2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin: *IN VITRO* Binding to Rat Liver Microsomes

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a highly toxic contaminant of the herbicide 2,4,5-T and certain chlorinated phenols, HIGGINBOTHAM, et al. (1968). It is also teratogenic, acnegenic and causes degenerative liver damage, ENVIRON. HEALTH PERSPECT, (1973). TCDD is a potent inducer of microsomal oxidative activity causing induction of aryl hydrocarbon hydroxylase with doses at the nmole/kg level, POLAND AND GLOVER (1974). Distribution and excretion studies of TCDD with rats indicate most of the administered TCDD is excreted in the feces with some storage in body fat and the liver. Furthermore, it appears that TCDD is preferentially localized in the endoplasmic reticulum of the liver. However, no metabolites of TCDD have been found from mammalian systems either in vivo or in vitro, PIPER, et al. (1973); VINOPAL AND CASIDA (1973); FRIES, et al. (1975). Using a rat hepatic microsomal system from phenobarbital-pretreated rats, we found that a major portion of incubated [^{14}C]-TCDD was not extracted with ethyl acetate, but was bound to microsomal macromolecules. We have now demonstrated that binding this TCDD was oxidative in nature and was induced by phenobarbital pretreatment.

MATERIALS AND METHODS

Hepatic microsomes from normal or phenobarbital-pretreated (3 days, 75 mg/kg, i.p.) male, Sprague-Dawley rats were prepared by standard procedures and incubated with [^{14}C]TCDD (supplied by Dow Chemical Company, Midland, Michigan) and an NADPH generating system in 6.0 ml of 0.1 M phosphate buffer at pH 7.4 for one hour (37^{o}C). For details of incubation mixture, see footnote, Table 1.

After incubation, the mixtures were extracted 2 times with equal volumes of ethyl acetate: centrifugation was used to complete the separation of phases. The aqueous phases contained precipitated solids which were collected on Millipore filters (0.45μ) and washed with distilled water. Precipitated solids on individual filters were dissolved in Soluene (Packard) and the total radioactivities in the solids fractions were determined by liquid scintillation counting. Aliquots of the organic and aqueous phases were also counted and all counts were corrected to dpm using [^{14}C]hexadecane as an internal standard.

Bulletin of Environmental Contamination & Toxicology,
Vol. 18, No. 1 © 1977 by Springer-Verlag New York Inc.

TABLE 1.

In vitro binding of [^{14}C]TCDD to precipitated solids of rat liver microsomes.

Incubation Mixture (N = 4)	Recovery of Added [^{14}C]TCDD (%)			
	Aqueous Mean±S.E.M.	Organic Mean±S.E.M.	Solids Mean±S.E.M.	Total Mean±S.E.M.
a/ Induced Microsomes	0.62±0.19	44.5±3.03	42.8±5.72	88.0±2.68
b/ Control I	0.30±0.10	87.1±2.28	2.9±0.31	90.3±2.15
c/ Control II	0.40±0.18	83.0±4.46	4.0±1.64	87.4±4.38
d/ Induced Microsomes + SKF-525A	0.28±0.05	91.3±0.78	3.4±0.61	95.0±1.32
e/ Induced Microsomes + GSH	0.62±0.08	89.6±3.14	2.2±0.40	92.4±3.38
f/ Normal Microsomes	0.25±0.03	82.5±2.25	7.0±2.51	89.8±2.00

a/ The incubation mixture contained 4.0 mg of microsomal protein from phenobarbital-pretreated rats, 1.8 µmoles of NADP, 18 µmoles of G-6-P, 0.4 Kornberg Units of G-6-P dehydrogenase, 38.4 ng (17.6 n Ci) of [^{14}C]TCDD added 5 µl of benzene, all in 6.0 ml 0.1 M phosphate buffer, pH 7.4. [^{14}C]TCDD was added after the microsomes because of the low water solubility of TCDD.

b/ Control I -- same as above except the microsomal fraction had been heat inactivated at 100°C for 10 min.

c/ Control II -- same as a/ without the NADPH generating system.

d/ Same as a/ plus 18 µmoles of SKF-525A added in 100 µl of ETOH.

e/ Same as a/ plus 18 µmoles of reduced glutathione (GSH).

f/ Same as a/ except microsomes were prepared from non-induced rats.

RESULTS AND DISCUSSION

Induced microsomes prepared from phenobarbital-pretreated rats bind over 40% of the added [^{14}C]TCDD to the solids fraction (Table 1). This binding to solids is mediated by a heat sensitive system and requires NADPH, as indicated by controls I and II, respectively. Added NADPH, in place of the NADPH generating system, produced a comparable level of TCDD-solids binding. A known mixed function oxidase inhibitor, SKF-525A, inhibited TCDD binding to control levels. Microsomes prepared from noninduced rats bound 7.0% of the added TCDD, but this value is considerably less than TCDD bound to the solids by phenobarbital-induced microsomes (42.8%). These results suggest that the binding of TCDD to the solids of the microsomal incubation mixture is mediated by a mixed function oxidase system. The observed binding was apparently not a covalent linkage since extraction of the incubation mixture with chloroform: methanol (9:1), removed the "bound" radioactivity and this material co-chromatographed with standard TCDD on tlc.

The time course of the binding of [^{14}C]-TCDD is represented in Figure 1. The binding reached a maximum between 45 and 60 min and decreased upon further incubation. The transitory nature of the binding, the apparent lack of a covalently bound product and the oxidative conditions necessary to produce the observed binding have been demonstrated. The mechanism of binding has not been determined, however.

The absence of phenolic and dihydro-diol metabolites would suggest that this binding is not mediated through an arene oxide intermediate. In addition, reduced glutathione (GSH), which reacts chemically and enzymatically with other arene oxides to form GSH conjugates, JERINA AND DALY (1974), does not form such conjugates with TCDD in this *in vitro* system, and indeed, GSH (3mM) inhibits the solids binding to control levels.

Another possibility is the oxidative formation of a TCDD cation radical through a one electron donation from TCDD to cytochrome P-450 in the normal ferric (Fe^{3+}) form to give cytochrome P-450 (Fe^{2+}) and the cation radical. Such a mechanism has recently been proposed by ROGAN *et al.* (1976) in the metabolic activation of polycyclic aromatic hydrocarbons. Electron spin resonace studies might cast further light on the mechanism of the reaction.

Another recent explanation of the basis for TCDD toxicity is the formation of charge transfer complexes (CROSBY, 1976). This suggestion rests upon theoretical calculation of charge distribution in the molecular orbitals of TCDD.

Although the detailed mechanism of binding has not been elucidated, we have demonstrated metabolic reactions are involved in the binding of TCDD. The role of this binding in the toxic,

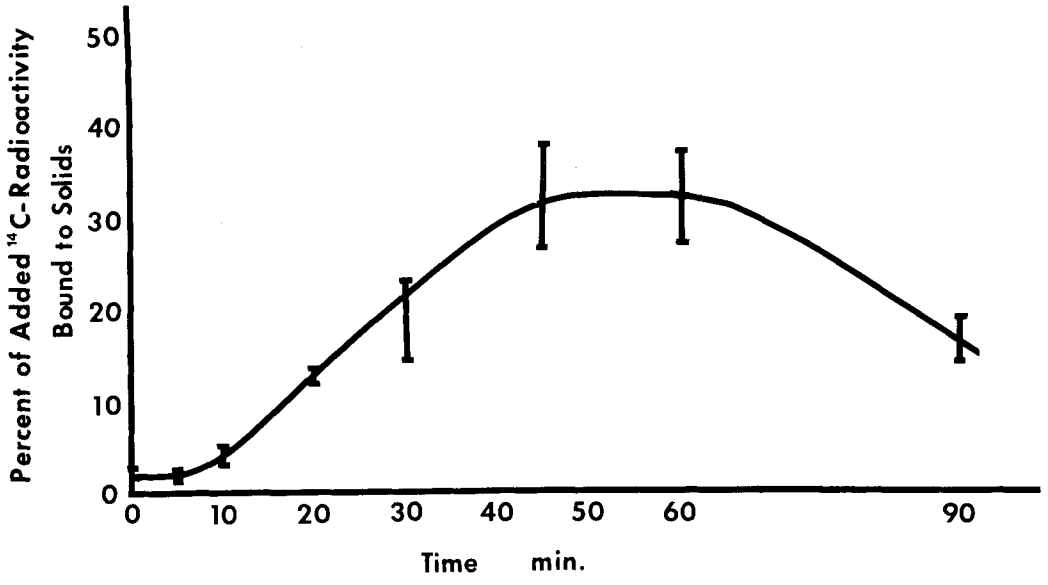

Fig. 1. Kinetics of [^{14}C]TCDD binding to microsomal solids upon incubation of [^{14}C]TCDD(0.02 mM) with microsomes from phenobarbital treated rats and an NADPH-generating system (see Table 1 for details). Incubations were terminated by extraction with ethyl acetate after the appropriate time intervals and solids bound ^{14}C was determined as described in the text. Each point represents 4 determinations $\pm$ S.E.M. except the 90 min value which represents only 2 values.

acnegenic, teratogenic or microsomal oxidase inducing properties of TCDD is unknown.

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Scientific article No. A2112, contribution No. 5069, of the Maryland Agricultural Experiment Station, Department of Entomology.

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