Absence of positive co-operativity in the binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to its cytosolic receptor protein

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The role of positive co-operativity in stabilizing the binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to the rat hepatic cytosolic TCDD receptor protein (Ah receptor) was investigated. The binding mechanism of TCDD was determined by kinetic means through equilibrium and saturation binding studies, and Scatchard and Hill plot analysis. In all studies, the slope of the Hill plot was close to 1.0, indicating the absence of positive co-operativity. Interpretation of the Scatchard plot was however complicated by the fact that both linear and nonlinear plots were experimentally obtained. The nonlinearity was shown to be an experimental artifact and a consequence not of co-operativity, but of high levels of nonspecific binding. The high level of nonspecific binding could be attributed to: (1) lipophilicity of the TCDD ligand, and (2) inefficient competition of receptor-bound [³H]TCDD. When nonspecific binding was minimized, the Scatchard slope was linear and in agreement with the Hill coefficient, thus indicating the lack of positive co-operativity in the binding of TCDD to the Ah receptor.

INTRODUCTION

The toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is in part mediated by the Ah (aryl hydrocarbon) receptor (Poland *et al.*, 1979, 1983; Poland & Knutson, 1982; Safe, 1984, 1986). The physicochemical characteristics of the Ah receptor are similar to those shown for the other steroid receptors (Poellinger *et al.*, 1985a; Okey *et al.*, 1979) and intracellularly, the steroids and TCDD are thought to elicit their biochemical and physiological/toxicological effects via a similar pathway (Poellinger *et al.*, 1985b; Gasiewicz & Rucci, 1984). These lipophilic ligands bind their cognate receptors noncovalently, reversibly and specifically. The binding induces a conformational change in the receptor protein that is associated with both an increase in ligand binding affinity and DNA binding affinity (Poland *et al.*, 1979, 1983; Safe, 1984, 1986; Muller *et al.*, 1985). Functionally, the receptors act as DNA binding proteins and are thought to elicit a complex, yet co-ordinated and characteristic, response by regulating the rate of induction of a number of select and diverse genes (Poellinger *et al.*, 1985b; Gasiewicz & Rucci, 1984; Jones *et al.*, 1985). For TCDD, toxicity is thought to be a result of a sustained alteration of gene expression (Poland & Knutson, 1982; Safe, 1984, 1986).

Recently, a molecular mechanism has been proposed for the oestrogen receptor which results in increased receptor–ligand complex stability and its prolonged nuclear retention (Sasson & Notides, 1983, 1984a,b). The mechanism is positive co-operativity, and strictly defined it is a site–site interaction of a multimer whereby conformational changes induced by ligand binding in one subunit mediate an increase in the binding affinity of the second subunit. Experimentally, evidence for co-

operativity is nonlinear Scatchard plots and Hill plots with slopes that are not equal to 1 (Notides *et al.*, 1981, 1985; Notides & Sasson, 1983). These kinetic analyses for the binding of oestrogen to its receptor demonstrated that, with sufficient receptor concentration, the binding was co-operative. From the dependency of co-operativity on receptor dimerization, it was assumed that monomer–monomer–receptor interactions were necessary to stabilize the higher affinity form of the activated oestrogen receptor (Notides *et al.*, 1981; Weichman & Notides, 1977). In contrast, the glucocorticoid receptor also appears to undergo conformational changes which result in increased ligand binding affinity; however this occurs in the absence of receptor dimerization (Lan *et al.*, 1984; Schmidt *et al.*, 1982; Ringold, 1985). Thus, dimerization is not an essential requirement for the stabilization of receptor–ligand complexes.

There are conflicting reports with respect to positive co-operativity of the Ah receptor in the literature, as both linear and nonlinear Scatchard plots of the binding of TCDD have been observed (Gasiewicz & Rucci, 1984; Guenther & Nebert, 1977; Okey *et al.*, 1979; Poellinger *et al.*, 1985b; Poland *et al.*, 1976). However, a large number of experimental artifacts have often been shown to introduce deviations of the Scatchard plot that have been erroneously attributed to positive co-operativity (Klotz, 1982; Ketelslegers *et al.*, 1984; Scheinberg, 1982; Chamnes & McGuire, 1975). Such experimental artifacts include: failure to achieve binding equilibrium, receptor degradation, impurity of the radioligand, incorrect separation of bound and free ligand, and high levels of nonspecific binding. This latter factor has been a serious limitation to the Scatchard analysis for the Ah receptor, since TCDD is very lipophilic and interacts nonspecifically and with low affinity with a large number of

Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran.

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non-receptor binding sites. Alternatively, other experimental conditions have been used which generate linear Scatchard plots using receptors with the potential to bind ligands co-operatively. A significant problem has been the failure to measure binding at sufficiently low ligand concentrations where nonlinearity attributable to co-operativity would be evident (Chamnes & McGuire, 1975; Ketelslegers *et al.*, 1984). In addition, if receptor dimerization is a requirement for positive co-operativity, such interactions may not be evident on the Scatchard plot if an insufficient concentration of receptor was used in the binding assay (Sasson & Notides, 1983, 1984a,b). With these constraints in mind, advantage was taken of the fact that young and old rats differ on the basis of levels of Ah receptor (Gasiewicz & Rucci, 1984; Poellinger *et al.*, 1985a) and levels of contaminating fat and thus, cytosol samples could be varied without biochemical manipulation with respect to both receptor concentration and number of nonspecific binding sites. Utilizing these differences in cytosol preparations, in-depth kinetic binding analyses were used to determine if TCDD binds co-operatively to the Ah receptor.

MATERIALS AND METHODS

Chemicals

[³H]TCDD (sp. radioactivity 50–52 Ci/mmol) was a gift from Dr. A. B. Okey (Division of Clinical Pharmacology, Hospital for Sick Children, Toronto, Ontario, Canada) and was determined by h.p.l.c. to be greater than 95% pure. 2,3,7,8-Tetrachlorodibenzofuran (TCDF) with a purity greater than 98% was synthesized in this laboratory by S. S. Hydroxyapatite was purchased from BioRad (BioGel HTP). Dimethyl sulphoxide and glycerol were obtained from Fisher Scientific; Hepes, dithiothreitol and Triton X-100 were obtained from Sigma. The scintillation fluid was purchased from National Diagnostics.

Animals

Male Wistar rats of various ages (3 weeks to greater than 3 months as indicated) were obtained from Harlan, Houston, TX, U.S.A. The animals were housed in polycarbonate cages, maintained on Ralston Purina rat chow and a daily 12 h diurnal light/dark cycle. The animals were killed by cervical dislocation and the livers perfused *in situ* with HEDG buffer [25 mM-Hepes, pH 7.4, 1.5 mM-EDTA, 1.0 mM-dithiothreitol and 10% (v/v) glycerol]. The livers were homogenized in buffer (1:3, w/v) and centrifuged at 100 000 *g* for 1 h. The resulting supernatant fraction was stored in liquid N₂ and usually cytosol samples less than 2 weeks old were used in the assays. The protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) and protein concentrations used in the assays ranged from 1 to 17 mg/ml.

Hydroxyapatite assays

The binding of TCDD to the Ah receptor was determined using the procedure of Gasiewicz & Neal (1982). Protein at a volume of 0.5 ml was incubated with ligand ranging in concentrations from 0.2 to 10 nM-[³H]TCDD. The [³H]TCDD and competitors were dissolved in dimethyl sulphoxide and added at a volume which never exceeded 2% of the final assay volume. Each

sample was assayed either in duplicate or triplicate. Nonspecific binding was determined by incubating parallel tubes with unlabelled TCDF (250–500-fold excess). Unless specified, the ligand and receptor were incubated at 20 °C for 2 h. After this time, 0.2 ml of the incubation mix was added to hydroxyapatite. The hydroxyapatite was washed repeatedly with HEDG buffer (1:3, w/v) until the pH reached 7.4. Following the addition of the incubation mixture, the hydroxyapatite/protein slurry was vortex-mixed and left on ice for 0.5 h. The tubes were shaken every 5 min. The volume of hydroxyapatite varied with protein concentration: 0.25 ml of hydroxyapatite was used at protein concentrations less than 5 mg/ml, 0.5 ml for protein concentrations ranging from 5 to 10 mg/ml and 1.5 ml for protein concentrations greater than 10 mg/ml. After 0.5 h, 1.0 ml of homogenizing buffer containing 1% Triton X-100 was added and the tubes were vortex-mixed and centrifuged at 3500 *g* for 0.5 min. The supernatant was poured off and the tubes were washed three more times; 2.0 ml of ethanol was added to the final hydroxyapatite precipitate and this was transferred to scintillation vials. Scintillation fluid (10 ml) was then added and the radioactivity was determined by liquid-scintillation counting (efficiency 30–50%).

Time course and receptor stability

The hydroxyapatite method outlined above was followed except the incubation time was varied from 0.5 to 24 h. To determine the stability of the receptor, protein without labelled ligand was preincubated for the same amount of time as protein containing a saturating concentration of labelled ligand. After the preincubation, a saturating concentration of [³H]TCDD was added and incubated at 20 °C for an additional 2 h. The amount of receptor degradation was defined as the amount of ligand binding which was lost after preincubation at sub-saturating ligand concentrations at 4 and 20 °C.

Scatchard plots

[³H]TCDD at concentrations of 0.2–10 nM were added to cytosol and the level of ligand bound was measured by the hydroxyapatite method. The specifically bound ligand was the difference between totally bound ligand and nonspecifically bound ligand (Scatchard, 1949). Linear regression was used to determine the slope of the nonlimiting portion of the Scatchard plot. The *K_d* and *R_{max}* values were determined from the derived equation.

RESULTS

Binding equilibria studies

Fig. 1 summarizes the time-course binding of [³H]TCDD to hepatic cytosol from 3–4-week-old male Wistar rats at protein concentrations of 2.0 and 6.0 mg/ml using a saturating concentration of the labelled ligand (5.2 nM). The binding curves indicated that equilibrium was reached after 2 h at 20 °C. The level of nonspecific binding was relatively similar at both protein concentrations, although the level of specific binding was greater at the higher concentration of protein and this reflected the concentrated level of receptor at this protein concentration. Incubation of a subsaturating concentration of labelled ligand (0.75 nM) with the cytosol containing high (6.0 mg/ml) and low

Fig. 1. Equilibrium binding of $[^3\text{H}]\text{TCDD}$ to the Ah receptor as measured using hepatic cytosol from 3–4-week-old rats

Hepatic cytosol was assayed for $[^3\text{H}]\text{TCDD}$ binding at 0.08, 0.5, 1, 2, 3, 4 and 5 h. The specific binding was the difference between total binding and nonspecific binding. The nonspecific binding was determined by incubating a 250–500-fold excess of unlabelled TCDF in addition to $[^3\text{H}]\text{TCDD}$. At a saturating concentration of $[^3\text{H}]\text{TCDD}$ (5.2 nM), specific binding was determined at a protein concentration of 2.0 mg/ml (●) and 6.0 mg/ml (○). The respective nonspecific binding is also shown (■, □). Each point represents the mean of $[^3\text{H}]\text{TCDD}$ binding from two or three determinations. The time to achieve equilibrium binding was similar for cytosols obtained from either young or old rats.

(2.0 mg/ml) protein concentrations gave binding curves (Fig. 2) which indicate that equilibrium is also attained after incubation for 2 h. The results further demonstrate that the levels of nonspecific and specific binding and the nonspecific/total binding ratios are identical at both high and low protein concentrations after incubation with a

Fig. 2. Equilibrium time-course of $[^3\text{H}]\text{TCDD}$ binding to the Ah receptor at a subsaturating ligand concentration (0.75 nM)

The specific (●) and nonspecific (■) binding was measured at a protein concentration of 2.0 mg/ml and also at 6.0 mg/ml (○ and □ respectively). Each point represents the mean value from two or three determinations.

subsaturating concentration of $[^3\text{H}]\text{TCDD}$. This is in contrast with the nonspecific/total binding ratios obtained at a saturating ligand concentration, which show a smaller ratio at a high protein concentration and a higher ratio at a low protein concentration (Fig. 1).

Binding studies with receptor from 3–4-week-old rats

A Scatchard plot for the binding of TCDD to the Ah receptor is shown in Fig. 3. The hepatic cytosol sample was obtained from immature male Wistar rats (3–4 weeks old). At a protein concentration of 1.7 mg/ml, the Scatchard slope is linear with a regression coefficient correlation of 0.91. The ligand concentrations used in the experiment ranged from 0.25 to 8 nM and the receptor was saturated at a ligand concentration of 2 nM. The binding affinity (K_d) was 0.29 nM and the maximum concentration of receptor (R_{max}) was 91 fmol/mg. When the data were transposed to the Hill plot (Fig. 4), the Hill coefficient (slope) was 1.1. This indicated a lack of positive co-operativity at this protein concentration.

The Scatchard plot at a higher protein concentration (6.0 mg/ml) was also linear with a regression correlation coefficient of 0.91 (Fig. 3). The receptor was saturated at a ligand concentration of 4.0 nM, the R_{max} value was 100 fmol/mg and the K_d was 1.25 nM. This K_d was significantly higher than the K_d obtained at the lower protein concentration. This protein-dependent difference in K_d value has been previously reported using other ligands and receptor proteins (Jacobs *et al.*, 1975; Dehaven & Mailman, 1983), and is attributed to methodology rather than a protein-concentration-dependent change in affinity. The Hill coefficient at the higher protein concentration (6.0 mg/ml) was identical

Fig. 3. Scatchard plot of $[^3\text{H}]\text{TCDD}$ bound specifically to the Ah receptor

Hepatic cytosol from 3–4-week-old male Wistar rats was incubated with concentrations of $[^3\text{H}]\text{TCDD}$ ranging from 0.25 to 10 nM. The maximum receptor concentration for this cytosol preparation was approx. 92 fmol/mg of protein. The protein concentrations used were 1.7 mg/ml (●) and 6.0 mg/ml (○). The dissociation constants found at each protein concentration were 0.29 nM and 0.87 nM, respectively. The r value obtained at a protein concentration of 1.7 mg/ml was 0.91; six different ligand concentrations assayed in duplicate were used to construct the plot. At a protein concentration of 6.0 mg/ml, the r value, derived from the slope comprising eight data points, assayed in duplicate, was also 0.91.

Fig. 4. Hill plot of [^3H]TCDD binding specifically to the Ah receptor

The plot is derived from the data shown in Fig. 3. The slope of the Hill plot was determined by linear regression and the Hill coefficient at a cytosolic protein concentration of both 1.7 mg/ml (●) and 6.0 mg/ml (○) was 1.1. Only the data values leading up to the point of saturation were used, since inclusion of post-saturation data points caused a biphasic skew in the Hill slope.

with the coefficient obtained at the lower protein concentration (Fig. 4).

Binding studies with receptor from mature rats

The Scatchard plot for the Ah receptor ligand binding data using protein obtained from a 12–17-week-old mature rat is illustrated in Fig. 5. At a concentration of both 2.4 mg/ml and 8.7 mg/ml, the Scatchard plot is

Fig. 5. Scatchard plot of specific [^3H]TCDD binding to the hepatic Ah receptor

The cytosol sample was obtained from mature (> 4 months old) male Wistar rats and the total receptor concentration was 30 fmol/mg of protein. The K_d obtained from the non-limiting portion of the Scatchard plot was 1.7 nM at a protein concentration of 2.4 mg/ml (●), and 1.8 nM at a protein concentration of 8.7 mg/ml (○). The r value obtained at the lower protein concentration ($n = 15$ data points determined in duplicate) was 0.74. The r value obtained at the higher protein concentration ($n = 11$, determined in duplicate) was 0.84.

Fig. 6. Hill plot of [^3H]TCDD binding to the Ah receptor

The plot was drawn by transforming the data shown in Fig. 5. However, only those points obtained up to receptor saturation were used. The Hill coefficient was 1.1 at a protein concentration of 2.4 mg/ml (●) and 8.7 mg/ml (○).

curvilinear. The K_d estimated from the limiting slope was approx. 2 nM, and the R_{max} value was approx. 33 fmol/mg of cytosolic protein. The decrease in receptor concentration was consistent with the reported age-dependent variations in rat hepatic receptor levels (Carlstedt-Duke *et al.*, 1979). While the nonlinear slope of the Scatchard plot was suggestive of positive co-operativity, the slope of the Hill plot (1.1) indicated the absence of a progressive increase in binding affinity (Fig. 6).

Binding studies with receptor from 10-week-old rats

The Scatchard plot of the receptor–ligand binding data using cytosol from 10-week-old rats was also curvilinear after incubation for 0.2, 2 and 5 h (Fig. 7). The nonlinearity of the Scatchard plots was observed after incubation of cytosol and labelled ligand for 0.5 h (nonequilibrium conditions) and after significant degradation of the receptor protein (5 h). Previous studies indicate that nonlinear Scatchard plots may result from measurements taken at nonequilibrium conditions or from degradation of the receptor (Notides *et al.*, 1981; Notides & Sasson, 1983; Chamnes & McGuire, 1975) and this was also observed in this study. However, these conditions were not responsible for the nonlinear Scatchard plots, since comparable results were obtained after incubation of the cytosolic receptor and labelled ligand for 2 h (Fig. 7). Moreover, the slopes of the Hill plots of the data obtained after the 0.5, 2 and 5 h incubations were 1.1 (results not shown).

Effects of nonspecific binding

Fig. 8 demonstrates the partial, irreversible binding of [^3H]TCDD to the Ah receptor. The concentration of protein used in this experiment was 2.0 mg/ml and the concentration of radiolabelled TCDD was 2.0 nM. Using these concentrations of ligand and protein, the level of totally bound [^3H]TCDD was 54.0 fmol/mg of protein. Nonspecific binding, determined by the simultaneous addition of [^3H]TCDD and a 250-fold excess of unlabelled TCDF, was 13.5 fmol/mg of protein. The

Fig. 7. Scatchard plot of $[^3\text{H}]\text{TCDD}$ binding to the Ah receptor as measured at three different equilibrium time points (Δ , 0.5 h; $\bullet$, 2 h; $\square$, 5 h)

The cytosol was prepared from rats that were 10 weeks old and used at a protein concentration of 8.4 mg/ml. At the specified time, 0.2 ml was removed from the incubation mix and added to a 1:3 (v/v) slurry of hydroxyapatite for 0.5 h. $[^3\text{H}]\text{TCDD}$ binding was determined by four subsequent washings of the hydroxyapatite/protein slurry with HEDG buffer (see the text) containing 1% Triton X-100. Each value shown is the mean of two determinations. For the 2 h binding plot, two additional data points are shown and reflect bound $[^3\text{H}]\text{TCDD}$ found at the two highest ligand concentrations. At similar ligand concentrations, the binding data obtained after 0.5 and 5 h were meaningless and therefore are not shown.

displacement of $[^3\text{H}]\text{TCDD}$ from the bound complex was determined by incubation of receptor protein and $[^3\text{H}]\text{TCDD}$ for 2 h followed by the addition of a 250-fold excess of unlabelled competitor for 1 h. Following this post-incubation with excess unlabelled TCDF, the level of bound $[^3\text{H}]\text{TCDD}$ was 48.6 fmol/mg. Thus, TCDF displaced only 10% of the radiolabelled TCDD. The experiment in which the additions of $[^3\text{H}]\text{TCDD}$ and TCDF were reversed was also carried out. The nonspecific binding determined for this experiment was 6.3 fmol/mg of protein. This represented a 2-fold reduction in the nonspecific binding compared with the results obtained after simultaneous co-incubation of $[^3\text{H}]\text{TCDD}$ and TCDF. We assumed that the decrease in nonspecific binding obtained on preincubation with unlabelled TCDF was a result of the unlabelled ligand binding to the Ah receptor, which blocked the binding of $[^3\text{H}]\text{TCDD}$. Therefore, it was concluded that the high nonspecifically bound $[^3\text{H}]\text{TCDD}$ observed in the competitive binding studies (i.e. $[^3\text{H}]\text{TCDD} + \text{TCDF}$) is due in part to significant levels of specifically bound labelled ligand.

The relationship between TCDD concentration, specific, nonspecific, and total binding is summarized in Fig. 9. The graph indicates that while the level of nonspecific binding varies directly with TCDD concentration, the levels of specific and total binding are not linearly related to the ligand concentration. For this reason, the

ratio of nonspecific to total or specific binding is not constant throughout the Scatchard assay and is variable at each ligand concentration. At low concentrations of TCDD, the ratio is relatively high because there is a low level of specific binding. As the concentration of ligand increases up to the point of receptor saturation, the proportion of bound receptor also increases and this is associated with a decrease in the nonspecific to total binding ratio. Since the specific binding is the difference between total binding and nonspecific binding, the influence of nonspecific binding on the Scatchard plot can be eliminated by plotting the levels of totally bound ligand instead of specifically bound ligand.

Fig. 10 shows a plot of the results previously reported in Fig. 5 in which the totally bound values are used and the resulting Scatchard plot was linear. Fig. 11 summarizes the Scatchard plots obtained after incubation of $[^3\text{H}]\text{TCDD}$ with rat hepatic cytosol and $[^3\text{H}]\text{TCDD}$ with the same cytosol preparation that had been preincubated for 1 h with TCDF in a 250-fold molar excess (as described in the legend to Fig. 8). The preincubation step with TCDF served to decrease the nonspecific binding of $[^3\text{H}]\text{TCDD}$ and the Scatchard plot of the resulting data was linear. In contrast, a Scatchard plot of the data obtained when the preincubation step was omitted was nonlinear. The slopes of the Hill plots of both sets of data (Fig. 11) were both 1.1 (results not shown).

Fig. 8. Irreversible binding of [³H]TCDD to the Ah receptor

Throughout this experiment, the following concentrations of protein, [³H]TCDD and unlabelled TCDF were used: 2.0 mg/ml, 2.0 nM and 500 nM, respectively. Initially, total and nonspecific binding were determined as outlined in the Materials and methods section and are shown in columns 1 and 2. To determine the displacement of receptor-bound [³H]TCDD, the radiolabelled ligand was added to cytosol for 2 h at 20 °C, at which time competing unlabelled ligand was added for an additional 1 h. Due to the irreversible nature of the receptor-[³H]TCDD interaction, the excess unlabelled TCDF displaced only 10% of the labelled ligand (see column 3). In the reverse experiment, unlabelled TCDF was added to cytosol for 1 h prior to the addition of [³H]TCDD and the level of nonspecific binding was determined (column 4). By preincubating initially with unlabelled ligand, the level of nonspecifically bound [³H]TCDD was decreased by 47%. Our interpretation is outlined in the Results section. The binding of [³H]TCDD and S.D. for each of four determinations of the total binding, nonspecific binding, 2 h pre-incubation and 2 h post-incubation in fmol/mg were 54.0 ± 3.54 , 13.5 ± 1.81 , 48.6 ± 3.56 and 6.30 ± 0.83 respectively.

DISCUSSION

TCDD appears to bind to the Ah receptor via a simple bimolecular binding process that does not induce co-operative changes in the receptor. The Scatchard plot is linear and the Hill coefficient is close to 1.0 when the specific binding is measured under conditions where the ratio of nonspecific to total binding is low. The occasional nonlinear Scatchard plot, coupled with a Hill coefficient of approx. 1, indicated that the nonlinearity resulted from a factor not related to co-operativity. The cause of the nonlinearity of the Scatchard plot appears to be an unproportionately high ratio of nonspecific to total binding. Since the concentration of TCDD usually determines the level of nonspecific binding, the variability of the ratio at a given concentration of TCDD arises largely from the fluctuation of the level of total binding, which is a function of the $R_{\max}$ value. The $R_{\max}$ value, or the maximum receptor concentration contained in the cytosol sample, is in turn variable because in the male rat, the Ah receptor, and thus the $R_{\max}$ value, decreases with age because of a developmental reduction of the

Fig. 9. Binding of [³H]TCDD to the hepatic Ah receptor at variable ligand concentrations

Total binding (Δ) was measured at eight ligand concentrations ranging from 0.25 to 9 nM. The level of nonspecific binding (■) was determined by a parallel incubation using a 250-fold molar excess of TCDF at each ligand concentration. The saturable specific binding (●) was determined to be the difference between total and nonspecific binding. The binding at each ligand concentration is the mean value of duplicate samples.

Fig. 10. Scatchard plot of total bound [³H]TCDD to the hepatic Ah receptor

The plot is derived from the data used to generate the Scatchard plot illustrated in Fig. 5. The protein concentrations used were 2.4 mg/ml (●) and 8.7 mg/ml (○). The [³H]TCDD binding shown is that binding measured without taking into account the levels of nonspecific binding. The regression lines shown in the Figure are linear, with regression coefficient correlations greater than 0.89. In both plots, only the data points of the binding up to the point of saturation are shown on this graph (eleven and nine determinations for the low and high protein concentrations respectively). High levels of nonspecific binding, while not affecting specific binding, result in a plateau of the nonlimiting portion of the graph which indicates the presence of a second, low-affinity site. This deviation from linearity, obtained at high ligand concentrations where the amount of [³H]TCDD bound is high, was omitted from this plot.

Fig. 11. Effect of preincubation with unlabelled ligand on the Scatchard plot

Hepatic cytosol (7.0 mg/ml) was incubated with a 250-fold molar excess of TCDF at 20 °C for 1 h (▲). At this time, [³H]TCDD was added for an additional 2 h. A parallel experiment was also run in which the preincubation step was omitted and the nonspecific binding determined after 2 h following the sequential addition of the labelled and unlabelled ligands (●).

expression of the receptor (Carlstedt-Duke *et al.*, 1979). Consequently, with cytosol preparations from older rats, the R_{max} value is low, the proportion of nonspecific to total binding is high, and the resulting Scatchard slope is usually skewed (Fig. 5). In contrast, cytosol from younger rats containing higher levels of the hepatic Ah receptor exhibit a lower nonspecific to total binding ratio, and the Scatchard plots are frequently linear (Fig. 3).

It has been shown for other receptors that biphasic ligand dissociation kinetics supply further evidence of co-operativity (Weichman & Notides, 1979; deBoer & Notides, 1981). The rate of ligand dissociation directly reflects the affinity of ligand-receptor binding. Since co-operativity acts to increase the affinity of binding, it is accompanied by a decrease in dissociation rate of the ligand from the receptor. As reported for the oestrogen receptor, two discrete K_d values differing by a factor of approx. 30 characterize the unbound and co-operatively-activated receptor, respectively (Notides *et al.*, 1985). Alternatively, monophasic ligand-receptor dissociation would indicate a simple binding affinity and would support the absence of co-operativity. However, for the Ah receptor, the dissociation kinetics could not be determined because the association of the TCDD-Ah receptor complex appears essentially irreversible (Fig. 8).

The molecular mechanism of co-operativity for the oestrogen receptor is thought to be receptor dimerization (Notides *et al.*, 1981; Notides & Sasson, 1983). Thus the extent of co-operativity, as defined by the Hill coefficient, increases as the concentration of oestrogen receptor increases. For the binding of TCDD to the Ah receptor, the Hill coefficient was similar at all receptor protein concentrations tested. For the oestrogen receptor, a critical concentration of receptor is required for co-operative interactions, which occur at receptor concentrations exceeding 1 nM (Notides *et al.*, 1985). At similar and higher receptor concentrations, no co-

operativity for the binding of TCDD to the Ah receptor was seen.

In retrospect, positive co-operativity results in an increased receptor binding affinity that is associated with a decrease in rate of ligand dissociation. It therefore acts to maintain the biologically active form of the receptor by stabilizing the receptor-ligand complex. Moreover, for the oestrogen receptor, co-operativity may also control the extent of hormonally-mediated transcription; to terminate transcriptional activity, the co-operative changes are reversed and the resulting decrease in binding affinity causes the ligand to dissociate from the receptor (Notides *et al.*, 1985). The Ah receptor does not exhibit detectable co-operativity. TCDD binds with an initially high affinity that is characterized by a low rate of receptor-ligand dissociation. This resulting tenacious association may act to prolong transcription, and the sustained alteration of gene expression (Poland *et al.*, 1983; Poland & Knutson, 1982) may be an important factor associated with the toxicity of TCDD.

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