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Studies on the Mechanism of Toxicity of the Chlorinated Dibenzo-p-dioxins*

by Alan Poland[†] and Edward Glover[†]

Introduction

Concern about the potential health hazards resulting from environmental pollution by the chlorinated dibenzo-p-dioxins and dibenzofurans arises from our recognition of the extraordinary potency of these compounds as toxins and teratogens and their inadvertant dispersion in the environment as contaminants of chlorinated phenolic products. Questions concerning the extent of environment contamination and those concerning the mechanism of toxicity produced by these compounds are at present unanswerable. Several papers in this symposium have presented the historical background which led to our current understanding and concern about this problem: (1) the "chick-edema" outbreaks caused by "toxic fats" in poultry feed and the eventual isolation and identification of a hexachlorinated dibenzo-p-dioxin; (2) the occurrence of acne among workers in several 2,4,5-T factories and recognition of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as the etiologic agent; (3) the NCI-commissioned study on the potential teratogenicity, carcinogenicity and mutagenicity of 2,4,5-T; (4) and the widespread use of Agent

Orange as a defoliant in Viet Nam and Cambodia.

The starting point of our studies was a report by Bleiberg and colleagues (1) that of 29 workers in a 2,4,5-T factory, all of whom had industrially acquired acne, 11 had porphyria and several had overt clinical porphyria cutanea tarda. TCDD has been shown to be the causative agent of the acne; however, the cause of the porphyria was uncertain. Porphyria cutanea tarda is an acquired defect of hepatic porphyrin metabolism characterized by an overproduction of porphyrins by the liver, increased urinary excretion of porphyrins, mechanical fragility and photosensitivity of the skin (blistering in areas exposed to sunlight), hyperpigmentation, and hirsutism. We restudied the factory 5 years later and found no evidence of porphyria in the employees (2). The fact that this syndrome abated following measures to reduce the formation TCDD and minimize employee exposure to this contaminant, suggested that TCDD might have been the causative agent of the industrial outbreak originally reported by Bleiberg et al. (1).

Methods and Materials

The halogenated dibenzo-p-dioxins and dibenzofurans and analyses of their purity were generously provided by Dr. A. Poland, Food and Drug Administration, Washington, D. C. and Mr. George Lynn, Dow Chemical Company, Midland, Michigan. In addition, Drs. J. Wade and A. Kende synthesized

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and analyzed a number of dibenzo-*p*-dioxins which were tested and reported elsewhere in this symposium.

Animals

Our experiments were performed in chick embryos which were routinely 15–20 days of age. The various dibenzo-*p*-dioxins were dissolved in *p*-dioxane, and 25 μ l of the solution was injected into the egg through a small hole punched in the air sac. Male Sprague-Dawley rats, 70–100 g, were used in some experiments.

Analysis

δ -Aminolevulinic acid synthetase activity was measured as previously reported (9). Aryl hydrocarbon hydroxylase was assayed essentially by the method of Gielen, Goujon, and Nebert (4). One unit of hydroxylase activity is defined as that amount of enzyme catalyzing the formation per minute at 37°C of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[*a*]pyrene. The assay was performed on the 10,000*g* supernatant, and results are expressed as units per milligram wet weight of liver.

Results

A variety of xenobiotics produce experimental hepatic porphyria and all have in common the ability to induce the initial and rate limiting enzyme in the heme biosynthesis pathway, δ -aminolevulinic acid synthetase (ALA synthetase). To test whether TCDD was in fact porphyrogenic, we administered the compound dissolved in 25 μ l *p*-dioxane to chick embryos. The embryos were sacrificed 48 hr later, and the ALA synthetase activity assayed in their livers. As seen in Figure 1, TCDD produced a dose-related increase in enzyme activity. As little as 4.66×10^{-12} mole egg (1.5 ng) produced a doubling enzyme activity and the highest level tested 1.55×10^{-8} mole/egg (0.5 μ g) produced a 85-fold induction. TCDD is more potent than any other inducer of ALA synthetase yet reported by at least three orders of magnitude, and, unlike most other porphyrogenic chemicals, induction is very prolonged, most likely a

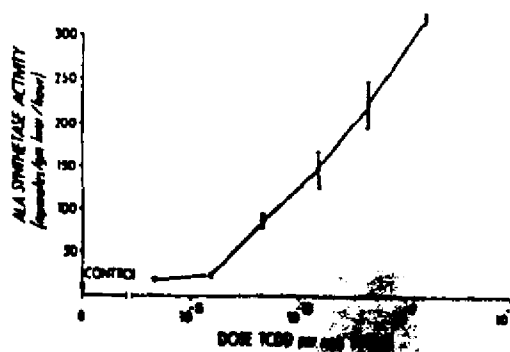


FIGURE 1. Logarithmic dose-response curve for the induction of ALA synthetase by TCDD. Chicken eggs of 17 days' gestation were injected with 25 μ l of solvent (control) or solvent containing various doses of TCDD, and hepatic enzyme activity was assayed 48 hr later. The points represent the mean $\pm$ standard error of three or four groups of pooled livers.

reflection of the long biological half-life of TCDD. We next screened a series of 15 halogenated dibenzo-*p*-dioxins for their ability to induce ALA synthetase. As seen in Figure 2, all the isomers which were inducers

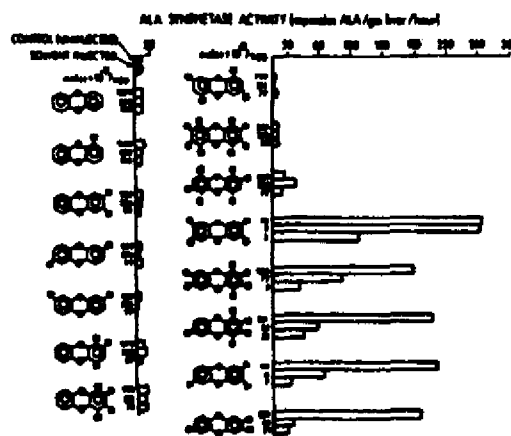


FIGURE 2. Structure-activity relationships of the halogenated dibenzo-*p*-dioxins: induction of ALA synthetase. Seventeen-day embryos were injected with the solvent (*p*-dioxane) or solvent containing the dioxin tested and enzyme activity was assayed 48 hr later. Uninjected control values ($n = 9$), do not differ appreciably from solvent injected controls ($n = 12$). Each bar for the test groups represents the value for a single group of three to five pooled livers.

had two common properties: (1) halogen atoms occupy at least three of the four lateral ring positions (2, 3, 7, and 8) and (2) there is at least one free, nonhalogenated carbon atom. Note that octachlorodibenzo-*p*-dioxin is inactive. The compounds that were not inducers were tested at 200–400 times the molar concentration of TCDD that produced a significant response.

To the extent that toxicologic data are available (5), all those dioxins which are at low doses lethal, teratogenic, or produce acne, also induce ALA synthetase, and those dioxins which are not potent toxins do not induce ALA synthetase. We have also tested a limited series of dibenzofurans; the unsubstituted compound and 2,8-dichloro and octachloro derivatives all fail to induce, and a mixture of di-, tri- and tetrachlorodibenzofurans is potent as an inducer of ALA synthetase. While the data are very limited, it appears the structure-activity relationship is similar in the dibenzofuran series.

As reported elsewhere in this symposium (6), TCDD does not induce ALA synthetase in several laboratory mammals. We have found it to be a poor inducer in the rat. This should not be interpreted to mean that the results obtained in the avian embryo have no relevance to man. For instance, many sex steroids appear to play a role in precipitating acute intermittent porphyria and porphyria cutanea tarda in man; however, while induction of ALA synthetase by these compounds can be shown in the chick embryo, it does not occur in the rat.

There is an empiric relationship observed by numerous investigators that many compounds which induce ALA synthetase also induce microsomal mixed-function oxygenase activity in the liver (also called the "drug metabolizing enzymes"). Two points are of note about this correlation: not all drugs induce both enzyme activities; also, the relationship many have a theoretical basis, in that heme is the prosthetic group of the terminal component of microsomal oxygenase, cytochrome P-450. The high concentration of cytochrome P-450 and rapid turnover relative to all other hepatic hemoproteins,

accounts for a large fraction of the total heme synthesized in the control liver. Some investigators have suggested that coordinate induction of ALA synthetase, the rate-limiting step in heme synthesis, and cytochrome P-450 and microsomal oxygenase activity may have a basis in providing the extra heme necessary for forming the new cytochrome P-450. Despite this not very satisfying teleologic explanation, there is a sizable literature reporting the concomitant induction of ALA synthetase and microsomal oxygenase activity.

We next studied the effect of TCDD on microsomal oxygenase. As a measure of this enzyme complex we choose to investigate aryl hydrocarbon hydroxylase activity, because aromatic hydroxylation is induced primarily by aromatic compounds, which in our view chemically resemble TCDD.

As seen in Figure 3, TCDD produces a dose-related induction of aryl hydrocarbon hydroxylase in chick embryo liver. At the lowest dose tested, 1.55×10^{-12} mole/egg (0.5 ng) there is a nearly twofold increase in enzyme activity, and maximal induction is produced

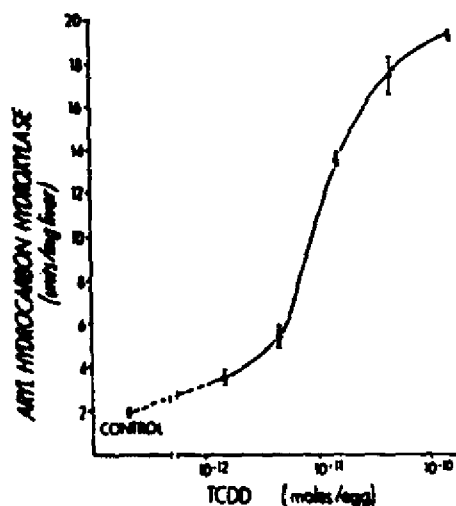


FIGURE 3. Logarithmic dose-response curve for the induction of aryl hydrocarbon hydroxylase. Eighteen-day embryos were injected with TCDD dissolved in *p*-dioxane or *p*-dioxane alone (control), and hepatic enzyme activity was assayed 24 hr later. Each point represents the mean $\pm$ standard error of four groups of pooled livers.

of TCDD at a dose of 3.11×10^{-10} mole/kg produces nearly a fivefold induction in rat liver enzyme activity (Fig. 5). Maximal induction of aryl hydrocarbon hydroxylase was produced by a dose of 3.11×10^{-8} mole/kg ($10 \mu\text{g/kg}$). It is estimated that the half maximal response is elicited by a dose of 8.5×10^{-10} mole/kg ($0.265 \mu\text{g/kg}$), roughly one hundredth the dose that kills 50% of rats (5). 3-Methylcholanthrene, a polycyclic hydrocarbon carcinogen is perhaps the most widely used compound as an inducer of aryl hydrocarbon hydroxylase. TCDD was found to be nearly 3×10^4 times as potent as 3-methylcholanthrene at inducing hydroxylase activity. Both drugs produce the same maximal degree of enzyme induction in rat liver, and the administration of both drugs together, each at a dose which produces maximal induction, elicits a response that is no greater than that produced by either drug alone.

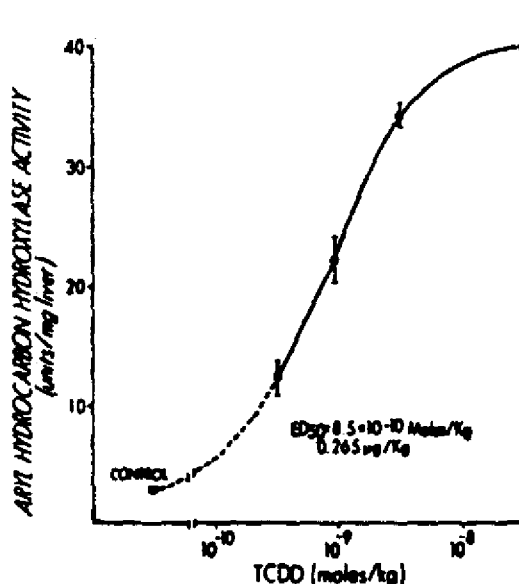


FIGURE 5. Logarithmic dose-response curve for the induction of aryl hydrocarbon hydroxylase in rat liver. Male Sprague-Dawley rats weighing about 80 g were injected intraperitoneally with TCDD dissolved in *p*-dioxane or *p*-dioxane alone (0.8 ml/kg), and hepatic aryl hydrocarbon hydroxylase activity was assayed 24 hr later. Each point is the mean $\pm$ standard error of five animals.

In Figure 6 we have plotted the results of the time course of induction produced by 3-methylcholanthrene (20 mg/kg in corn oil), TCDD ($10 \mu\text{g/kg}$ in *p*-dioxane) and control rats (receiving either corn oil or *p*-dioxane). Aryl hydrocarbon hydroxylase is induced to the same extent at 1 and 4 days by maximally inducing doses of each drug. However, by 8 days, hydroxylase activity in the 3-methylcholanthrene-treated rats returned to control levels, while induction persisted in the TCDD-treated rats for over 1 month. Accompanying the induction of aryl hydrocarbon hydroxylase by both drugs the following changes were noted: an increase in the total CO-binding microsomal cytochrome (cytochrome P-450 and P-448), a shift in the CO-maximum peak by difference spectroscopy from 450 nm to 448 nm , and a shift in the ratios of the peaks observed with ethyl isocyanide as a ligand by difference spectroscopy. These changes are interpreted to mean both 3-methylcholanthrene and TCDD

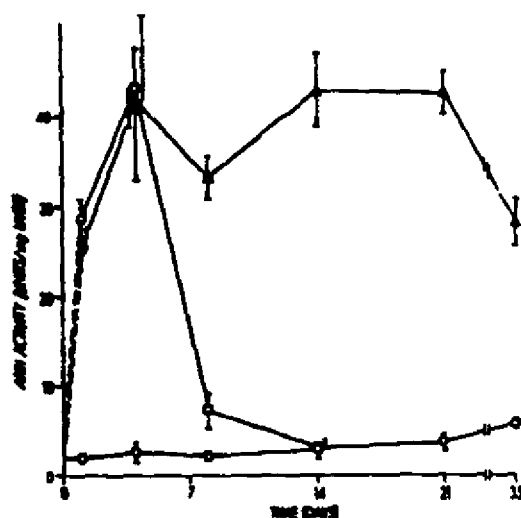


FIGURE 6. Time course of aryl hydrocarbon hydroxylase induction in rat liver following TCDD and 3-methylcholanthrene administration. Male Sprague-Dawley rats, weighing about 80 g were given a single injection of TCDD in *p*-dioxane ($10 \mu\text{g/kg}$), 3-methylcholanthrene in corn oil (20 mg/kg), or the solvents alone. Each point represents the mean $\pm$ standard error of four rats.

induce the formation of a new type of cytochrome P-450 which contains its heme iron in a high-spin state. We have also found that both TCDD and methylcholanthrene produce induction of aryl hydrocarbon hydroxylase in a number of extrahepatic tissues.

In summary, TCDD appears to be similar to 3-methylcholanthrene as an inducer of microsomal oxygenase differing primarily in potency (by four orders of magnitude) and duration of action. The persistent induction following TCDD administration is probably a result of its long biological half-life.

Discussion

The results of our investigation demonstrate TCDD is a potent inducer of ALA synthetase and aryl hydrocarbon hydroxylase in the chick embryo liver. There is a perfect correlation between those dioxins which induce both enzymes and the toxicity data, to the extent the data are available on the various dioxins. The structure-activity relationship reveals that all dioxins which are potent inducers have halogens at three of the four lateral ring positions and at least one nonhalogenated carbon atom. The sensitivity of induction of aryl hydrocarbon hydroxylase by TCDD and other toxic dioxins suggests this response might be a very valuable screening bioassay to detect the presence of the toxic dioxins in commercial products or environmental samples. It should be emphasized that the nonspecificity of the response makes it imperative that one extract the samples tested to remove polycyclic hydrocarbons, and the test is only collaborative, not definitive for TCDD and related dibenzo-*p*-dioxins.

Now, I should like to turn to the broader question of the mechanism of toxic action produced by TCDD. Any proposed mechanism of toxicity must account for several observations about TCDD (1). TCDD is a nearly planar, highly lipophilic, and a rather chemically unreactive molecule, which possesses remarkable biologic potency, and hence specificity (2). There are very large differences in susceptibility of different species to TCDD, as presented by Schwetz (5). The oral

LD₅₀ in the guinea pig is one thousandth that of the dog (3). There is a very sharp structure-activity relationship among the dioxins. The oral LD₅₀ values of the 2,7-dichloro and octachloro derivatives are greater than 10⁴ times that of TCDD in the rat (4). TCDD seems to produce hepatic cell necrosis, and liver insufficiency is the presumed cause of death in the rat. However, multiorgan involvement in the rat has been reported at this symposium and elsewhere. Furthermore, as reported by Vos and Moore and colleagues (7, 8), hepatic necrosis is minimal in the mouse and guinea pig and perhaps insufficient to account for death. Thus we must account for the different pattern of histologic damage in different species (5). TCDD is remarkably slow in its toxic action leading to death. Regardless of dosage, animals die weeks after a single administration of TCDD (6). TCDD is an extraordinarily potent teratogen in a number of species (7). Finally, our investigations suggest that all dioxins which are potent toxins, as acneogens, teratogens, producing mortality or chick edema, also are potent inducers of aryl hydrocarbon hydroxylase activity. This enzyme complex is present and inducible in a number of tissues and is responsible for the aromatic hydroxylation of many xenobiotics.

It is useful to examine the proposed mechanism of toxicity for other aromatic or halogenated aromatic compounds that, like TCDD, are chemically relatively unreactive and highly lipophilic. The two most extensively investigated models are the liver necrosis produced by halogenated benzenes and the carcinogenesis produced by polycyclic hydrocarbons (9). Briefly the literature can be summarized as follows. The parent compound is metabolized to a very reactive arene oxide intermediate. This intermediate may then chemically rearrange to a phenol, be further metabolized to a dihydrodiol or glutathione conjugate, or, react chemically to covalently bind to various cellular macromolecules which act as nucleophiles. In the case of bromobenzene centrolobular liver necrosis, the epoxide is believed to attach to proteins, and in the case of the polycyclic

hydrocarbon carcinogenesis the critical event is believed to be the binding of the "K-region" epoxide to DNA.

We propose a similar model for the toxicity of TCDD. The parent compound enters the cell and binds to some induction-receptor site which initiates the events which ultimately lead to the formation of more aryl hydrocarbon hydroxylase activity. TCDD is recognized by a second site in the cell, the enzyme-active center of microsomal oxygenase, and converted to a reactive metabolite, possibly an epoxide. The conversion of TCDD to its reactive metabolite is the rate-limiting step in dioxin metabolism, and this step is increased by the induction of aryl hydrocarbon hydroxylase. Some of these reactive metabolite molecules bind to cellular macromolecules producing some impairment of function which gradually produces cell death.

It is useful to examine this hypothesis in light of the known facts concerning TCDD toxicity. The large difference in species susceptibility to TCDD might be explained by the differences in the rate of metabolism of TCDD. The multiple organ damage produced by TCDD and variable pattern of histologic damage in different species might be explained by the relative rate of formation and further inactivation of the reactive metabolite in different organs. (3) As pointed out by Gehring (10), there is some evidence, far from unequivocal, that the administration of C¹⁴-TCDD results in unextractable radioactivity in rat liver. (4) The teratogenic effect of TCDD may be a result of mutagenesis by intercalation of the parent compound into DNA (11) or by intercalation and covalent binding of the metabolite, analogous to the acridine and aminofluorene compounds (12, 13).

This hypothesis is highly speculative and presented only to encourage further investigation. The major assumptions remain unsupported: (1) TCDD is metabolized, and (2) the metabolite covalently binds to some cellular constituent. The demonstration that both these events do or do not occur must await the synthesis of radioactive TCDD of high specific activity.

The chlorinated dibenzo-p-dioxins are worthy of much greater investigation, not only because the potential public health hazard they pose, but also the remarkable potency and sharply defined structure-activity relationship they demonstrate suggests an uncommon specificity of action. Ultimately, TCDD, like other potent toxins, (i.e., botulinus toxin, tetrodotoxin, organic phosphates) may become a useful biologic tool.

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