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Chlorinated Dibenzo-p-dioxins: Potent Inducers of δ-Aminolevulinic Acid Synthetase and Aryl Hydrocarbon Hydroxylase

II. A Study of the Structure-Activity Relationship

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SUMMARY

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, an extremely potent toxin and teratogen formed during the commercial synthesis of the herbicide 2,4,5-trichlorophenoxyacetic acid, is a potent inducer of δ-aminolevulinic acid synthetase and aryl hydrocarbon hydroxylase in chick embryo liver. The induction of hydroxylase activity is more sensitive to low doses of the toxin than is the induction of δ-aminolevulinic acid synthetase; 2-fold induction is produced by 1.55 pmoles/egg (0.5 ng), and maximal induction by 155 pmoles/egg. Coordinate expression of the two enzymes is also dissociable by the administration of cycloheximide, which selectively inhibits induction of the synthetase. Fifteen halogenated dibenzo-*p*-dioxins were screened for their ability to induce the two enzymes, and a well-defined structure-activity relationship emerged; all the congeners that induced both enzymes had halogen atoms at a minimum of three of the four lateral ring positions and contained at least one nonhalogenated ring position. There is a perfect correspondence between the whole animal toxicity data on the dibenzo-*p*-dioxin congeners and their ability to induce both enzymes.

INTRODUCTION

In the past 15 years a number of lines of investigation have uncovered the extraordinary toxic potency of certain chlorinated dibenzo-*p*-dioxins and dibenzofurans, which are formed as contaminants in the commercial synthesis of a number of chlorinated phenols (1–4). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, a contaminant formed during the synthesis of the herbicide 2,4,5-trichlorophenoxyacetic acid, is the best studied of these compounds. TCDD[1] is perhaps the most toxic small molecule known; the oral

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[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; ALA, δ-aminolevulinic acid.

LD_{50} in guinea pigs is 1 μg/kg (3 nmoles/kg) (4). Several distinctive features characterize the toxicity of TCDD: (a) death is delayed at least 3 weeks after a single oral dose in all laboratory animals; (b) there is more than a 1000-fold difference in the susceptibility of different species; and (c) histological examination reveals multiple organ damage according to a species-dependent pattern. In the rat gradual liver necrosis (5, 6) is the apparent cause of death. In addition to its lethality, TCDD produces teratogenesis and embryotoxicity, a syndrome of edema in the newborn chicken, and acne when dermally applied to rabbits' ears or human skin (4).

In 1964 Bleiberg *et al.* (7) reported industrially acquired acne (chloracne) and porphyria cutanea tarda in several workers in a 2,4,5-T factory. The acne has been shown to be a direct result of TCDD exposure (8–10). Porphyria cutanea tarda is an acquired defect in hepatic porphyrin synthesis characterized by increased porphyrin accumulation in the liver, increased urinary porphyrin excretion, and photosensitivity and mechanical fragility of the skin (11). The cause of porphyria in these factory workers was uncertain. Upon reinvestigation of the factory 5 years later, we discovered that the syndrome had cleared in all the workers after reduction of TCDD contamination (12).

Hepatic prophyria can be produced in animals by a number of compounds, all of which have in common the capacity to induce[2] the initial enzyme in the heme pathway, δ-aminolevulinic acid synthetase (13, 14). This mitochondrial enzyme is the rate-limiting step in the formation of heme. We have recently shown that TCDD is a potent inducer of ALA synthetase in chick embryo liver and thus was the likely cause of the porphyria observed in the workers in the 2,4,5-T factory (15).

Many drugs that induce ALA synthetase also induce hepatic microsomal mixed-function oxygenase (16, 17). Microsomal oxygenase is a membrane-bound enzyme complex responsible for the oxidative metabolism of a variety of xenobiotics and several endogenous compounds (18). The active enzyme site of this enzyme complex is a hemoprotein, cytochrome P-450.[3] There is considerable evidence that this cytochrome consists of a mixture of at least two hemoproteins and that the type of cytochrome present determines substrate specificity (19, 20).

We now report studies on the effects of the halogenated dibenzo-*p*-dioxins on ALA synthetase and microsomal oxygenase in chick embryo liver. As a measure of microsomal oxygenase activity we measured aryl hydrocarbon hydroxylase,[4] because preliminary studies revealed that TCDD, like the polycylic hydrocarbons, selectively induced this enzyme activity. The chick embryo liver was employed because (a) the avian embryo is the animal model most sensitive to the toxic effects of TCDD (24), (b) induction of ALA synthetase in chick embryo liver is well characterized and very sensitive to xenobiotics (25), (c) the egg is a closed system, reducing the risk of laboratory contamination by excretion, and (d) the avian embryo is inexpensive permitting large screening experiments.

The structure-activity relationship of a series of 15 halogenated dibenzo-*p*-dioxins was examined for their ability to induce ALA synthetase and aryl hydrocarbon hydroxylase. The ability of the various

[2] The term enzyme induction is used to denote a relative increase in the rates of synthesis *de novo* or activation of enzyme activity from preexisting moieties, or both, compared to the rate of enzyme degradation. Since ALA synthetase and aryl hydrocarbon hydroxylase are membrane-bound, and the latter enzyme is a multicomponent system, there are technical difficulties in attempting to distinguish between enzyme synthesis *de novo* and activation. Thus induction here signifies only the rate of accumulation of enzyme activity.

[3] In this paper cytochrome P-450 refers to all species of the carbon monoxide-binding microsomal hemoprotein, both P-450 and P_1-450 (P-448).

[4] Aryl hydrocarbon hydroxylase has also been called benzo[*a*]pyrene hydroxylase, but since it metabolizes a number of polycyclic hydrocarbons to phenolic derivatives, the more general name is preferred. The true reaction product is an epoxide (21, 22), which is further metabolized or rearranges to the phenolic derivative, the product measured (23). Perhaps the most appropriate nomenclature would be aryl hydrocarbon epoxidase.

dioxins[5] to evoke enzyme induction is correlated with toxicity data.

MATERIALS AND METHODS

Dr. Albert Pohland, Chief of the Biotoxin Section of the Division of Chemistry and Physics, Food and Drug Administration, Washington, D. C., generously provided the compounds listed below. Much of the information on their synthesis and analysis can be found in ref. 26. The congeners possess very large differences in activity, posing the problem of whether a small response produced by a high dose of a compound is due to weak intrinsic activity or to trace contamination (less than 1%) by a very active congener. Many of the analogues are similar in physical properties and difficult to separate. Finally, some of the dibenzo-*p*-dioxins have only been synthesized once, and there are no physical constants in the literature with which to compare them. For these reasons each dibenzo-*p*-dioxin congener is identified below by lot number, degree of purity, and method of analysis: 1-chloro (F-1041), 2,3-dichloro (F-1043), 2,7-dichloro (F-908), 1,2,4-trichloro (F-989), 1,2,3,4-tetrachloro (F-990), 2,3,7,8-tetrachloro (F-883), 1,2,3,4,6,7,8,9-octachloro (F-912), and 2,8-dichloro (F-988). The structure of each compound was substantiated by method of synthesis, by appropriate infrared, ultraviolet, electron spin resonance, and mass spectra, and by demonstration of purity greater than 99% by gas-liquid chromatography with electron capture detection. The 2,8-dichloro analogue, while more than 99% pure on gas-liquid chromatography, had an X-ray diffraction pattern revealing two different crystal structures.

In addition, Dr. Pohland provided other samples, of lesser purity: 1,2,3,5,7-pentachloro (F-1053) and 1,2,3,4,7,8-hexachloro (F-1055) were more than 95% pure by gas-liquid chromatography; 1,2,4,6,7,9-hexachloro (F-1061) was only 90% pure by gas-liquid chromatography; the 2,3,7-trichloro compound (F-909A) was only available as a mixture of 60% (w/w) 2,3,7-trichloro and 40% 2,7-dichloro; and the 2,3,7-tribromodioxin (F-991) was 94% pure, containing 5% 2,7-dibromo and 1% 2,3,7,8-tetrabromo congeners by gas-liquid chromatography.

Mr. George Lynn and Dr. W. W. Muelder, of Dow Chemical Company, Midland, Mich., generously provided dibenzo-*p*-dioxin (8A13-9:144), which was 98% pure by gas-liquid chromatography.

NADPH, Tris, cycloheximide, benzo[*a*]pyrene, bovine serum albumin, and quinine sulfate were purchased from Sigma Chemical Company. The benzo[*a*]pyrene was recrystallized from warm benzene by the addition of methanol. Actinomycin D was purchased from Calbiochem. Dr. Harry Gelboin, of the National Cancer Institute, kindly provided 3-hydroxybenzo[*a*]pyrene. All other reagents were of reagent grade.

Precautions in handling halogenated dibenzo-p-dioxins. A small quantity of each test compound (less than 1 mg) was weighed on an electric balance, dissolved in *p*-dioxane in a screw-cap tube wrapped in aluminum foil to prevent photodecomposition (27), and then stored under a well-ventilated hood. Great care was taken in handling these compounds, especially in weighing the solid; precautions included the use of disposable gloves and disposable coverings for the work surfaces, and the cleaning of all glassware in contact with a high concentration of the compounds, first with chloroform and then with a concentrated sulfuric acid–potassium dichromate solution. Whenever possible, disposable plastic ware was used.

Treatment of animals. Fertilized white Leghorn chicken eggs were purchased from Babcock Farms, Ithaca, N. Y., or SPAFAS, Inc., Norwich, Conn. The eggs were incubated at 37° with 70% or greater humidity and used between the 15th and 20th days of gestation (total gestation period, 21 days).

Test compounds were administered into the air sac through a small hole punched in the shell. The halogenated dibenzo-*p*-dioxins were injected in a volume of 25 μl of *p*-dioxane (unless otherwise noted), using a Hamilton microliter syringe. *p*-Dioxane (25 μl) was injected into control eggs, which did not display enzyme activities significantly different from untreated eggs. The hole in the shell was covered with cellophane tape to prevent dehydration, and the

[5] Dioxin is used to denote any of the halogenated dibenzo-*p*-dioxins.

eggs were returned to the incubator until the embryos were killed.

Preliminary experiments revealed that cycloheximide and actinomycin D, when administered into the air sac or yolk sac, were only partially effective in preventing enzyme induction. Therefore these compounds were administered intravenously by the method of Finkelstein (28). The eggs were "candled" to visualize the vascular supply to the shell membrane. Then, with a dentist's drill equipped with a $\frac{5}{8}$-inch abrasive disc, a 3 × 3 mm piece of shell was removed over a prominent vein, with care taken not to injure the underlying membrane. The eggs were returned to the incubator for 1 hr to harden the shell membrane. Using a 27-gauge needle and tuberculin syringe, 0.1 ml of 0.9% Nacl containing actinomycin D or cycloheximide was injected into the vein. TCDD was administered by the usual route into the air sac, and both sites of injection were covered by cellophane tape to prevent dehydration. (We are grateful to Dr. Urs Meyer, Department of Medicine, University of California Medical School, San Francisco, for recommending this technique.)

Enzyme assays. The livers from three to five similarly treated animals were pooled, weighed, and homogenized in isotonic KCl and then centrifuged at 10,000 × *g* for 20 min. ALA synthetase was assayed by resuspension of the 10,000 × *g* pellet, as previously described (15). Each point was the average of triplicate determinations.

Aryl hydrocrbon hydroxylase was assayed by the method of Gielen, Goujon, and Nebert (23), modified slightly. The reaction was run in a total volume of 1.05 ml containing 50 μmoles of Tris-HCl (pH 7.2), 1 mg of bovine serum albumin, 0.5 μmole of NADPH, 3.0 μmoles of $MgCl_2$, 0.1 ml of 10,000 × *g* liver supernatant equivalent to 5 mg of liver, and 100 nmoles of benzo[*a*]pyrene in 50 μl of methanol, added to start the assay. After incubation for 10 min at 37° in a shaker water bath the reaction was terminated by the addition of 1.0 ml of cold acetone and 3.25 ml of hexane. The mixture was reincubated at 37° for 10 min to extract the benzo[*a*]pyrene and metabolites. A 1-ml sample of the 3.3-ml organic phase was extracted with 3.0 ml of 1 N NaOH, and the alkali-extractable metabolites were examined in an Aminco-Bowman spectrophotofluorometer (model 4-8202 SPF); fluorescence corresponding to 3-hydroxybenzo[*a*]pyrene has an activation peak at 395 nm and an emission maximum at 520 nm. The fluorescence of a sample blank, to which the benzo[*a*]pyrene was added after the acetone and hexane, was subtracted from the fluorescence of each experimental sample. The fluorometer was calibrated with a standard solution of quinine sulfate or 3-hydroxybenzo[*a*]pyrene. One unit of aryl hydrocarbon hydroxylase activity was defined as that amount of enzyme catalyzing the formation per minute at 37° of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[*a*]pyrene. Activity was expressed as units per milligram of liver, wet weight. Each assay was performed twice; duplicate results normally varied less than 10%. The assay was linear for enzyme concentration and time under the conditions employed. The assay was always performed at pH 7.2 (37°) in Tris buffer at the optimal pH for hydroxylase activity in embryos treated with TCDD or 3-methylcholanthrene. In the control chick liver, hydroxylase activity had an optimal activity at pH 6.6 and enzyme activity was about 25% lower at pH 7.2.

RESULTS

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin is a symmetrical, nearly planar molecule; all 4 chlorine atoms are indistinguishable from one another (Fig. 1). TCDD was found to be a potent inducer of aryl hydrocarbon hydroxylase activity in chick embryo liver (Fig. 2). A plot of the enzyme activity vs. log administered dose gave a sigmoidal curve, with half-maximal induction at 10 pmoles/egg (3.2 ng). Even at the lowest dose tested, 1.55 pmoles/egg (0.5 ng), TCDD nearly doubled enzyme activity. Maximal induction of aryl hydrocarbon hydroxylase, approximately a 10-fold increase above control levels, was produced by 155 pmoles/egg. TCDD is over 10^4 times more potent than 3-methylcholanthrene on a molar basis in inducing aryl hydrocarbon hydroxylase.[6]

[6] A. Poland and E. Glover, manuscript in preparation.

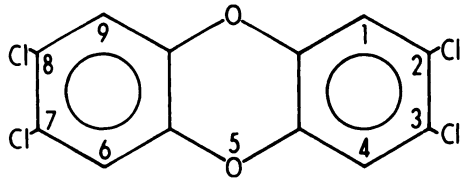

FIG. 1. *Structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin*

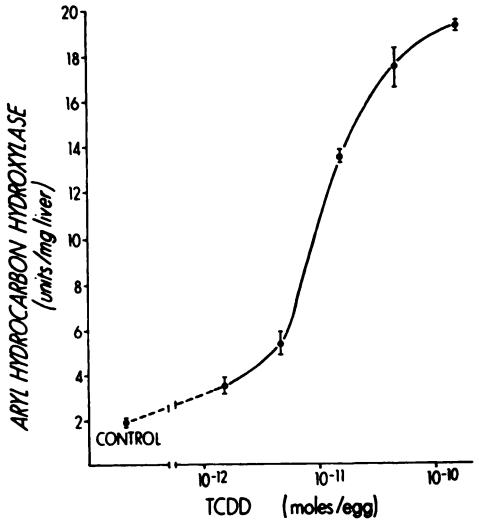

FIG. 2. *Log dose-response curve for induction of aryl hydrocarbon hydroxylase*

TCDD dissolved in *p*-dioxane, or *p*-dioxane alone (control), was injected into 18-day embryos, and hepatic enzyme activity was assayed 24 hr later. Each point represents the mean ± standard error of four groups of pooled livers (three livers per group).

We previously reported that TCDD was a potent inducer of ALA synthetase in chick embryo liver (15). It should be noted that induction of hydroxylase activity is more sensitive to TCDD than ALA synthetase, and that a dose of 50 ng/egg of TCDD produces maximal hydroxylase induction whereas at least 10 times this dose is necessary for maximal induction of ALA synthetase (15).

The time course of hydroxylase induction by TCDD is shown in Fig. 3. After a short lag period there is a continuous increase in enzyme activity, which reaches a maximum at 18 hr. Enzyme induction by TCDD is prolonged for at least 5 days (Fig. 3B), in contrast to the less sustained induction we have observed with 3-methylcholanthrene This persistent induction of aryl hydrocarbon hydroxylase is similar to the prolonged induction of ALA synthetase (15) and most likely results from the long biological half-life of TCDD (29).

Structure-activity relationship. We compared the abilities of a series of 15 congeners of dibenzo-*p*-dioxin to induce aryl hydrocarbon hydroxylase. The dose of TCDD employed was 47 pmoles/egg, which evoked nearly maximal induction (Fig. 2). The other dioxins were tested at 1, 10, or 100 times this dose, and the results are expressed as the mean ± standard error of four groups of pooled livers (Fig. 4). All compounds capable of inducing hydroxylase activity did so at 47 or 470 pmoles/egg, and all the dioxins that failed to stimulate enzyme activity produced no change at 4.7 nmoles/egg. The one exception was 1,2,4,6,7,9-hexachlorodibenzo-*p*-dioxin, which at the highest dose tested produced a modest increase in hydroxylase activity. This compound was only 90% pure by gas-liquid chromatography, and it was impossible to distinguish between weak potency intrinsic in the compound and contamination with a potent inducing congener. Contamination with 0.1% (w/w) TCDD would account for the observed response, and the question will remain unsettled until a purer sample of this hexachlorodioxin becomes available.

Another way to examine the sharply defined dioxin structure required to induce aryl hydrocarbon hydroxylase is to see whether high doses of the inactive congeners will block the induction produced by TCDD. That is, do these inactive compounds bind to the induction receptor site and act as antagonists? To test this, we first showed that high doses of the 2,7-dichloro, 1,3,6,8-tetrachloro, and octachloro congeners produced no significant induction of hydroxylase activity (Table 1). The 1,3,6,8-tetrachloro congener, at 1000 times the dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, roughly doubled enzyme activity. It is again impossible to distinguish between weak potency of the parent compound and trace contamination with a potent analogue. Next these three inactive compounds were administered to chick embryos, followed 2 hr later by TCDD.

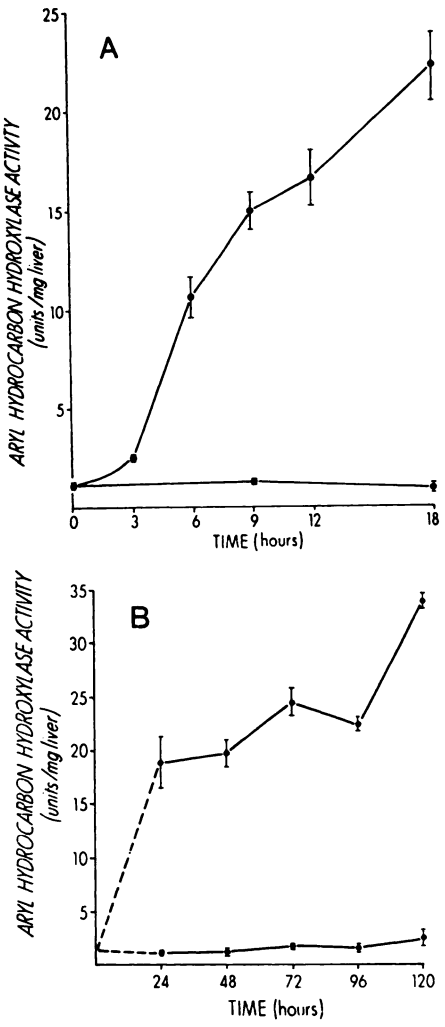

FIG. 3. *Time course of induction of aryl hydrocarbon hydroxylase*

A. Twenty-day-old embryos were given 466 pmoles (150 ng) of TCDD per egg, dissolved in *p*-dioxane, or received just the solvent, and the hepatic enzyme activity was assayed at the indicated time intervals.

B. Fifteen-day embryos were treated with the solvent or 150 ng of TCDD, and hepatic enzyme activity was assayed 1–5 days later. The embryos were staggered in their incubation ages, so that all embryos were killed and their enzyme activity assayed on the same day. The points are the means ± standard errors of four groups of pooled livers (three or four livers per group).

The induction by TCDD alone did not significantly differ from that produced in the animals previously treated with the inactive dibenzo-*p*-dioxins and then TCDD.

The dibenzofuran ring is very similar to that of dibenzo-*p*-dioxin. Some chlorinated dibenzofurans, mostly tetrachloro and pentachloro compounds, which arise as contaminants during the synthesis of 2,4,5-T (9), and polychlorobiophenyls (30, 31) have been shown to produce hepatotoxicity and acne. We have found that the dibenzofuran ring and the 2,8-dichloro-, 3,7-dichloro-, and octachlorodibenzofurans have little or no capacity to induce aryl hydrocarbon hydroxylase, but that 2,3,7,8-tetrachlorodibenzofuran is a very good inducer, roughly as potent as TCDD.[7]

Induction of ALA synthetase. Each of the 15 dioxin congeners was tested at at least three dose levels for its ability to induce hepatic ALA synthetase in the chick embryo. As shown in Fig. 5, the structure-activity relationship for induction of ALA synthetase was identical with that observed for the induction of aryl hydrocarbon hydroxylase. The inactive dioxins failed to elicit any enzyme induction at 200–400 times the dose of TCDD (30 pmoles/egg) that evoked a substantial increase in ALA synthetase.

In the structure-activity studies shown in Figs. 4 and 5, it can be seen that TCDD is the most potent of the dioxins. Other experiments showed that all the dioxins which are inducers produce the same maximal response; i.e., they differ only in potency, not in intrinsic activity.

Inhibition of induction of ALA synthetase and aryl hydrocarbon hydroxylase by actinomycin D and cycloheximide. The stimulation of both ALA synthetase and microsomal oxygenase by a variety of foreign compounds has been shown to be preventable by the administration of inhibitors of RNA and protein synthesis. These studies have contributed to the idea that the increase in the activities of both enzymes represents induction, although it is not yet possible to distinguish between protein synthesis of these enzymes *de novo* and activation of preexisting components.

Table 2 shows the results of an experiment in which cycloheximide and actinomycin D

[7] A. Poland, J. Wade, and A. Kende, unpublished observations.

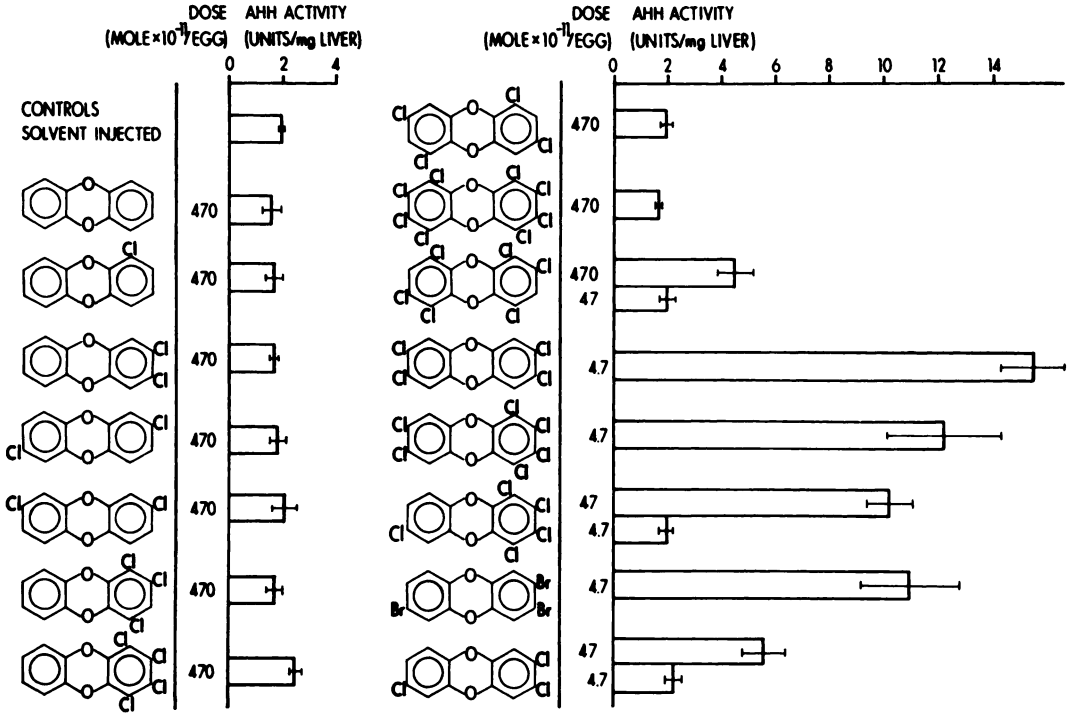

FIG. 4. *Structure-activity relationship of halogenated dibenzo-p-dioxins: induction of aryl hydrocarbon hydroxylase (AHH) activity in the chick embryo liver*

Eighteen-day embryos received 25 μl of *p*-dioxane or *p*-dioxane containing the test compound, and enzyme activity was assayed 24 hrs later. The purity of each compound is discussed under MATERIALS AND METHODS. Each bar represents the mean ± standard error of four groups of pooled livers, except for the solvent-treated controls and TCDD, where $n = 12$. Three to five livers were pooled per group.

TABLE 1

Aryl hydrocarbon hydroxylase induction by TCDD and competition by other halogenated dibenzo-p-dioxins in chick embryo

A total of 40 μl of *p*-dioxane containing various dioxins was injected into 18-day chick embryos. In groups 8–10 the inactive dioxin was given 2 hr before TCDD. Twenty-four hours later the embryos were killed, and the livers were pooled and assayed for enzyme activity. Results are means ± standard errors of four or five groups of pooled livers, with three to five livers per group.

Treatment	Dose	Comparative molar ratio (to TCDD dose)	Aryl hydrocarbon hydroxylase activity
	pmoles/egg		*units/mg liver*
1. Control (*p*-dioxane)			2.14 ± 0.29
2. 2,7-Dichloro-DD[a]	46,600	1000	2.62 ± 0.08
3. 1,3,6,8-Tetrachloro-DD	46,660	1000	5.19 ± 0.90
4. Octachloro-DD	18,600	400	2.84 ± 0.33
5. TCDD	46.6	1	24.84 ± 2.16
6. Control (*p*-dioxane)			1.58 ± 0.14
7. TCDD	46.6	1	14.85 ± 1.38
8. 2.7-Dichloro-DD + TCDD	46,600 + 46.6	1000:1	14.75 ± 1.02
9. 1,3,6,8-Tetrachloro-DD + TCDD	46,600 + 46.6	1000:1	13.34 ± 0.82
10. Octachloro-DD + TCDD	18,600 + 46.6	400:1	14.36 ± 0.85

[a] Dibenzo-*p*-dioxin.

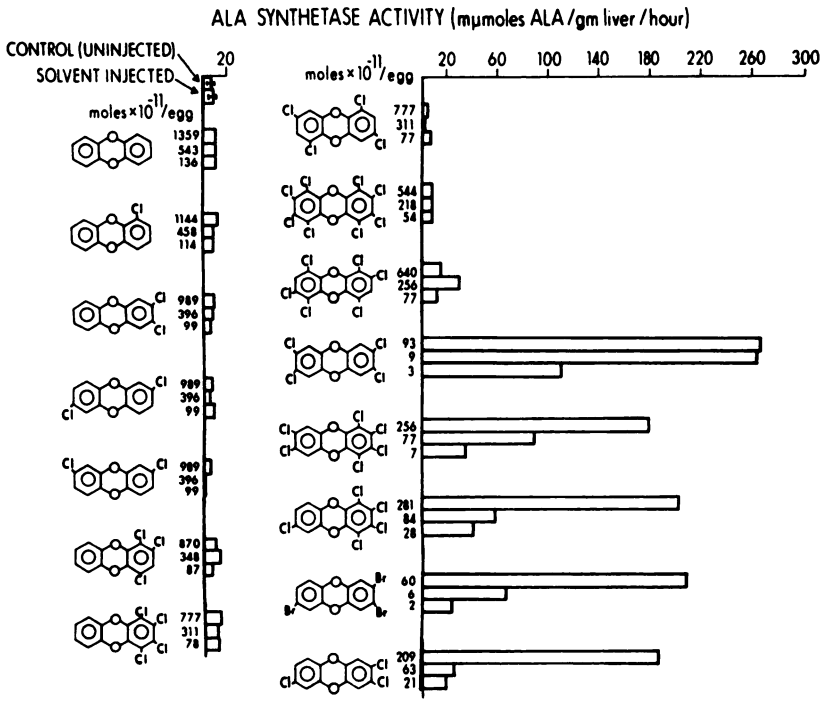

FIG. 5. *Structure-activity relationship of halogenated dibenzo-p-dioxins: induction of ALA synthetase*
Seventeen-day embryos received the solvent alone or containing the dibenzo-*p*-dioxin tested, and the enzyme activity was assayed 48 hr later. Values for untreated controls (n = 9) did not differ appreciably from controls receiving solvent (n = 12). Each bar for the test groups represents a single group of three to five pooled livers.

were administered intravenously to eggs just prior to the dose of TCDD. Eleven hours later the embryos were removed, and both ALA synthetase and aryl hydrocarbon hydroxylase activities were assayed in each liver. TCDD-treated embryos showed a 13-fold rise in ALA synthetase and nearly a 6-fold increase in hydroxylase activity. The induction of both enzymes was completely inhibited by prior administration of actinomycin D, supporting other investigations which suggested that RNA synthesis is necessary for the induction of both enzymes. More interesting is the finding that the induction of ALA synthetase was sensitive to inhibition by cycloheximide, and induction of hydroxylase activity relatively insensitive. At doses of 10 and 25 μg of cycloheximide, the induction of ALA synthetase was completely suppressed; however, induction of hydroxylase activity in the same livers was inhibited only 18% and 28%, respectively. It is improbable that this result can be explained by the difference in half-lives between ALA synthetase and aryl hydrocarbon hydroxylase or their respective mRNAs, because cycloheximide was administered before TCDD. The induction of ALA synthetase, and resultant increase in heme synthesis, is not essential for the induction of aryl hydrocarbon hydroxylase.

DISCUSSION

TCDD is a potent inducer of both ALA synthetase and aryl hydrocarbon hydroxylase in chick embryo liver. TCDD differs from other compounds that have been reported to induce either or both enzymes in three aspects. (a) It is at least 1000 times more potent than any other compound yet

TABLE 2

Effect of actinomycin D and cycloheximide on induction of ALA synthetase and aryl hydrocarbon hydroxylase by TCDD

Fertilized eggs of 18 days' gestation received intravenously 0.1 ml of 0.9% NaCl (control, TCDD) or 0.1 ml of 0.9% NaCl containing actinomycin D or cycloheximide. Fifteen minutes later 25 μl of *p*-dioxane (controls) or 466 pmoles of TCDD in 25 μl of *p*-dioxane was injected into the air sac. Eleven hours later the embryos were killed, and ALA synthetase and aryl hydrocarbon hydroxylase were assayed simultaneously in single livers. Results are means ± standard errors of the number of embryos shown.

Treatment	*n*	ALA synthetase	Aryl hydrocarbon hydroxylase
		nmoles ALA/g/hr	*units/mg*
Control	5	19.0 ± 6.7	1.31 ± 0.15
TCDD	5	261.9 ± 43.9	7.36 ± 0.77
TCDD + actinomycin D, 30 μg	4	6.3 ± 5.1	1.17 ± 0.14
TCDD + cycloheximide, 5 μg	5	29.8 ± 5.8	7.89 ± 0.70
TCDD + cycloheximide, 10 μg	5	9.9 ± 2.3	6.27 ± 1.11
TCDD + cycloheximide, 25 μg	5	6.2 ± 3.5	5.68 ± 0.37

tested. (b) It has a prolonged duration of action: both enzymes are still induced in chick embryo liver 5 days after a single dose, presumably a reflection of the persistence of TCDD, which has been reported to have a long biological half-life (29). We have found a single dose of TCDD to induce hepatic aryl hydrocarbon hydroxylase in the rat after more than 35 days.[6] (c) The series of halogenated dibenzo-*p*-dioxins tested have a sharply defined structure-activity relationship.

Structure-activity relationship. The induction of ALA synthetase and microsomal oxygenase by a wide variety of compounds has prompted numerous structure-activity studies (13, 32–37). Although within any particular series of compounds certain properties are common to inducing congeners, comparison of all the compounds known to induce reveals no common attributes except that most are lipophilic at physiological pH (14, 18).

In the series of halogenated dibenzo-*p*-dioxins tested, five compounds were found to be potent inducers of both ALA synthetase and aryl hydrocarbon hydroxylase. The dioxins that were inducers had two common properties: chlorine or bromine atoms occupy at least three of the four lateral ring positions (Nos. 2, 3, 7, and 8), and there is at least 1 nonhalogenated carbon atom. The fully chlorinated octachlorodibenzo-*p*-dioxin did not induce either enzyme. One compound, 1,2,4,6,7,9-hexachlorodibenzo-*p*-dioxin, behaved exceptionally in that at high doses it produced a modest induction of aryl hydrocarbon hydroxylase. However, because this congener was only 90% pure it is impossible to distinguish between weak potency of this dioxin and contamination with a potent inducing congener.

We have recently synthesized and tested a number of dioxin isomers which support this interpretation of the structure-activity relationship.[7] From this larger series it was found that substitutions at the lateral ring positions are active in the order Br > Cl > F > NO_2.

To elicit the induction of aryl hydrocarbon hydroxylase, we assume that the dioxins combine with some site, the "induction receptor," and this interaction initiates the event or events leading to the formation of more aryl hydrocarbon hydroxylase activity. No assumptions are made about this "induction receptor" with respect to its subcellular location, the nature of its binding to TCDD, or whether this drug-receptor interaction is the same as that which initiates the induction of ALA synthetase. The dioxin congeners that did not induce aryl hydrocarbon hydroxylase or ALA synthetase were devoid of agonist activity at 100–1000 times the dose of TCDD used, and high does of the inactive dioxins did not block the induction of hydroxylase activity produced by TCDD. Thus the inactive dioxins act neither as weak agonists nor as antagonists to TCDD, suggesting that they have little affinity for the induction receptor site. Potency, structural specificity, and apparent relative

resistance to metabolic degradation (38) make the dioxins ideal tools to explore the site(s) at which foreign compounds must interact to initiate the induction of aryl hydrocarbon hydroxylase and ALA synthetase. Among the unanswered questions about the structure-activity relationship among the dioxins are the importance of the oxygen atoms: would the 2,3,7,8-tetrachloro derivatives of other tricyclic planar ring systems be active? And does the interaction of the TCDD molecule with the hypothetical induction receptor depend on the steric configuration of the molecule or primarily on the electron distribution?

Some chlorinated dibenzofurans have been reported to be toxic contaminants of 2,4,5-T (9) and the polychlorinated biphenyls (30, 31). It is noteworthy that dibenzofuran and dibenzo-*p*-dioxin are closely related tricyclic planar molecules, and that chlorinated congeners of both rings have been reported to cause acne and hepatotoxicity and follow a similar structure-activity pattern in their ability to induce aryl hydrocarbon hydroxylase and ALA synthetase. These data suggest that the mechanism of toxic action may be identical for the chlorinated dibenzo-*p*-dioxins and dibenzofurans.

Of practical import is the relationship between the toxicity of the various halogenated congeners of dibenzo-*p*-dioxins and their ability to induce aryl hydrocarbon hydroxylase and ALA synthetase. TCDD and other toxic dioxins produce several toxic responses in addition to lethality: embryotoxicity and teratogenesis, edema in the newborn chick, and acne when applied to rabbits' ears. The available data (4) suggest that any dioxin that elicits one of these responses evokes them all. The toxicity data on the various dioxin congeners correspond perfectly with the ability of these compounds to induce ALA synthetase and aryl hydrocarbon hydroxylase. For instance, the oral LD_{50} of TCDD in the rat is 22 μg/kg, and the 2,7-dichloro- and octachlorodioxins —both inactive as inducers—have LD_{50} values of over 1 g/kg. Similarly, the unhalogenated ring, 2,7-dichloro-, 2,3-dichloro-, 2,8-dichloro-, 1,2,4-trichloro-, 1,2,3,4-tetrachloro-, and octachlorodioxins—all of which failed to cause acne—also produced no enzyme induction. However, TCDD, 2,3,7-tribromo-, 2,3,7-trichloro-, and mixtures of various hexachlorodioxins were potent inducers as well as acneogens.

The empirical correspondence between the capacity of various dioxins to produce toxicity and evoke enzyme induction suggests that enzyme induction may be involved in the mechanism of toxic action. Furthermore, this correspondence suggests that enzyme induction may be used as a simple confirmatory bioassay to detect the presence of the toxic dioxins and contaminants in the environment.

Relationship between induction of ALA synthetase and aryl hydrocarbon hydroxylase. Of the many structurally diverse compounds that induce hepatic microsomal oxygenase, most of the drugs concurrently induce ALA synthetase. Since the turnover of cytochrome P-450 accounts for a major fraction of the total hepatic heme synthesis in the basal state (16), it has been asserted that induction of microsomal oxygenase activity must be accompanied by induction of ALA synthetase to provide the extra heme necessary for the induced apocytochrome P-450 (17).

In the present investigation, the identical structure-activity relationships obtained with the dioxin congeners for the induction of ALA synthetase and aryl hydrocarbon hydroxylase support the idea of some type of coordinate gene linkage (13, 39–41). However, several observations contradict this simple interpretation. First are the different dose-response curves for induction of these two enzymes by TCDD; the induction of hydroxylase activity is more sensitive to low doses of TCDD and maximal induction is produced by a dose of 155 pmoles/egg, whereas even at a 10-fold greater dose ALA synthetase induction is not maximal. In the chick embryo, moreover, the administration of 3-methylcholanthrene produces marked induction of hydroxylase activity and has almost no effect on ALA synthetase.[8] Finally, the induction of ALA synthetase is completely prevented by a dose of cycloheximide that only slightly inhibits hy-

[8] A. Poland and E. Glover, unpublished observations.

droxylase expression. In the chick embryo microsomal oxygenase can be induced in the complete absence of induction of ALA synthetase (and hence the presumed absence of augmented heme synthesis).

The above data demonstrate that the concurrent expression of ALA synthetase and aryl hydrocarbon hydroxylase is dissociable under certain conditions. The often coordinated induction of these enzymes may result from some loose gene linkage (41) or tightly coupled gene transcription and subsequent post-transcriptional control mechanisms, which have been described for the expression of both enzymes (42, 43).

We believe that further investigation of TCDD is warranted on two accounts. First, the presence of the chlorinated dibenzo-*p*-dioxins and dibenzofurans as contaminants in our environment poses an incalculable potential health hazard which can only be assessed when we have some more basic information on the degree of pollution and the nature of the biological toxicity. Second, the unparalleled potency of the dioxins as inducers of ALA synthetase and aryl hydrocarbon hydroxylase makes them extremely valuable in the study of enzyme induction. Elucidating the mechanism of action of TCDD may increase our knowledge in such diverse areas as regulation of gene expression, mechanisms of hepatotoxicity, and teratogenesis.

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