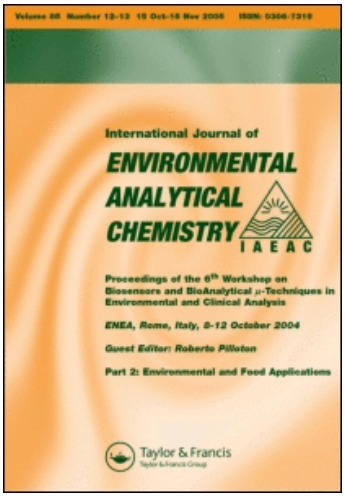

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A Screening Assay for the Tetrachlorodibenzo-p-dioxin Receptor using the [^{125}I]iodovaleramide Derivative of Trichlorodibenzo-p-dioxin as the Binding Ligand

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A Screening Assay for the Tetrachlorodibenzo-*p*-dioxin Receptor using the [^{125}I]iodovaleramide Derivative of Trichlorodibenzo-*p*-dioxin as the Binding Ligand

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A relatively simple assay method for the putative cytosolic 'receptor' that binds 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds is described. The assay is based on specific binding of [^{125}I]dioxin to cytosol 'receptor' protein. Saturation is ensured by competition experiments in which unlabeled TCDD and other competitors displace the radiolabeled ligand from specific binding sites. This assay has been applied to estimation of levels of 'receptor' in cytosol.

KEY WORDS: TCDD receptor, dextran-charcoal, specific binding.

The induction of drug metabolizing enzymes such as aryl hydrocarbon hydroxylase by polycyclic aromatic compounds, including 3-methylcholanthrene, β-naphthoflavone, and benzo[a]pyrene, has been well characterized over the years.[1–3] It has been shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most potent inducers of aryl hydrocarbon hydroxylase and related enzyme activities.[4–6] The induction of aryl hydrocarbon hydroxylase activity by TCDD and other polycyclic aromatic

compounds is regulated by a single genetic locus.[3,7,8] It was postulated[7-10] that TCDD and other polycyclic aromatic compounds bind with receptor protein to form a ligand-receptor complex in the initial step of the mechanism of induction. Then this ligand-receptor complex presumably translocates to the nucleus where it may act as a derepressor. Poland, *et al.*[9] have identified a high affinity, stereospecific, low capacity receptor protein in liver cytosol of mice.

Different assay methods have been developed for the cytosolic receptor protein, all based on high affinity binding of TCDD. Poland, *et al.*[9] used an assay method based on correcting for nonspecific binding of [^{3}H]TCDD after dextran-charcoal treatment. Sucrose density gradient analysis following dextran-charcoal treatment[11,12] and isoelectric focusing in polyacrylamide gels following dextran-charcoal treatment[13] have also been used in attempts to increase specificity. All of these methods were developed using [^{3}H]TCDD as a radioactive ligand. None of these assays are completely acceptable as routine screening methods because they are extremely time consuming and inconvenient. The assay method proposed in this paper using pure, high specific activity 1-N-([^{125}I]-5-iodovaleramido)-3,7,8-trichlorodibenzo-*p*-dioxin ([^{125}I]dioxin) is intended as an alternative assay method for estimation of receptor levels for screening or range finding purposes, column monitoring during purification, etc. Since the present assay shows less non-specific binding than previous assays, specificity is not sacrificed.

EXPERIMENTAL

Materials

1 - N - ([^{125}I] - 5 - iodovaleramido) - 3, 7, 8 - trichlorodibenzo - *p* - dioxin (specific activity 78 Ci/mmol) was prepared as described previously.[14] Briefly, 1-amino-3,7,8,-trichlorodibenzo-*p*-dioxin was converted to 5-bromovaleramide derivative and the bromine was subsequently replaced with iodine. The unlabeled iodo-derivative was then treated with carrier free Na^{125}I to form the [^{125}I]dioxin. Unlabeled 2,3,7,8-TCDD was prepared by the method of Elvidge.[15] Other dibenzo-*p*-dioxin and dibenzofuran standards were kindly provided by Dr. John Moore of this Institute. Activated charcoal was purchased from

Sigma Chemical Co., (St. Louis, Mo.). Dextran T500 and Sephadex G-100 were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). Sodium [^{125}I]iodide with a specific activity of 17 Ci/mg was obtained from New England Nuclear.

Buffer

The assay buffer ("KTE buffer") contained 0.2 M of KCl, 20 mM Tris-HCl, 1 mM Na-EDTA and the pH was adjusted to 7.4 with HCl.

Preparation of cytosol

Mice (C57BL/6J) and rats (Sprague–Dawley) were killed by decapitation, the livers were dissected out, minced, and homogenized with 3 volume of KTE buffer at 0°C in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at $10{,}000 \times g$ for 20 min, and the resulting supernatant fraction was then centrifuged at $105{,}000 \times g$ for 1 hr. The supernatant fraction was carefully pipetted out. This cytosol fraction was further diluted with KTE buffer. The protein concentration was determined by the method of Lowry *et al.*[16] with bovine serum albumin as standard. Fresh cytosol preparations were used in all experiments.

Radioactivity measurement

Radioactivity was measured with a Packard Model 5210 Auto Gamma scintillation spectrometer. Column fractions were collected into 13×100 mm tubes and the radioactivity was measured directly from these tubes. A Packard Model 7230 Radiochromatogram Scanner was used for TLC plates.

Binding assay

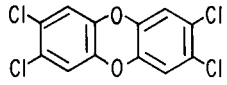
All assays were performed in duplicate. In the assay, each tube containing [^{125}I]dioxin (2×10^4 to 2×10^5 dpm, 59–590 pg) with or without various potential inhibitors was incubated with a final volume of 1 ml of cytosol fraction diluted to contain 150 μg of protein. The [^{125}I]dioxin and other lipophilic compounds were dissolved or suspended in 20 μl of KTE buffer containing 1%

Cutscum (Fisher Scientific Co., Raleigh, N.C.) prior to adding the cytosol. The mixture was incubated in a shaking water bath for 2 hrs at 4°C. After incubation, 0.2 ml of the mixture was removed to measure the total radioactivity. Another 0.4 ml of the mixture was removed and mixed with 0.2 ml of charcoal/dextran suspension (3% activated charcoal and 0.03% dextran T 500, w/v). The tubes were agitated with a Vortex mixer and placed in a shaker bath for 5 min at 4°C, and centrifuged at 3,000 × g for 10 min. Aliquots (0.3 ml) of the supernatant were carefully removed, and radioactivity was determined. For the determination of TCDD specific binding, [^{125}I]dioxin (0.2 nM) was incubated in duplicate preparations with and without an 800-fold excess of unlabeled TCDD and the unbound radioligand removed by charcoal/dextran adsorption. Under these conditions, TCDD receptor sites are blocked by the large excess of unlabeled TCDD, so that the radioactivity remaining in the supernatant fraction after the charcoal adsorption represents non-specific binding of [^{125}I] dioxin to low affinity, high capacity (i.e. relatively non-saturable) binding sites. Specific binding is taken as the difference between [^{125}I]dioxin bound in the absence of unlabeled TCDD and [^{125}I]dioxin bound in the presence of a large excess of unlabelled TCDD.

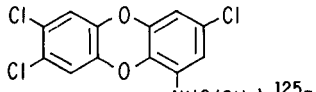
RESULTS AND DISCUSSION

The use of [^{125}I]dioxin (Figure 1) for the receptor binding assay has several advantages over [^{3}H]TCDD: (a) The material can be obtained in radiochemically pure form without contamination by other isomers; (b) the problem of tritium exchange during assay is avoided; and (c) higher specific activities than that used in the present work (78 Ci/mmol) can easily be obtained. When [^{125}I]dioxin bound cytosol was analyzed on a Sephadex G-100 column following treatment with dextran-charcoal, a radioactive peak appeared in the void column (Figure 2). Addition of excess unlabeled TCDD to the cytosol displaced most of the radioactivity in this peak indicating that most of the binding observed is saturable.

The solubility of TCDD and related compounds in aqueous systems is very low. The detergent Cutscum, as 1% solution in buffer, was used to solubilize TCDD and other hydrophobic compounds.



2,3,7,8 - Tetrachlorodibenzo - p - dioxin
(TCDD)



1-N-([^{125}I] -5- iodovaleramido)-3,7,8 -trichloro-
dibenzo-p-dioxin (^{125}I-Dioxin)

FIGURE 1 Chemical structures of TCDD and radio-ligand [^{125}I]dioxin.

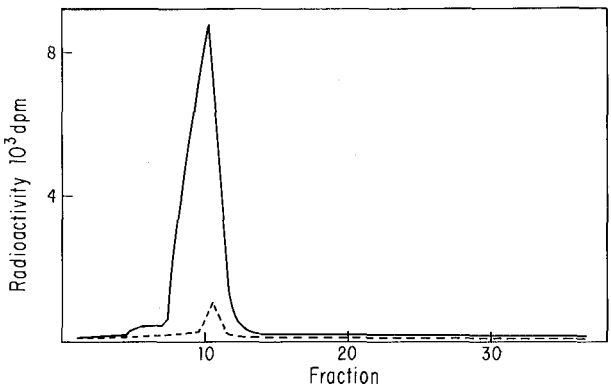


FIGURE 2 Detection of specific [^{125}I]dioxin binding. Hepatic cytosol was incubated with [^{125}I]dioxin in the absence (solid line) or in the presence (dashed line) of 800-fold excess of unlabeled TCDD. Following dextran-charcoal treatment, it was chromatographed on Sephadex G-100 column and eluted with 0.1 M KCl buffer, pH 7.4.

This system has been successfully used in the radioimmunoassay of TCDD without interferring with binding.[14] Under these conditions, [^{125}I]dioxin is not free in solution but held in micelles of the nonionic detergent.[14] Proteins which bind TCDD with low affinity in previous receptor assays, causing a major amount (up to 98%) of

nonspecific binding, are apparently unable to compete with the Cutscum in the present assay. As was the case in the radioimmunoassay for TCDD,[14] however, protein with a high affinity for TCDD, whether antibody or receptor, is able to compete effectively with the detergent micelles. In pilot experiments not described in detail we found that dextran-charcoal easily removed TCDD and [^{125}I]dioxin from Cutscum micelles.

Unlabeled competitors were incubated to compete with the [^{125}I]dioxin binding. The results of competitive binding between [^{125}I]dioxin and other compounds expressed as per cent relative to TCDD are shown in Table I. Phenobarbital showed essentially no competitive binding to the [^{125}I]dioxin binding species. However, 3,4,5,3′,4′,5′-hexachlorobiphenyl and 3-methlcholanthrene were capable of displacing [^{125}I]dioxin from specific binding sites. Unlabeled TCDD had the highest affinity for the binding site of the compounds tested. The data in Table I are in agreement with the findings of Bandiera *et al.*,[17] who found that the competitive binding of 3-methylcholanthrene was of the same order of magnitude as that of TCDD in spite of the great difference in their abilities to induce cytochrome P-448 *in vitro*.

TABLE I

Competition with [^{125}I]dioxin binding to rat hepatic cytosol receptor.

Ligand	Relative inhibition[a]
TCDD	100
3,4,5,3′,4′,5′-Hexachlorobiphenyl	45
3-Methylcholanthrene	87
Phenobarbital	8

[a]Inhibition by 10^{-9} M of test compound relative to inhibition by 10^{-9} M of TCDD set equal to 100.

The specific binding of [^{125}I]dioxin as a function of cytosol protein concentration is shown in Figure 3. The binding was linear up to a protein concentration of 150 μg per assay tube; beyond this point [^{125}I]dioxin was no longer available in excess.

The present assay indicated that hepatic cytosol from Sprague–Dawley rats 3–6 months old contained approximately 18 fmoles of

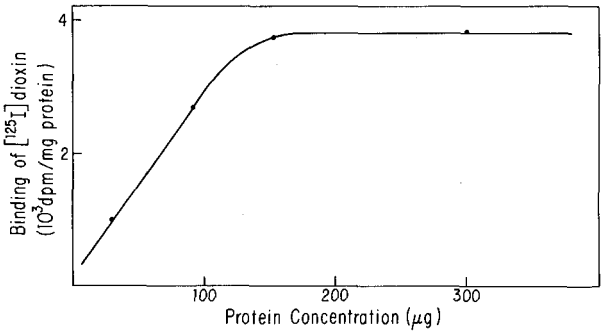


FIGURE 3 Effect of cytosol protein concentration on the specific binding of [^{125}I]dioxin to the receptor. Conditions are described under "Materials and Methods".

receptor per mg of cytosol protein; the corresponding value for C57BL/6J mice was 94 fmoles per mg protein. This is in good agreement with Carstedt–Duke who reported 14 fmol/mg protein for Sprague–Dawley rats,[18] Poland *et al.* who reported 84 fmol/mg protein in C57BL/6J mice[9] and Okey *et al.*[12] has reported 60 fmol/mg protein also in C57BL/6J mice. Young rats reportedly have higher receptor levels than old rats,[19] so exact comparisons of levels reported in the literature may be misleading.

The assay described here yields values for receptor levels comparable to those given by assays used previously. However, the present receptor assay exhibits much less nonspecific interference (less than 10% *versus* 98%) than the original method described by Poland *et al.*,[9] and is much faster and simpler than assays requiring sucrose density gradient centrifugation[12] or electrofocusing.[18] The relative standard deviation ($\pm 10\%$) for direct screening of cytosol is typical of most competitive binding assays and quite adequate for a rapid screening. The absolute accuracy of the assay can not be evaluated until pure receptor protein becomes available.

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