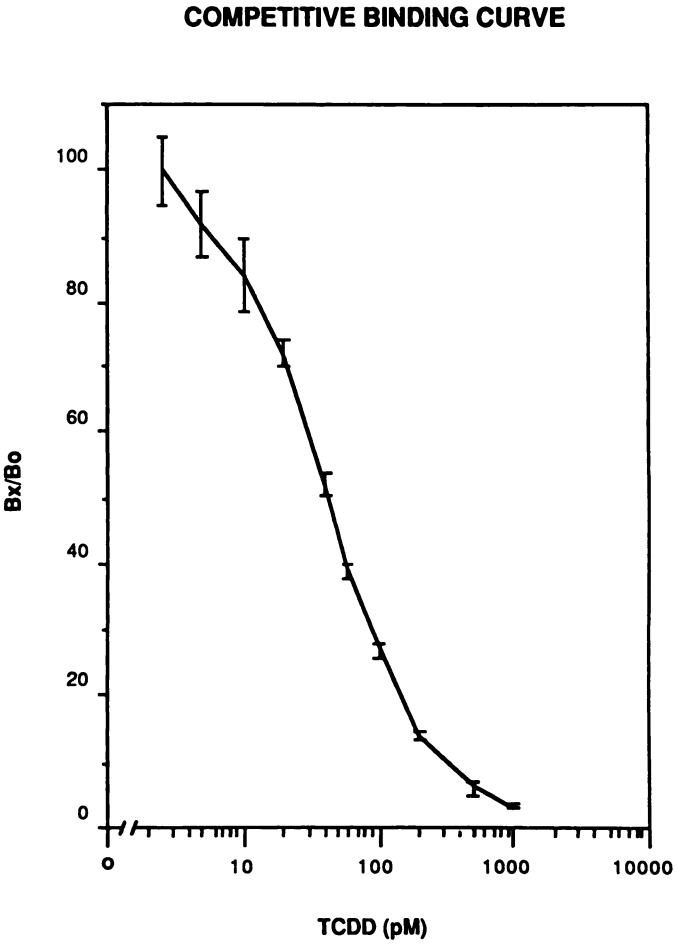

Fig. 4. Competitive binding assay. Standard curve with TCDD as competing ligand. Assay was run, as described in text, with increasing concentrations of TCDD, a total radioligand concentration of 7.2 pM (added 8.2 pM), and a receptor concentration of 18 pM. Incubation was for 16 hr at 4°. Ordinate: B_x/B_0, specifically bound radioligand in the presence of a given concentration of TCDD divided by specifically bound radioligand in the absence of TCDD. Abscissa: concentration of TCDD (picomolar concentration). The first data point is the interpolated concentration of B_0, as calculated by the four parameter logistic model (see text). For this standard curve; $EC_{20} = 13$ pM, $EC_{50} = 40$ pM, $EC_{80} = 138$ pM, B_0 divided by total radioligand = 0.36, NSB divided by total radioligand = 0.06.

TABLE 2

Quality control parameters of the competitive binding assay standard curve

The standard curve for TCDD competition with radioligand for the *Ah* receptor was determined as described under "Materials and Methods." Quality control parameters were compared from assays using freshly prepared radioligand ($n = 9$), and using radioligand after 75% radiodecay (120 days, $n = 1$). Values are means ± standard deviation. All parameters are described in the text. ND, not determined.

Parameter	"New" Radioligand No Radiodecay	Radioligand after 75% Radiodecay
B_0/L_T	0.36 ± 0.06	0.34
NSB/L_T	0.06 ± 0.01	0.06
EC_{20} (pM)	14 ± 3	14
EC_{50} (pM)	41 ± 3	45
EC_{80} (pM)	140 ± 20	160
Logit slope	1.3 ± 0.1	1.2
45 pM TCDD (control pool)	42 ± 6	48
40 pM [^{14}C]TCDD	40 ± 2	ND

TABLE 3

Relative binding potencies for ligands of the Ah receptor

The competitive binding assay was performed using nonradiolabeled congeners, under standard conditions as described under "Materials and Methods." The $[R]_T$ was 20 pM, and $[L]_T$ = 7–7.6 pM. The true K_D of the radioligand was assumed to be 6.5 pM (18). The EC_{50} is the total ligand concentration which produced a 50% reduction in the specific binding of the radioligand; the relative binding potency is $EC_{50(competitor)}/EC_{50(TCDD)}$. The K_C for each compound was determined by the method of Linden (Eqs. 5 and 6; Ref. 24) and the relative K_C = K_C competitor/K_C TCDD.

Competitor	EC_{50}	Relative EC_{50}	K_C	Relative K_C
	pM		pM	
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	41	1	6.8	1
2-Iodo-7,8-Dibromodibenzo-*p*-dioxin	58	1.4	11	1.7
2,3,7,8-Tetrachlorodibenzofuran	85	2.1	18	2.6
2,3-Dibromodibenzo-*p*-dioxin	580	14	140	20
2,3-Dichlorodibenzo-*p*-dioxin	6400	160	1600	230

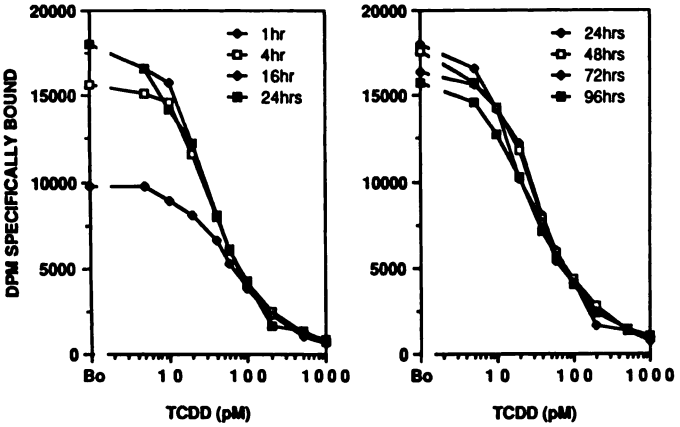

Fig. 5. Effect of incubation time on the competition binding curve. The assay was run as described in the text, with increasing concentrations of TCDD, a total radioligand concentration of 7.5 pM and a receptor concentration of 18 pM. The competition binding curves were analyzed after varying incubation times (key is inset) at 4°. Ordinate: radioligand specifically bound in disintegrations per milliliter. Abscissa: concentration of unlabeled TCDD (picomolar concentration). Each value is the result of triplicate determinations.

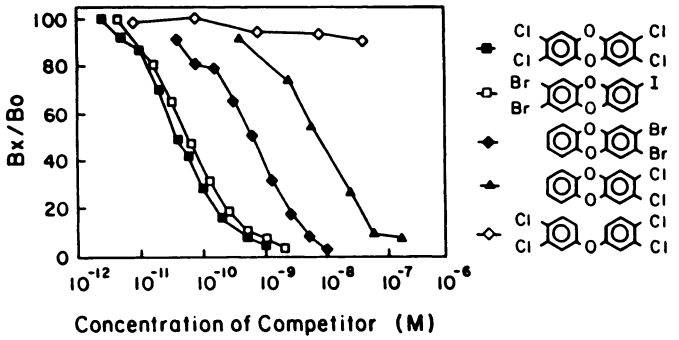

Fig. 6. Competition by various compounds for the specific binding of [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin to the *Ah* receptor. The assay conditions are identical to those described in Fig. 5. All values are the result of quadruplicate determinations. Compounds were dissolved in dimethyl sulfoxide and added to the incubation in 5 μl total volume.

ing of [^{14}C]TCDD. Three independent analyses of a [^{14}C]TCDD standard, quantified by β-scintillation spectrometry to a concentration of 40 pM, gave a value of 40 ± 2 pM from the standard curve (Table 2).

^{125}I decays to tellurium with a half-life of 60 days. Radiodecay of the iodinated ligand may produce unlabeled 2,3-dibromodibenzo-*p*-dioxin in the radioligand stock solution. The K_D of the 2,3-dibromodibenzo-*p*-dioxin was estimated by competitive binding to be approximately 140 pM (see below). We tested whether radioligand decay products might compete for receptor binding and alter the competitive binding curve. Assays using the stock solution of radioligand 120 days after its synthesis (75% of ^{125}I radiodecay), showed no significant deviation in the quality control parameters compared to freshly prepared radioligand (Table 2). Thus, repurification of the radioligand by high performance liquid chromatography does not appear necessary for at least 120 days after synthesis.

Using the standard assay conditions outlined above, competitive binding curves were generated for a variety of halogenated-dioxin congeners (examples are given in Fig. 6). The relative binding affinities of these congeners, as indicated by estimates of EC_{50} and K_D (Table 3), are in agreement with relative binding affinities, and biological potencies which have been published previously [i.e., TCDD > TCDBF > 2,3-dichlorodibenzo-*p*-dioxin > 3,4,3′,4′-tetrachlorobiphenylether (10, 25)]. Additionally, testosterone, phenobarbital, thyroxine, estradiol, cortisol, and pregnenalone-16α-carbonitrile at levels as high as 0.8 μM (i.e., 10^5 times the concentration of radioligand) did not compete with the radioligand for *Ah* receptor occupancy (data not shown).

Discussion

In this report, we describe a sensitive competitive binding assay for the detection and characterization of ligands of the *Ah* receptor. Since the sensitivity of such an assay is a function of the binding affinity and specific activity of the radioligand, considerable attention has been devoted to the selection, labeling, and binding kinetics of this compound, [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin (18).

The higher specific acitivity of this radioligand (2176 Ci/mmol) and the greater counting efficiency of γ-emission over β-emission increases the ratio of counts per minute per femtomole of radioligand about 2 orders of magnitude as compared to [^{3}H]TCDD (58 Ci/mmol). The ^{125}I-labeled ligand offers other minor advantages: 1) it has high radiochemical purity resulting from the easy separation of the iodinated product from the starting material, reaction intermediates, and radiodecay products, 2) specific activity is determined as that of the Na[^{125}I] used for synthesis, and 3) after 75% radiodecay, a stock solution of the ^{125}I-ligand produces the same competitive binding curve, indicating that radiodecay products do not significantly affect receptor-ligand binding (Table 2).

In a previous report (18), we described the effects of protein concentration on the quantification of "free" radioligand and on the calculations of K_D and $[R]_T$. We suggested that at protein concentrations above 70 μg/ml, "nonspecifically bound" radioligand is misclassified as "free" radioligand, leading to significant overestimation of K_D by Scatchard analysis. By minimzing misclassification errors we calculated the K_D of [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin to be approximately 6.5 pM. For the competitive binding assay, a concentration of 150 μg/ml was employed to ensure ligand solubility. At this protein concentration Scatchard analysis of the saturation binding isotherm yielded an apparent K_D of 16 pM (Fig. 1), suggesting that some misclassification of nonspecifically bound radioligand as free radioligand is occurring. The effect of this misclassification appears to be minimal, as the calculation of K_C for 2-iodo-7,8-

dibromodibenzo-*p*-dioxin (i.e., 11 pM) is only 1.7-fold greater than the previously determined estimate of K_D for the ^{125}I analogue (i.e., 6.5 pM). This 1.7-fold overestimation of K_C suggests that the true K_C for TCDD is approximately 4 pM, 100-fold lower than previous estimates for this compound (25).

It has been previously proposed (32) and demonstrated (33) that a competitive binding assay, using the *Ah* receptor and radioligand, could be used to screen for the presence of competing ligands present in the environment. The extraordinary sensitivity of the present assay, attributable to the high specific activity of the radioligand, revised estimate of receptor affinity, and optimization of assay sensitivity, make it feasible to screen environmental samples for TCDD and related halogenated aromatic hydrocarbons: e.g., chlorinated dibenzo-*p*-dioxin, -dibenzofuran, and -biphenyl isomers. Environmental samples may also contain polycyclic aromatic hydrocarbons, many of which are also ligands of the *Ah* receptor. These compounds can be eliminated from analysis by the appropriate sample cleanup. The method of sample cleanup depends on the sample matrix (e.g., biological tissue, soil, water, etc.) and the presence of interfering substances. The competitive binding assay does not provide chemical identification of competing ligands, but an estimate of their concentrations as "TCDD-binding equivalents" (see Rationale and Table 3). Samples judged to have sufficient concentrations of TCDD-binding equivalents, could be subjected to mass spectrometry for chemical identification.

Finally, one of the most exciting uses of this competitive binding assay, is to screen biological tissue extracts for the postulated endogenous ligand of the *Ah* receptor.

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