MOLECULAR PHARMACOLOGY, 18, 571–580

1,4-Bis[2-(3,5-Dichloropyridyloxy)]Benzene, a Potent Phenobarbital-Like Inducer of Microsomal Monooxygenase Activity

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Received March 28, 1980; Accepted June 16, 1980

SUMMARY

POLAND, A., I. MAK, E. GLOVER, R. J. BOATMAN, F. H. EBETINO AND A. S. KENDE. 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene, a potent phenobarbital-like inducer of microsomal monooxygenase activity. *Mol. Pharmacol.* **18:** 571–580 (1980).

Sodium phenobarbital, diphenylhydantoin, dichlorophenobarbital, chlordane, mirex, and dieldrin, compounds which produce a similar pleiotropic response in the liver, were compared for their potency to induce hepatic aminopyrine *N*-demethylase activity in B6D2F$_1$/J mice. The ED$_{50}$, the dose which produces one-half the maximum enzyme induction when administered daily for 3 days, for phenobarbital is 1.07×10^{-4} mol/kg/day, and for the most potent of these compounds, dieldrin, the ED$_{50}$ is 8.0×10^{-6} mol/kg/day. A new phenobarbital-like compound, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, (TCPOBOP), is 650 times as potent as phenobarbital, with an ED$_{50}$ of 1.63×10^{-7} mol/kg/day. Maximally effective doses of phenobarbital and TCPOBOP, administered alone or in combination, induce hepatic aminopyrine *N*-demethylase activity, cytochrome *P*-450, and NADPH–cytochrome *c* reductase to the same extent. The cytochrome(s) *P*-450 induced by these three treatments is similar by CO-difference spectra, ethyl isocyanide difference spectra, and SDS–polyacrylamide gel electrophoresis of liver microsomes. Both phenobarbital and TCPOBOP produce an increase in liver weight, proliferation of the smooth endoplasmic reticulum, and induction of microsomal epoxide hydrolase and cytosolic glutathione *S*-transferase activities. Both compounds induce microsomal monooxygenase activity in the proximal intestines, but not in kidney or skin. A single maximally effective dose of TCPOBOP (3 mg/kg) induces hepatic aminopyrine *N*-demethylase and microsomal epoxide hydrolase activities in B6D2F$_1$/J mice for over 20 weeks. Following the administration of a single dose of ^{3}H-TCPOBOP, radioactivity accumulates and is stored primarily in the adipose tissue and to a lesser extent in the liver. The radioactivity extracted from adipose tissue, 30 days after administration of the ^{3}H-TCPOBOP, chromatographed as the parent compound. The hepatic concentration of TCPOBOP, after the administration of an ED$_{50}$ dose, was estimated to be 1×10^{-7} M. TCPOBOP is a potent and long-acting phenobarbital-like inducer, a faithful mimic of this pleiotropic response for all measures tested. Despite the lack of a discernable structure–activity relationship among the numerous foreign compounds which evoke the phenobarbital pleiotropic response, the existence of a potent agonist, i.e., TCPOBOP, suggests that this response may be mediated through a specific recognition site.

INTRODUCTION

Induction of the hepatic microsomal monooxygenase enzyme system and a coordinate increase in several other liver enzymes are produced by the administration of a remarkable variety of foreign chemicals (1, 2). The liver microsomal monooxygenase system, which metabolizes most lipophilic foreign compounds, consists of a flavoprotein, NADPH–cytochrome *P*-450 reductase, and a group of hemoproteins, cytochromes *P*-450. Several distinct species of cytochrome *P*-450 have been identified and

Supported in part by National Institute of Environmental Health Sciences Grant R01-ES0-1884, and National Cancer Institute Program Project Grant P01-CA-2284 and Core Grant CA-07175.

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0026-895X/80/060571-10$02.00/0

purified from rat and rabbit liver (3, 4) and shown to differ in primary structure, substrate specificity, and genetic control.

Most compounds which induce hepatic monooxygenase activity produce one of two patterns of response:[3] Phenobarbital is the prototype of one class, and 3-methylcholanthrene is the prototype of the other group. Phenobarbital increases liver weight, stimulates proliferation of the smooth endoplasmic reticulum, and induces NADPH–cytochrome *P*-450 reductase and one or more species of cytochrome *P*-450. The increase in monooxygenase activity is directed toward a wide variety of substrates. In addition, phenobarbital stimulates a number of other enzyme activities, e.g., UDP–glucuronosyl transferase (9), glutathione-*S*-transferase (10), ϕ-aldehyde dehydrogenase (11), and microsomal epoxide hydrolase (12) activities.[4] In contrast, the administration of 3-methylcholanthrene produces a smaller increase in the proliferation of the endoplasmic reticulum, and little or no increase in the reductase, and induces different species of cytochrome *P*-450, and the associated monooxygenase activity is directed toward a narrower spectrum of cytochrome *P*-450. 3-Methylcholanthrene also stimulates a number of nonmonooxygenase enzymes, e.g., DT-diaphorase (13) and τ-aldehyde dehydrogenase (11). For other foreign chemicals, which are considered as either phenobarbital-like or 3-methylcholanthrene-like, usually on the basis of their induction of microsomal monooxygenase activity, it is assumed, but infrequently documented that these compounds are faithful mimetics of the entire pleiotropic response produced by the prototype compounds.

For the expression of each of these pleiotropic responses, it seems reasonable to hypothesize the existence of a recognition site, a receptor, which, when it binds to the appropriate phenobarbital-like or 3-methylcholanthrene-like agonist, mediates the ensuing coordinated induction response. It has been possible, recently, to demonstrate and characterize the receptor for 3-methylcholanthrene-like compounds in the cytosol of mouse and rat liver (14) and show that the receptor mediates the specific nuclear uptake of the inducing compound (15). This advance is largely attributable to the availability of a potent agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),[5] which is four orders of magnitude more potent than 3-methylcholanthrene ($ED_{50} = 1 \times 10^{-9}$ mol/kg)[3] and binds to the cytosol receptor with a high affinity ($K_D = 0.27$ nM).

At present there is little evidence for the existence of a receptor for the phenobarbital response. In pharmacological theory, one hypothesizes the existence of a receptor on the basis of several indirect lines of evidence: (1) a reasonably potent, biologically active compound which produces a graded dose–response relationship; (2) among a group of related compounds, some discernable structure–activity relationship; (3) some evidence of tissue specificity for the response; and (4) ideally, the existence of a competitive antagonist. The induction of monooxygenase activity by phenobarbital-like compounds is dose related, and the response is largely confined to the liver and small intestines (16). However, none of the other criteria for a receptor have been satisfied. All of the compounds which evoke a phenobarbital-like response appear to be very weak agonists. For phenobarbital-like compounds, an estimate of the dose which produces one-half the maximum induction for some measure of the pleiotropic response (ED_{50}) is usually not available; however, their lack of potency is evident from the observation that these compounds are usually administered at 10^{-4} to 10^{-3} mol/kg/daily (or as a single dose if the compound has a long biological half-life). There is no discernable structure–activity relationship among the remarkable variety of compounds which evoke this response: cyclic, nitrogen-containing compounds (e.g., barbiturates and hydantoins), simple halogenated aromatic hydrocarbons (e.g., 1,2,4-tribromobenzene and 2,4,5,2′,4′,5′-hexachlorobiphenyl), complex halogenated cyclic compounds (e.g., dieldrin and mirex), and alkanes and substituted alkanes (e.g., hexane (17) and 1,1,1-trichloroethane (18)).

This apparent lack of structure–activity relationship among compounds which produce a phenobarbital-like pleiotropic response may be due to the minimal potency of these compounds. One approach to understanding the mechanism by which these diverse agents produce this characteristic response is to search for a potent phenobarbital-like inducer. The availability of a potent agonist and evidence of a structure–activity relationship among its closely related congeners would support the hypothesis that the response is receptor mediated. In this report we describe a potent and long-acting phenobarbital-like compound, 1,4-bis[2-(3,5-dichloropyridyloxyl)]benzene, and compare it to phenobarbital.

MATERIALS AND METHODS

Materials

Dieldrin, chlordane, and mirex were purchased from the RFR Corp., Hope, R.I. Diphenylhydantoin was a gift from the Parke–Davis Co., Detroit, Mich. Benzphetamine was a gift of the Upjohn Co., Kalamazoo, Mich. Dichlorophenobarbital was a gift of the Lilly Co., Indianapolis, Ind. 7-Ethoxycoumarin, 7-hydroxycoumarin, 4-dimethylaminoantipyrene, and *trans*-styrene oxide were purchased from Aldrich Chemical Co., Milwaukee, Wis. Cytochrome *c*, glutathione, monosodium glucose-6-phosphate, glucose-6-phosphate dehydrogenase (from torula yeast), NADH, NADP, and NADPH were purchased

[3] In addition to the two "classical" responses typified by phenobarbital and 3-methylcholanthrene, several other distinct patterns of hepatic enzyme induction produced by other compounds have been reported recently: pregnenolone 16α-carbonitrile (5), isosafrole (6) 2(3)-tertbutyl-4-methoxyphenol (7), and ethanol (8).

[4] The increase in these enzyme activities is referred to in this paper as induction, but true enzyme induction, de novo protein synthesis, has been demonstrated for only a few of these enzymes.

[5] Abbreviations used: ED_{50}—the dose of a drug which produces one-half the maximal response; TCPOBOP—1,4-bis[2-(3,5-dichloropyridoxy)]benzene; dieldrin—1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*endo*-*exo*1,4:5,8-dimethanonaphthalene; chlordane—1,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan; dichlorophenobarbital—5-(3,4-dichlorophenyl)-5-ethyl barbituric acid; mirex—dodecachlorooctahydro-1,3,4-metheno-1H-cyclobuto[*cd*]pentalene; TCDD—2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

from Sigma Chemical Co., St. Louis, Mo. 1-Chloro-2,4-dinitrobenzene was purchased from Eastman Organic Chemicals, Rochester, N. Y. [*Dimethyl*-^{14}C]aminopyrine (30 mCi/mmol) and [7-^{3}H]*trans*-styrene oxide (30 mCi/mmol) were purchased from Amersham Searle, Arlington Heights, Ill. Protosol was purchased from New England Nuclear Corp., Boston, Mass., and RIA-Solve II scintillation cocktail was purchased from Research Products International Corp., Elk Grove Village, Ill. LK5DF silica gel plates were bought from Kontes Glass Co., Vineland, N. J.

Syntheses

1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP). 2,3,5-Trichloropyridine (4.47 g) and hydroquinone (1.36 g) were dissolved in 10 ml of dimethylsulfoxide heated at 65°C, and a 50% aqueous solution of 30 mM sodium hydroxide was added over 30 min. The temperature was increased to 110°C and maintained for 2 h. The mixture was cooled to room temperature, then combined with 100 ml of a 10% solution of sodium hydroxide, and the resulting solid was filtered. The solid was washed with water, air-dried, and recrystallized once from carbon tetrachloride to yield 1.90 g (36%), mp 157–159°C, NMR ($CDCl_3$), δ 7.15 (4H, s), 7.72 (2H, d, $J = 3H_z$), and 7.92 (2H, s, $J = 3H_z$); the mass spectrum gave a molecular ion for $C_{16}H_7O_4N_3Cl_4$.

1,4-Bis[2-(3,5-dichloropyridyloxy)]-2-nitrobenzene. To TCPOBOP (1.12 g) dissolved in 20 ml of concentrated sulfuric acid, cooled in an ice bath, was added 30 ml of cold concentrated nitric acid, and the solution was stirred and permitted to warm to room temperature over 2 h. The reaction mixture was poured into 500 ml of water and extracted twice with 125 ml of ethyl acetate. The organic extracts were combined, washed four times with an equal volume of water, dried, and evaporated to yield 1.17 g (94%) of a nearly pure nitro derivative as a light yellow solid. Recrystallization from ethyl acetate–hexanes gave a pure 2-nitro compound, mp 157–158.5°C.

Calc for C, 42.99%; H, 1.58%; and N, 9.40%. Found for C, 42.97%; H, 1.73%; and N, 9.47%.

1,4-Bis[2-(3,5-dichloropyridyloxy)]-2-aminobenzene. A solution of 1.17 g of the nitro compound in 30 ml of ethyl acetate containing 250 mg of 5% palladium on charcoal was reduced under 1 atm of hydrogen gas until no further gas uptake was observed. The solution was filtered through celite to remove the catalyst and evaporated to give 1.05 g of nearly pure amine (96%), mp 169–170°C, on recrystallization from $CHCl_3$ ether.

Calc for C, 46.08%; H, 2.18%; and N, 10.07%. Found for C, 46.38%; H, 2.26%; and N, 10.30%.

1,4-Bis[2-(3,5-dichloropyridyloxy)]-2-iodobenzene. To an ice bath-cooled solution of 1.05 g of the above amine compound in 10 ml of concentrated sulfuric acid was a solution of 422 mg of sodium nitrite dissolved in 7 ml of concentrated sulfuric acid. The mixture was stirred with cooling for 1.5 h and then 10 ml of cooled, saturated potassium iodide solution was added dropwise over a 30-min period. The mixture was stirred and permitted to warm to room temperature overnight and then poured into a stirred mixture of ethyl acetate (100 ml) and water (400 ml). The separated organic layer was washed successively with three portions (70 ml) of dilute sodium bisulfite, once with water, and once with a saturated salt solution. The organic phase was dried (Na_2SO_4) and evaporated. The red-brown oily solid residue was dissolved in chloroform and adsorbed into 10 g of silica gel. This silica gel was placed at the top of a 2.5 × 40-cm column of silica gel and saturated with CCl_4. The column was first eluted with 600 ml of carbon tetrachloride followed by 500 ml of chloroform, which removed 358 mg of intermediate-polarity material. Recrystallization of this material four times from CCl_4 yielded 148 mg (11%) of the pure iodo compound; mp 172.5–174°C, NMR ($CDCl_3$), δ 7.16 (2H, m), 7d.60 (1H, dd, $J = 2$ and $4H_z$), 7.71 (2H, m), 7.85 (1H, d, $J = 2H_z$), and 7.90 (1H, d, $J = 2H_z$); mass spectrum *m/e* 530, 528, and 526.

Calc for C, 36.40%; H, 1.34%; N, 5.31%; and I, 24.03%. Found for C, 36.37%; H, 1.23%; N, 5.417%; and I, 35.69%.

1,4-Bis[2-(3,5-dichloropyridyloxy)]-[2-^{3}H]benzene. The iodo compound (8.6 mg) was dissolved in 2 ml of ethyl acetate which had been dried over anhydrous $MgSO_4$. To this solution was added 20 mg of 5% palladium on a charcoal catalyst. The reaction was stirred overnight at room temperature under an atmosphere of 25 Ci of tritium gas. The labile tritium was removed *in vacuo*, by dissolving the compound in ethyl acetate and then adding ethanol as a protonic solvent. After filtration of the catalyst, ethyl acetate and ethanol were again added and again the compound was taken to dryness *in vacuo*. The compound was dissolved in 10 ml of ethyl acetate. This reductive dehalogenation was performed by New England Nuclear, Boston, Mass. The yield was 395 mCi of radiolabeled compound.

One-half of the sample (200 mCi) was evaporated to dryness, redissolved in toluene, and applied to a silica gel 60 column (34-ml volume). The sample was eluted with toluene and the peak radioactive fractions were pooled. On tlc the purified tritiated compound comigrated with unlabeled TCPOBOP. Analysis by HPLC equipped with a radioactive detector indicated that the compound was 93 to 96% radiochemically pure. The specific activity of ^{3}H-TCPOBOP was 24.2 Ci/mmol.

Animals

B6D2F$_1$/J mice were either purchased from Jackson Laboratory, Bar Harbor, Maine, or bred in our laboratory by mating C57BL/6J female and DBA2J male mice obtained from the same institution. The mice were housed in plastic cages with hardwood bedding or in hanging wire cages and permitted unlimited access to food (Wayne Mouse Breeder Blox, Allied Mills, Chicago, Ill.) and water.

Both male and female mice, usually between 6 and 15 weeks of age, were used, but in a given experiment we used mice of only one sex and of the same age. All compounds were administered intraperitoneally. Sodium phenobarbital was administered dissolved in saline, and TCPOBOP was dissolved in corn oil.

Cellular Fractionation

Liver and other tissues were weighed, homogenized in 4 vol of 1.15% potassium chloride, and centrifuged at 10,000*g* for 20 min. The 10,000*g* supernatant fraction was

centrifuged for 1 h at 100,000*g*. For spectral studies the microsomal pellet was resuspended in isotonic potassium chloride and recentrifuged at 100,000*g* for 1 h.

Assays of Microsomal Monooxygenase Activity

All monooxygenase activities were measured on the 10,000*g* supernatant fraction and expressed as moles of product formed per minute per wet weight of tissue.

Aminopyrine *N*-demethylase activity was routinely assayed in a manner similar to the method of Cochin and Axelrod (19) in which the formaldehyde product formed is converted to a chromogen by the Hantzch reaction (20). The total incubation volume was 2 ml, containing 0.5 μmol NADP, 0.5 μmol NADH, 15 μmol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 10 μmol $MgCl_2$, 20 μmol semicarbazide, 260 μmol potassium hydrogen phosphate buffer, pH 7.4, and 20 μmol aminopyrine. The 10,000*g* supernatant fraction equivalent to 10 and 20 mg wet weight of liver from induced and control animals, respectively, was added to the reaction mixture and incubated for 10 min at 37°C.

Benzphetamine *N*-demethylase activity in the liver was assayed under identical conditions as aminopyrine *N*-demethylase with the substitution of 1 mM benzphetamine.

Aryl hydrocarbon hydroxylase activity was assayed by the rate of formation of the 3-hydroxy metabolite of benzo[*a*]pyrene determined fluorimetrically as previously described (21).

To measure the lower monooxygenase activity found in nonhepatic tissues, two sensitive assays were used. Aminopyrine *N*-demethylase activity was measured using the radiometric assay previously described (22). The 10,000*g* supernatant fraction of the proximal intestines (2 to 3 in. in length) equivalent to 20 mg wet weight of tissue was incubated at pH 8.0 with ^{14}C-aminopyrine (5×10^5 dpm, 0.25 mM) in a total volume of 0.5 ml.

7-Ethoxycoumarin *O*-deethylase activity was measured as previously described (23) at pH 7.5, with a substrate concentration of 0.5 mM. For adult mouse liver, the 10,000*g* supernatant fraction equivalent to 1 mg wet weight was incubated for 10 min. For fetal liver and adult extrahepatic tissue, the postmitochondrial supernatant fraction equivalent to 10 mg of tissue was incubated for 15 to 30 min.

Under the conditions described above, all of the assays were found to be linear with enzyme concentration and time.

Glutathione-S-transferase activity. This was measured in the hepatic cytosol using 1-chloro-2,4-dinitrobenzene as a substrate by the method of Habig *et al.* (24).

Microsomal epoxide hydrolase. This was assayed in liver microsomes using [7-^{3}H]styrene oxide as a substrate by the method of Oesch *et al.* (12) with the modifications of Seidegard *et al.* (25) and Jerina *et al.* (26) as we have recently described (27).

NADPH–cytochrome c reductase activity. This was measured in liver microsomes by the method of Masters *et al.* (28).

Cytochrome P-450. This was measured by the method of Omura and Sato (29) as the reduced carbon monoxide vs reduced difference spectrum in a Cary 118C recording spectrophotometer. The Soret maximum was measured to the nearest 0.1 nm. The ethyl isocyanide difference spectrum of cytochrome *P*-450 was measured in liver microsomes in 0.1 M potassium phosphate buffer, pH 7.4, reduced with sodium dithionite (30). The final ethyl isocyanide concentration was 6.6 mM.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. SDS–polyacrylamide gel electrophoresis of liver microsomes was performed in slab gels using an upper stacking gel of 3% and a lower gel of 7.5% acrylamide by the method of Laemmli (31) and the gel was stained with Coomassie blue (32).

Quantification of Tissue Radioactivity

From mice administered ^{3}H-TCPOBOP, heparinized blood was obtained by orbital sinus puncture and samples of inguinal fat, liver, proximal intestines, lung, brain, and kidney were taken. Weighed tissue samples of approximately 100 mg were added to 0.5 ml of water and 1 ml of Protosol in a scintillation vial and heated overnight at 55°C. One hundred-microliter samples of blood were treated identically and then bleached with the addition of 250 μl of a 20% benzoyl peroxide solution. To all samples were added 100 μg of glacial acetic acid and 10 ml of RIA-Solve II scintillation cocktail. Counting efficiency ranged from as low as 15% for blood to 40% for fat.

Extraction and Identification of Radioactivity from Adipose Tissue

Adipose tissue obtained from mice 10 and 30 days after the administration of ^{3}H-TCPOBOP (7.5×10^{-6} mol/kg, 2.15×10^7 dpm/μmol) was pooled and stored at −15°C. One gram of fat was homogenized in 20 ml of ethanol, 10 ml of 1 N potassium hydroxide in ethanol added, and the mixture boiled at 85°C for 15 min. To the cooled, saponified solution, 4 ml of water was added and the solution extracted three times with 30 ml of hexane. The pooled hexane extracts were evaporated with nitrogen to 20 ml, then back extracted twice with 20 ml of a 0.1 N sodium hydroxide, 1 M sodium chloride solution, and the organic layer was evaporated to near-dryness. The residue was dissolved in 500 μl of hexane and 100-μl samples were spotted on LK5DF silica gel thin-layer plates (Kontes Glass Co., Vineland, N. J.) and developed in acetone or toluene. The plates were then scraped at 1-cm intervals and the radioactivity was quantified. ^{3}H-TCPOBOP has an R_f of 0.5 in toluene and 0.79 in acetone.

Proteins. Proteins were determined by the method of Lowry *et al.* (32) using bovine serum albumin as a standard.

Calculations. The potency (ED_{50}) of phenobarbital and TCPOBOP to induce aminopyrine *N*-demethylase activity was estimated from dose–response data by a computer program. Three separate dose–response curves were analyzed for each compound, and their potency was estimated as the mean of the logarithm of individual estimates.

RESULTS

The induction of hepatic aminopyrine *N*-demethylase activity in B6D2F$_1$/J mice was selected as our standard measure of the phenobarbital response. We compared the potency of sodium phenobarbital and five other compounds, reported to be relatively potent phenobarbital-like inducers, to simulate this hepatic monooxygenase activity. Each compound was administered daily for 3 days at four or five dosage levels, and 24 h after the last dose, enzyme activity was determined. Hepatic aminopyrine *N*-demethylase activity was maximally induced to 7 to 12 times the activity in control animals. Sodium phenobarbital produced a dose-related increase in enzyme activity, with a half-maximally inducing dose of 1.07×10^{-4} mol/kg/day (27.3 mg/kg/day) (Table 1). The five other compounds tested were all more potent than phenobarbital. Dieldrin, the most potent, had an ED_{50} of 8.0×10^{-6} mol/kg/day. All of these compounds appeared to induce hepatic aminopyrine *N*-demethylase activity to approximately the same maximum. No further experiments were performed to determine if all these compounds evoke qualitatively the same pleiotropic response, because of their lack of potency.

In Fig. 1 is shown the structure of 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, abbreviated TCPOBOP (for tetrachloro-pyridine-O-benzene-O-pyridine). We compared the potency of this compound and sodium phenobarbital to induce hepatic aminopyrine *N*-demethylase activity. As shown in Fig. 2, both compounds produced parallel dose–response curves, with nearly the same maximal response (see below). Based on three dose–response experiments for each compound, the best estimate of the ED_{50} for TCPOBOP is 1.63×10^{-7} mol/kg/day, and for phenobarbital, the best estimate of the ED_{50} is 1.07×10^{-4} mol/kg/day. TCPOBOP is approximately 650 times more potent than phenobarbital. The dose–response curves for TCPOBOP given as a single dose on day 1 and as daily divided doses for 3 days are the same.

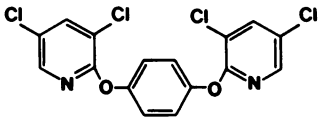

FIG. 1. *The structure of 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP)*

If TCPOBOP is a phenobarbital-like agonist, then it should evoke all of the pleiotropic effects produced by phenobarbital. We compared the effects of both drugs on various measures of the hepatic microsomal monooxygenase system in B6D2F$_1$/J mice (Table 2). A single maximal inducing dose of TCPOBOP, 7.46×10^{-6} mol/kg (3 mg/kg), produced an 11-fold increase in aminopyrine *N*-demethylase activity; a maximally tolerated regimen of phenobarbital, 100 mg/kg/day for 3 days, produced only 85 to 90% of this response. The combined administration of phenobarbital and TCPOBOP produced no greater response than that of TCPOBOP administered alone. Both compounds, administered alone or administered together, produced a modest induction (5- to 6-fold) of aryl hydrocarbon hydroxylase activity. Both phenobarbital and TCPOBOP administered alone, or in combination, produced the same increase in total microsomal cytochrome *P*-450, which had the same maxima by CO-difference spectra (450.0 to 450.3 nm) and similar ethyl isocyanide-difference spectra; and these three treatment groups had a similar, approximately 2-fold increase in NADPH–cytochrome *c* reductase activity. By contrast, TCDD, a 3-methylcholanthrene-like inducer, produced a large increase in aryl hydrocarbon hydroxylase activity, but only a small increase in aminopyrine *N*-demethylase activity, little or no change in NADPH–cytochrome *c* reductase activity, and a qualitatively different type of cytochrome *P*-450 with a maximum in the CO-difference spectrum of 448.3 nm and an altered ethyl isocyanide difference spectrum. Phenobarbital and TCPOBOP produced a comparable increase in the ratio of liver to body weight and a proliferation in

TABLE 1

Relative potency of selected inducers of hepatic aminopyrine-N-demethylase activity

B6D2F$_1$/J female mice, all of the same age in any given experiment, and between 6 and 15 weeks old, were administered the test compound at four or five different dose levels, by intraperitoneal injection daily for 3 days. Dichlorophenobarbital was dissolved in a dilute aqueous solution of sodium hydroxide, sodium phenobarbital was dissolved in 0.9% aqueous sodium chloride, and the other compounds were dissolved in corn oil. Each experiment included the appropriate solvent-injected controls and a group of mice injected with sodium phenobarbital at a dose of 100 or 120 mg/kg/day. Five mice were used for each group. Twenty-four hours after the last injection the animals were killed and hepatic aminopyrine *N*-demethylase activity was assayed as described in Materials and Methods. The mean ± SE of enzyme activity at each dose level was plotted versus the logarithm of dosage level and the ED_{50} estimated graphically.

	ED_{50}	Relative potency
	mol/kg/day	
Phenobarbital	1.1×10^{-4}	1.0
Diphenylhydantoin	5.4×10^{-5}	2.0
Dichlorophenobarbital	2.2×10^{-5}	5.0
Chlordane	1.6×10^{-5}	6.9
Mirex	1.2×10^{-5}	9.2
Dieldrin	8.0×10^{-6}	13.8

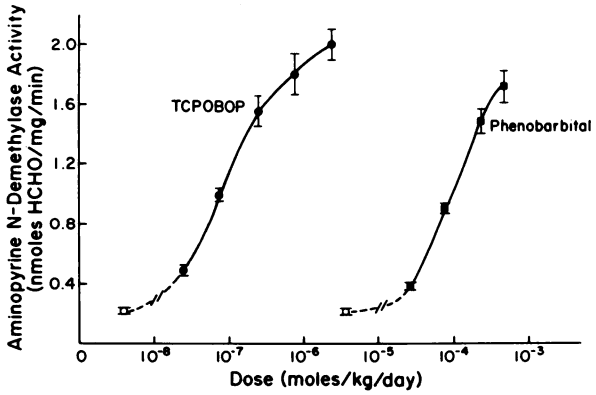

FIG. 2. *B6D2F$_1$/J female mice 6 weeks of age were injected intraperitoneally daily for 3 days with sodium phenobarbital in saline or TCPOBOP in corn oil, or the appropriate vehicle alone, and then hepatic enzyme activity was assayed*

Each point represents the mean ± SE of a group of five mice.

TABLE 2

The effect of phenobarbital and TCPOBOP on hepatic microsomal monooxygenase activities in B6D2F$_1$/J mice

B6D2F$_1$/J mice were administered a single intraperitoneal dose of corn oil (control), 7.5 ml/kg, TCPOBOP, 3 mg/kg dissolved in corn oil, or TCDD, 96.6 μg/kg dissolved in *p*-dioxane (0.4 ml/kg). Sodium phenobarbital (100 mg/kg dissolved in 0.9% NaCl solution) was given daily for 3 days. Seventy-two hours after the initial injection, the animals were killed, and the hepatic activities measured. The results are a composite of two experiments: Aminopyrine *N*-demethylase and aryl hydrocarbon hydroxylase activities were measured in one experiment using 10-week-old male mice, and cytochrome *P*-450 spectral measurements and NADPH–cytochrome *c* reductase in a separate experiment using 10-week-old female mice. The values are the means ± SE of determinations on five animals.

	Aminopyrine *N*-demethylase act.	Aryl hydrocarbon hydroxylase act.	Cytochrome *P*-450	Cytochrome *P*-450 ethyl isocyanide spectrum	NADPH–cytochrome *c* reductase
	nmol/mg/min	*nmol/mg/min*	*nmol/mg*	*ΔOD 455–490/433–490*	*nmol/mg/min*
Control	0.24 ± 0.01	12.8 ± 0.5	0.62 ± 0.04[a]	0.251 ± 0.014[a]	89.5 ± 4[a]
Phenobarbital	2.38 ± 0.10	59.3 ± 2.1	2.44 ± 0.20	0.202 ± 0.011	162 ± 5
TCPOBOP	2.70 ± 0.07	79.4 ± 2.5	2.45 ± 0.08	0.226 ± 0.012	193 ± 11
Phenobarbital + TCPOBOP	2.66 ± 0.13	72.6 ± 2.7	2.44 ± 0.21	0.226 ± 0.012	182 ± 4
TCDD	0.55 ± 0.11	201 ± 5.8	1.97 ± 0.11	0.700 ± 0.059	100 ± 7

[a] Mean ± SE of four animals.

the smooth endoplasmic reticulum as seen by electron microscopy (data not presented).

In data not shown, we found that a single maximally inducing dose of TCPOBOP (3 mg/kg) and a maximally tolerated regimen of sodium phenobarbital (100 mg/kg/day for 3 days) both produced an 11-fold increase in hepatic benzphetamine *N*-demethylase activity in B6D2F$_1$/J mice. Pregnenolone 16α-carbonitrile (25 mg/kg/day for 3 days), a distinctly different type of inducer (4), produced only a 4-fold increase in this monooxygenase activity. Hepatic microsomes from B6D2F$_1$/J mice treated with TCPOBOP, sodium phenobarbital, or the combined administration of the two drugs gave indistinguishable electrophoretic patterns on SDS–polyacrylamide gels.

We compared the effects of phenobarbital and TCPOBOP administration on two other enzyme activities in the liver of B6D2F$_1$/J mice: microsomal epoxide hydrolase activity (measured using [7-^{3}H]styrene oxide as a substrate) and cytosolic glutathione *S*-transferase activity (using 1-chloro-2,4-dinitrobenzene as a substrate). As shown in Table 3, a single dose of TCPOBOP induced a 3-fold increase in microsomal epoxide hydrolase and a 4-fold increase in glutathione-*S*-transferase activity, significantly greater than the 2-fold increase in both activities produced by a daily dose of 100 mg/kg of sodium phenobarbital. In a second experiment, in which sodium phenobarbital was administered at 100 mg/kg initially, 120 mg/kg the second day, and then 150 mg/kg for 5 days, phenobarbital induced an induction comparable to that of TCPOBOP (data not shown).

TABLE 3

The effect of phenobarbital and TCPOBOP on glutathione-S-transferase and epoxide hydrolase activities in mouse liver

B6D2F$_1$/J female mice, 10 weeks old, were administered a single intraperitoneal dose of corn oil (control), 7.5 ml/kg, or TCPOBOP, 3 mg/kg in corn oil, or sodium phenobarbital, 100 mg/kg dissolved in saline, daily for 8 days. Nine days after the initial dosage, the animals were killed and epoxide hydrolase activity was measured in microsomes as the rate of styrene epoxide hydration per mg microsomal protein per min, and glutathione-*S*-transferase activity was measured as the rate of 1-chloro-2,4-dinitrobenzene conjugation per mg cytosol protein per min. Each value is the mean ± SE of determinations on five animals.

	Epoxide hydrolase activity	Glutathione-*S*-transferase activity
	nmol/mg/min	*nmol/mg/min*
Control	6.19 ± 3.8	1.20 ± 0.09
Phenobarbital	14.6 ± 0.6	2.57 ± 0.14
TCPOBOP	21.6 ± 0.7	5.23 ± 0.39
Phenobarbital + TCPOBOP	23.3 ± 0.9	5.72 ± 0.15

We compared the effects of phenobarbital and TCPOBOP on the induction of microsomal monooxygenase activity in the liver and in extrahepatic tissues in B6D2F$_1$/J mice (Table 4). While the maximally tolerated dose regimen of phenobarbital (100 mg/kg/day for 3 days) is almost as effective as TCPOBOP (3 mg/kg) at inducing aminopyrine-*N*-demethylase activity in the liver, TCPOBOP is more effective in the intestines. TCPOBOP (30 mg/kg) produced a 40-fold increase in aminopyrine *N*-demethylase activity and a 30-fold increase in 7-ethoxycoumarin *O*-deethylase activity in the intestines. Neither drug produced any increase in microsomal monooxygenase activity in the lung, while a small increase in enzyme activity (1.5- to 2.5-fold) was observed with phenobarbital and TCPOBOP in the kidney.

When painted on the back of shaven B6D2F$_1$/J mice, neither TCPOBOP nor mirex produced any stimulation of 7-ethoxycoumarin *O*-deethylase activity in the skin (Table 5). However, both of these lipophilic compounds were sufficiently absorbed from the skin to produce substantial induction of hepatic microsomal monooxygenase activity. In fact TCPOBOP is approximately equipotent at inducing hepatic aminopyrine *N*-demethylase by dermal painting as by intraperitoneal administration.

As seen in Table 6, a single intraperitoneal injection of TCPOBOP (30 mg/kg) to pregnant mice (14 days of gestation) induced 7-ethoxycoumarin *O*-deethylase activity in both maternal and fetal liver.

We compared the time course of induction of hepatic aminopyrine *N*-demethylase activity by TCPOBOP and phenobarbital, and the fall in enzyme activity after drug withdrawal (Fig. 3). A single maximally inducing dose of TCPOBOP (3 mg/kg) produced a maximal response by 48 h, and this induced enzyme activity persisted for the duration of the experiment (12 days). The daily admin-

TABLE 4

Effect of phenobarbital and TCPOBOP on monooxygenase activity in various tissues

Twelve-week-old female B6D2F$_1$/J were administered a single intraperitoneal dose of corn oil (control), 7.5 ml/kg, or TCPOBOP, 3 or 30 mg/kg in corn oil, or sodium phenobarbital, 100 mg/kg dissolved in saline, daily for 3 days. The animals were sacrificed 72 h after initial dosing and their tissues assayed for monooxygenase activity. Aminopyrine *N*-demethylase activity was assayed in the liver by the colorimetric measurement of HCHO formation, and in the intestines by the use of ^{14}C-aminopyrine. The values are the means ± SE of determinations on five animals.

	Aminopyrine-*N*-demethylase activity		7-Ethoxycoumarin *O*-deethylase activity		
	Liver	Intestines	Intestines	Lung	Kidney
	nmol/mg/min	*pmol/mg/min*		*pmol/mg/min*	
Control	0.32 ± 0.01	0.70 ± 0.16	1.98 ± 0.59	1.15 ± 0.14	0.33 ± 0.04
Phenobarbital, 100 mg/kg × 3 days	2.33 ± 0.15	9.0 ± 3.0	15.6 ± 3.0	1.02 ± 0.14	0.33 ± 0.04
TCPOBOP, 3 mg/kg	2.79 ± 0.13	20.3 ± 4.5	50.7 ± 0.5	—	—
TCPOBOP, 30 mg/kg	2.74 ± 0.13	33.9 ± 6.5	65.3 ± 9.7	0.79 ± 0.13	0.50 ± 0.05
TCPOBOP, 30 mg/kg, + phenobarbital, 100 mg/kg, × 3 days	2.91 ± 0.08	41.6 ± 3.0	54.2 ± 7.4	1.35 ± 0.11	0.64 ± 0.10

istration of phenobarbital (100 mg/kg) evoked its maximal response by 72 h. The maximal induction produced by phenobarbital was only about 90% of that produced by TCPOBOP. After 5 days, the administration of phenobarbital was stopped (day 0 in Fig. 3), and the enzyme activity rapidly declined, returning to near-control levels in 5 days.

We further studied the prolonged induction response produced by TCPOBOP (Fig. 4). Following a single dose of the compound (3 mg/kg), hepatic aminopyrine *N*-demethylase activity was induced approximately 8-fold on days 10 and 17, and then enzyme activity gradually decreased over the next 5 months, but was still nearly four times the control activity 143 days after the administration of TCPOBOP. We also measured the time course of the increase in the ratio of liver to body weight (Fig. 4B) and the induction of hepatic microsomal epoxide hydrolase activity (Fig. 4C) produced by a single dose of TCPOBOP. These effects of TCPOBOP administration declined more rapidly than the induction of aminopyrine *N*-demethylase activity, but remained above the control levels over the 5 months of the experiment.

The prolonged biological response observed after a single dose of TCPOBOP is a reflection of the long biological half-life of TCPOBOP. Following a single intraperitoneal administration of ^{3}H-TCPOBOP (3 mg/kg, 5.34 × 10^5 dpm/μg), animals were killed at 1, 3, 10, and 30 days, and the radioactivity was quantified in a variety of organs. As seen in Fig. 5, the radioactivity accumulated to the greatest extent in adipose tissue, approximately an order of magnitude greater then the liver concentration and 100-fold higher than that in the blood. Accumulation of radioactivity in kidney, intestines, lung, and brain was intermediate between that of liver and blood. Adipose tissue samples obtained from mice 10 and 30 days after the administration of ^{3}H-TCPOBOP were hydrolyzed in alkali and then extracted with hexane. This procedure recovered 88.0 ± 2.3% (N = 4) of the tissue radioactivity. Virtually all of the extracted radioactivity, when spotted on silica G thin-layer plates, chromatographed in two solvents with the same R_f as ^{3}H-TCPOBOP. Thus, the majority of the radioactivity in adipose tissue at 10 and 30 days represents the parent compound.

We wished to estimate the hepatic concentration of TCPOBOP following a dose which produces one-half the maximal induction of hepatic aminopyrine *N*-demethylase. B6D2F$_1$/J mice were administered a single intraperitoneal injection of TCPOBOP at three dose levels containing a constant amount of radiolabeled compound. The animals were sacrificed 3 days later, the radioactivity

TABLE 5

The effect of skin painting TCPOBOP, mirex, and TCDD on monooxygenase activity in skin and liver

B6D2F$_1$/J female mice, weighing approximately 20 g, were shaved on their backs and painted with 25 μl of acetone or 25 μl of a solution of the test compound dissolved in acetone. The acetone solutions of the test compounds—TCPOBOP, 1 mg/ml, and mirex, 20 mg/ml—were applied daily for 3 days; TCDD, 100 μg/ml, was applied only once. One group of mice was administered a single intraperitoneal dose of TCPOBOP (3 mg/kg) in corn oil. The animals were killed 72 h after the initial dosing. The skin from the treated area was removed, then scraped with a razor blade to remove largely epidermal cells, and the cells removed were homogenized. 7-Ethoxycoumarin *O*-deethylase activity was assayed in skin homogenate and expressed per mg of protein. Each value is the mean ± SE of determinations in five animals.

Treatment	Estimated total dose	Skin, 7-ethoxycoumarin *O*-deethylase activity	Liver, aminopyrine *N*-demethylase activity
	mg/kg	*pmol/mg/min*	*nmol/mg/min*
Control, skin painting	—	6.83 ± 0.37	0.36 ± 0.01
TCPOBOP, skin painting	3.75	7.14 ± 1.52	3.06 ± 0.10
Mirex, skin painting	75.0	7.50 ± 0.89	1.98 ± 0.15
TCDD, skin painting	0.125	190.1 ± 12.4	0.66 ± 0.03
TCPOBOP, parenteral	3.0	—	2.97 ± 0.18

TABLE 6

Transplacental induction of monooxygenase activity by TCPOBOP

C57BL/6J female mice were mated with DBA/2J male mice, and the date of insemination was determined by vaginal plugs. Fourteen-day-pregnant mice received a single subcutaneous injection of corn oil (7.5 ml/kg) or TCPOBOP (30 mg/kg) in corn oil. The animals were sacrificed 4 days later, and hepatic enzyme activity was determined in maternal liver and a pooled sample of fetal livers.

	N	Hepatic ethoxycoumarin *O*-deethylase activity (nmol/mg/min)	
		Maternal liver	Fetal liver
Control	5	27.4 ± 6.2	0.31 ± 0.04
TCPOBOP	6	291.0 ± 20.2	2.21 ± 0.37

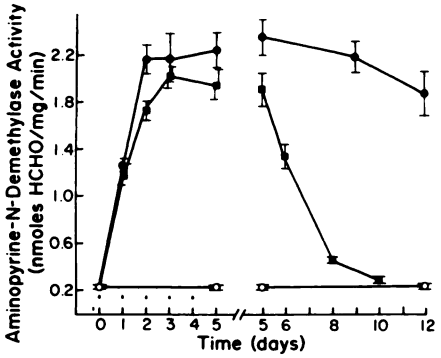

FIG. 3. *B6D2F$_1$/J female mice 12 to 15 weeks of age were administered a single dose of corn on day 0* (○), *a single dose of TCPOBOP (3 mg/kg) dissolved in corn oil on day 0* (●), *or sodium phenobarbital (120 mg/kg) daily for 5 days* (■)

The arrows at the bottom indicate the doses of phenobarbital. At the right is a second experiment in which the animals received identical treatment regimens for the first 5 days, and then the daily administration of phenobarbital was stopped. The animals were sacrificed at the indicated times and hepatic enzyme activity was assayed. Each point represents the mean ± SE of determinations on five animals.

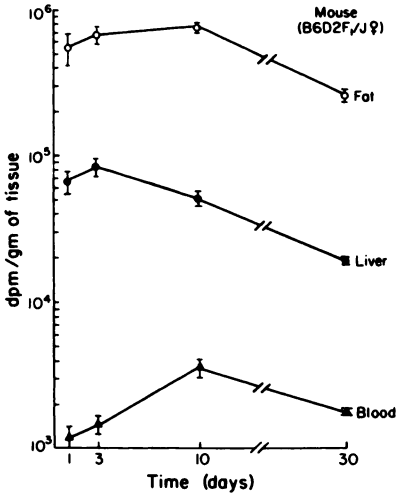

FIG. 5. *B6D2F$_1$/J, 9-week-old, female mice were administered a single intraperitoneal injection of 7.5 × 10^{-6} mol/kg of ^{3}H-TCPOBOP (5.34 × 10^5 dpm/μg)*

The animals were killed at subsequent time periods and the radioactivity in various tissues was quantified. Each point is the mean ± SE of determinations on five or six animals.

in samples of liver and adipose tissue was quantified, and the tissue concentrations of TCPOBOP were plotted versus the dose of the compound administered. As seen in Fig. 6, over the 2500-fold dose range, there is a linear relationship between the tissue concentration and the administered dose. At the ED_{50} dose, 4.9 × 10^{-7} mol/kg, the hepatic concentration is approximately 1 × 10^{-10} mol/g, or 1 × 10^{-7} M.

DISCUSSION

In this report, we have presented evidence that TCPOBOP is a faithful mimic of phenobarbital for all of the responses measured. In B6D2F$_1$/J mice, both compounds produce a comparable liver enlargement, hypertrophy of the smooth endoplasmic reticulum, induction of cytochrome *P*-450 and associated monooxygenase activities, and induction of NADPH–cytochrome *c* reductase, microsomal epoxide hydrolase, and glutathione *S*-transferase activities. Both compounds produce a dose-related induction of hepatic aminopyrine *N*-demethylase activity, with parallel dose–response curves. The half-maximal inducing dose of TCPOBOP, ED_{50}, is 1.63 × 10^{-7} mol/kg/day; and for phenobarbital the ED_{50} is 1.07 × 10^{-4} mol/kg/day (both drugs administered daily for 3 days). TCPOBOP is 650 times as potent as phenobarbital in producing this response. The maximal increase in hepatic aminopyrine *N*-demethylase activity produced by TCPOBOP was consistently 10 to 15% greater than the maximal response produced by phenobarbital. This is to be expected, since the maximally tolerated dose of phenobarbital (100 to 120 mg/kg/day) is only 4.5 times the ED_{50} dose. The combined administration of a maximally tolerated regimen of phenobarbital and a maximally ef-

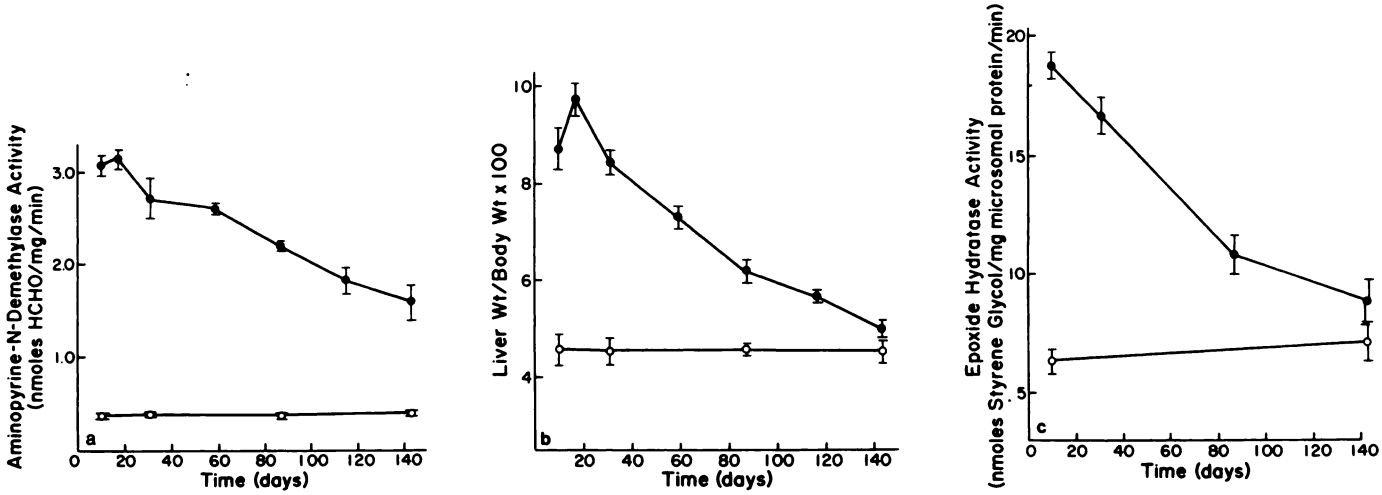

FIG. 4. *B6D2F$_1$/J female mice were administered a single intraperitoneal dose of TCPOBOP (3 mg/kg) dissolved in corn oil* (●) *or the vehicle alone* (○)

(A) Time course of hepatic aminopyrine *N*-demethylase activity. (B) Time course of liver/body weight ratio. (C) Time course of microsomal epoxide hydrolase activity.

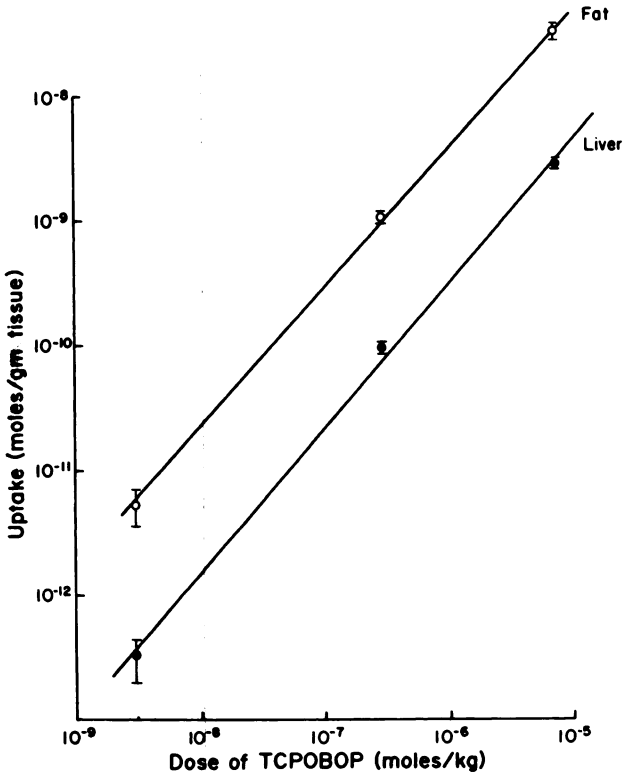

FIG. 6. *B6D2F$_1$/J mice, 9-week-old females, were administered a single intraperitoneal injectioin of TCPOBOP, at three dose levels, 3 × 10^{-9}, 3 × 10^{-7}, and 7.5 × 10^{-6} mol/kg, containing a constant amount of ^{3}H-TCPOBOP (1.6 × 10^8 dpm/kg)*

Seventy-two hours later, the animals were killed and the radioactivity in their liver and adipose tissue was quantified. Each point represents the mean ± SE of values for four mice.

fective dose of TCPOBOP produced no greater response than that evoked by TCPOBOP alone. Following a single dose of TCPOBOP (3 mg/kg), hepatic aminopyrine *N*-demethylase and microsomal epoxide hydrolase activities and the ratio of liver to body weight remained elevated over control levels for 20 weeks.

The prolonged biological response produced by TCPOBOP is attributable to the persistence of the parent compound (Fig. 5). TCPOBOP is primarily stored in the adipose tissue, and the hepatic concentration following an ED_{50} dose (4.9×10^{-7} mol/kg) is estimated to be 1×10^{-7} mol/kg. If this pleiotropic response is receptor mediated, then the liver concentration of TCPOBOP (1×10^{-7} M) at the half-maximal inducing dose is an estimate of the binding affinity of the receptor.

Central to understanding the mechanism of the phenobarbital pleiotropic response is explaining how a large group of compounds which have no apparent chemical similarity all produce a common effect. Three hypotheses may be advanced. (1) This response is *not* mediated through a receptor, a specific, saturable, high-affinity binding site, but phenobarbital-like inducers exert their action through some common physical properties (analogous to the mechanism of general anesthesia produced by anesthetics). (2) All of these compounds are reasonably lipophilic, and most, if not all, appear to be substrates for hepatic microsomal monooxygenase enzyme complex. This has led to the suggestion that it is the binding and/or metabolism of these compounds to cytochrome *P*-450 that is the signal for initiating the pleiotropic response (17, 34), i.e., the receptor for induction is the enzyme active site. While this is probably the most widely favored hypothesis, one correlate of this model remains to be explained. If the "receptor" is a particular cytochrome *P*-450, and pleiotropic response is proportional to receptor occupation, then as induction procedes, there will be an increasing amount of receptor. An inducing compound with a long biological half-life (e.g., TCPOBOP and mirex) should produce an ever-increasing response, which is not observed. Of course one might hypothesize that some step other than receptor occupation becomes rate limiting, and this would lead to a plateauing of the response. (3) All phenobarbital-like compounds do bind to a common receptor, a specific, saturable recognition site, which is distinct from cytochrome *P*-450 or any of the other products of the pleiotropic response. Since all of the known inducers (with the exception of TCPOBOP) are such weak agonists, it is difficult to discern any structure–activity relationship, which may be obvious only with more potent series of compounds.

At present, there is no compelling evidence to substantiate any of these hypotheses. TCPOBOP represents the first phenobarbital-like agonist of any substantial potency ($ED_{50} = 4.9 \times 10^{-7}$ mol/kg). For any drug-induced response, the availability of a potent agonist always suggests that the effect is mediated through a receptor. TCPOBOP should provide a valuable tool for further study of the phenobarbital pleiotropic response.

ACKNOWLEDGMENT

We gratefully acknowledge the help of Dr. Norman Drinkwater for writing a computer program for analyzing the dose–response curves and potency ratio.

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