0026-895X/89/060831-08$02.00/0

MOLECULAR PHARMACOLOGY, 35:831-838

Inducible Bilirubin-Degrading System in the Microsomal Fraction of Rat Liver

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Received July 26, 1988; Accepted February 23, 1989

SUMMARY

The hypothesis that treatment of Gunn rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) activates an alternative pathway of bilirubin disposal, involving an induced form of cytochrome P-450 [*Proc. Natl. Acad. Sci. USA* 75:682–685 (1978)], has been investigated by studying the mechanisms of bilirubin oxidation in chemical model systems and in liver microsomal systems *in vitro*. Hematin, copper sulfate, and the iron chelate of EDTA were all active in promoting degradation of bilirubin in the presence of hydrogen peroxide. Evidence was obtained for a microsomal bilirubin-degrading system that could be induced in the liver by treating either rats or chick embryos with TCDD, β-naphthoflavone, or 3,4,3′,4′-tetrachlorobiphenyl (3,4-TCB) *in vivo*. The activity of this system required NADPH and oxygen and was markedly stimulated by addition of 3,4-TCB (a planar polyhalogenated biphenyl) and much less markedly by the nonplanar analogue 2,4,2′,4′-tetrachlorobiphenyl. These two biphenyls were also inhibitory towards the 7-ethoxyresorufin *O*-deethylase activity of the induced microsomes and here again the nonplanar analogue was markedly less active. Dose-response experiments for stimulation of bilirubin breakdown and inhibition of 7-ethoxyresorufin *O*-deethylase activity after addition of 3,4-TCB *in vitro* showed both effects to be caused by similar concentrations of the biphenyl. These results suggest that a polyhalogenated chemical may interact with TCDD-induced microsomes, inhibit their monooxygenase activity, and stimulate production of a bilirubin-degrading species.

Bilirubin, the ultimate product of heme degradation in humans and other mammals, is conjugated in the liver mostly with glucuronic acid before its excretion into the bile (1). Under conditions in which the conjugating ability is impaired because of a genetic defect of the UDP-glucuronyl transferase enzyme, an alternative pathway of bilirubin disposal has been suggested to contribute significantly to the maintenance of bilirubin homeostasis. Schmid and Hammaker (2) were the first to provide evidence for such an alternative pathway both in the Gunn rat and in humans with a similar genetic defect of the conjugating enzymes (the Crigler-Najjar syndrome). Polar oxidative derivatives of bilirubin are excreted in excess under these conditions (3, 4) and the involvement of bilirubin oxidases in this alternative disposal mechanism has been suggested (2, 3); however, the nature of the enzymatic system responsible and its tissue and intracellular location are still obscure.

A poorly defined bilirubin oxidase has been described in the mitochondrial fraction from rat and human brain (5). This oxidase could catalyze the degradation of unconjugated bilirubin to diazo-negative products in the presence of oxygen, but the identity of the oxidative species involved has not yet been defined. Neither has the physiological significance of this system been established. Similarly active systems have been more recently reported for liver (6), again in the mitochondrial fraction, and in the homogenates of the intestine and kidney (7). Kaul *et al.* (8) and Cuypers *et al.* (9) have considered the possible involvement of superoxide anion and of lipid peroxides in bilirubin oxidative breakdown.

An important development in the field has been the demonstration that treatment of Gunn rats with a powerful inducer of liver monooxygenase enzymes (TCDD) lowers the abnormally high levels of blood plasma bilirubin and stimulates bilirubin catabolism to certain polar metabolites (10, 11), an effect more recently extended to the similarly acting inducers 3-methylcholanthrene and β-naphthoflavone (12). These authors have suggested that the microsomal mixed function oxidase of the induced cytochrome P-448 is responsible for the above effects, but no direct evidence that microsomes can catalyze bilirubin degradation has so far been produced.

We have now investigated the role of active oxygen species in bilirubin breakdown with model oxidative systems *in vitro* and found evidence for the involvement of hydrogen peroxide; in addition to H_2O_2, a heme compound or a non-heme form of chelated iron appears to be required for bilirubin oxidation. Evidence has also been obtained for a microsomal bilirubin-

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3,4-TCB, 3,4,3′,4′-tetrachlorobiphenyl; 2,4-TCB, 2,4,2′,4′-tetrachlorobiphenyl; DMSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin-*O*-deethylase.

degrading system, which can be induced by treating either rats or chick embryos with TCDD or similar polyhalogenated inducers *in vivo* and may involve the microsomal cytochrome P-450/NADPH-dependent reductase system.

Materials and Methods

Animals. Rats (body weight, 200–220 g) homozygous for the Gunn genetic trait (13) were obtained from the Royal Free Hospital Medical School (London) and were used after a period of at least 1 week in a temperature-controlled environment on either Labsure 41B or RM expanded No. 3 breeding diet (both diets were obtained from W. Lillico & Son, Ltd., Betchworth, Surrey, UK). No significant differences were found in either the concentration of plasma bilirubin or the enzymatic activity of microsomes isolated from rats on the two diets. Treatment with TCDD involved a single intraperitoneal injection of 10 μg/kg of body weight (7.5 μg/ml in corn oil); control animals received oil alone.

Before treatment and 3 days thereafter, blood was drawn from the tail vein of each individual rat into a heparinized 0.1-ml pipette for bilirubin estimation and an additional sample of blood was analyzed at time of killing. Liver microsomes were prepared from female rats of the Gunn (jaundiced) and LAC:P (Wistar-derived, nonjaundiced) strains, some treated with TCDD (10 or 300 μg/kg) 3 days before killing. The body weight of the LAC:P rats was 160–220 g. The microsomes were isolated by differential centrifugation (14), washed with 1.15% KCl, and stored frozen (at −70°), as a pellet overlaid with 0.5 ml of the KCl solution, until needed. The enzyme systems under study were found to be stable under these conditions of storage for at least 4 months.

Chicken embryos, 16 days of age, of the Rhode Island Red × White Rock strain, were obtained from Orchard Farm (Pinner, Middlesex, UK) and liver microsomes were prepared 24 hr after a single dose of 3,4-TCB [145 μg/embryo (15)] or β-naphthoflavone [3 mg/embryo (16)], the former dissolved in 10 μl of 1,4-dioxan and the latter in 100 μl of DMSO, both given by injection through the air sac into the fluids surrounding the embryo. The livers were perfused *in situ* by injecting 5 ml of ice-cold phosphate-buffered saline (0.14 M NaCl/8 mM sodium phosphate buffer, pH 7.3) through the heart and microsomes were then obtained and stored as described above for rats.

Analytical procedures. Blood plasma bilirubin was usually measured, by a direct spectrophotometric assay, by scanning the spectrum of a dilute plasma solution and correcting for any heme contamination, as suggested by White *et al.* (17). In two experiments the colorimetric method of Michaëlsson (18) was also employed and the results of the two techniques were found to be in very good agreement, whether the Gunn rats had been treated with TCDD or not.

Proteins were measured by the method of Lowry *et al.* (19) and cytochrome P-450, according to Omura and Sato (20).

Source of special chemicals. NADH (type III), NADPH (type III), crystallized human albumin, and bilirubin were obtained from Sigma Chemical Co (Poole, Dorset, UK); hemin was a kind gift from Dr. S. Sassa (The Rockefeller University, New York, NY); 7-ethyoxyresorufin was from Pierce Chemical Co (Rockford, IL); 2,4-TCB was from Analabs (New Haven, CT); 3,4-TCB was from Ultra Scientific (Hope, RI); and β-naphthoflavone was obtained from Aldrich Chemical Co (Gillingham, Dorset, UK). TCDD was synthesized in our laboratories by a method described previously (21).

Experiments with model chemical systems. Bilirubin oxidation by authentic hydrogen peroxide was studied by monitoring the loss of absorbance at 450 nm (approximately the absorption maximum of bilirubin) in disposable plastic cuvettes (incubated in the presence of air in the thermostatically controlled, 28°, compartment of a Cary 2200 spectrophotometer). The incubation mixture contained, in a total volume of 3.5 ml, the following components, with final concentrations in parentheses: Tris·HCl, pH 8.2 (100 mM); bilirubin (10 μM), freshly dissolved and added in DMSO (1.4 μl/ml); KCl (26 mM); and either methemalbumin, copper sulphate, or iron-EDTA chelate as a catalyst. The stock methemalbumin dilution was prepared according to the method of Tenhunen *et al.* (22), using human albumin and contained 2.23 mM hematin; a suitable solution in 1.15% KCl was then added to the incubation mixture to give the final concentration of hematin indicated in the text. Iron-EDTA chelate was prepared by mixing $FeCl_3$ and EDTA in a 1:2.5 molar ratio and the final concentrations used for both iron and chelator are given in the text.

Experiments with isolated liver microsomes. The rate of metabolism of bilirubin by isolated microsomes was studied at 28° in disposable plastic cuvettes incubated in presence of air, as described above for the oxidation in chemical systems. The total volume of the incubation mixture was 3.5 ml and the following components were present (with final concentrations in parentheses): Tris·HCl buffer, pH 8.2 (0.1 M); KCl (26 mM); microsomes from control or induced livers (corresponding to a concentration of cytochrome P-450 ranging from 32 to 260 pmol/ml); DMSO (2.8 μl/ml) with or without the drug to be tested; and, in some experiments, EDTA (2 mM). After a preliminary incubation of 5 min, either NADPH or NADH (73 μM) were added to both sample and reference cuvettes, followed immediately thereafter by addition of bilirubin (a fresh solution in DMSO) to the sample cuvette only. The decrease in absorbance at 450 nm was then followed; rates given refer to the initial rates observed and are expressed as pmol of bilirubin disappearing/min, using a ϵmM = 55.8, obtained experimentally under the conditions of the assay. The EROD activity of microsomes was measured by a slight modification of the method of Burke and Mayer (23), by following the rate of increase of fluorescence in a cuvette incubated in the presence of air in the thermostatically controlled compartment (28°) of a Perkin Elmer LS-5 luminescence spectrometer. The incubation mixture contained, in a total volume of 2.25 ml, the following components: Tris·HCl buffer, pH 7.4 (47.3 mM); 7-ethoxyresorufin (0.4 μM) added in DMSO (4.4 μl/ml of incubation); and microsomes, delivering 7.8 to 32 pmol of cytochrome P-450/ml of incubation. The excitation and emission wavelengths were 522 nm and 586 nm, respectively. After a preliminary incubation of 1 min, the reaction was initiated by addition of NADPH to a final concentration of 125 μM. When the inhibition of EROD activity caused by addition of chlorinated biphenyls was studied, a lower concentration of 7-ethoxyresorufin was also employed (0.125 μM). Initial rates of uninhibited enzyme activity were found to be linearly related to amount of microsomes taken with both concentrations of substrate.

Results and Discussion

Hydrogen peroxide-dependent breakdown of bilirubin in model systems: role of hematin, non-heme iron, and copper. Bilirubin undergoes a very slow autoxidation in air (24) and Brodersen and Bartels (5) have reported a very rapid destruction of bilirubin by hydrogen peroxide in the presence of lactoperoxidase and other hemoproteins. We now show that heme itself is markedly active in catalyzing bilirubin degradation by hydrogen peroxide, a catalytic property shared by copper sulfate and, to a lesser extent, by the iron chelate of EDTA (Table 1). A 160-fold stimulation of the hydrogen peroxide-dependent rate of bilirubin oxidation was obtained with 64 nM hematin, compared with only a 6.7-fold stimulation seen with a much greater molar concentration (57 μM) of Fe-EDTA. In contrast to either hematin, copper sulfate, or Fe-EDTA, EDTA itself, added on its own, decreased the effect of hydrogen peroxide on bilirubin degradation.

It will be noticed from Table 1 that bilirubin breakdown could also be observed in the absence of added hydrogen peroxide; this hydrogen peroxide-independent rate was again inhibited by the addition of EDTA alone and stimulated by the addition of copper sulfate or Fe-EDTA, whereas hematin was inactive in this respect. The marked stimulation of bilirubin

TABLE 1

Oxidation of bilirubin by hydrogen peroxide: role of hematin, Fe-EDTA, and copper

Bilirubin (35 nmol) was added to a spectrophotometric cuvette containing, in a total volume of 3.5 ml, the various components given in Materials and Methods and the further additions listed below. The rate of oxidation of bilirubin was calculated from the decrease in absorbance at 450 nm and the values given refer to the maximum rate observed (averages ± standard errors of the number of observations in parentheses). Recording was started immediately after addition of either H_2O_2 or equivalent volumes of H_2O, except in the experiments with copper, when recording was initiated by addition of copper, and, after a linear rate was established H_2O_2 was added to the same samples to obtain a new rate given below under H_2O_2 (2.5 mM) plus $CuSO_4$ (57 μM). Similar results were obtained when the reaction was started by adding, in quick succession, Fe (or Cu), H_2O_2, and bilirubin (in this order) and the recording was initiated immediately thereafter.

H_2O_2	Other additions	Rate of bilirubin oxidation	Increase in rate attributable to H_2O_2
mM		*pmol/min*	
None	None	24.1 ± 3.5 (3)	
2.5	None	74.7 ± 9.2 (4)[a]	50.6
None	EDTA (142 μM)	11.0 ± 0.3 (3)[b]	
2.5	EDTA (142 μM)	22.2 ± 2.3 (5)[b]	11.2
None	Hematin (64 nM)	27.6 ± 2.2 (3)	
2.5	Hematin (64 nM)	8330.0 ± 111.0 (6)[a]	8300.0
None	Fe (57 μM) in EDTA (142 μM)	147.2 ± 8.2 (5)	
2.5	Fe (57 μM) in EDTA (142 μM)	485.0 ± 19.3 (4)[a]	338.0
2.5	Fe (57 μM) in EDTA (2 mM)	459.0 ± 18.6 (3)	
None	$CuSO_4$ (57 μM)	2590.0 ± 110.0 (5)	
2.5	$CuSO_4$ (57 μM)	8200.0 ± 182.0 (5)[a]	5610.0
2.5	$CuSO_4$ in EDTA (2 mM)	33.0 ± 3.6 (4)[b]	

[a] $p < 0.01$, when compared with corresponding values obtained without H_2O_2.
[b] $p < 0.01$, when compared with corresponding values obtained without EDTA.

breakdown caused by copper (both in the absence and in the presence of exogenous hydrogen peroxide) could be almost completely prevented by EDTA (unlike those caused by iron), suggesting that the EDTA chelate of iron possesses prooxidant activity, whereas the EDTA chelate of copper does not. We conclude that copper sulfate and Fe-EDTA can not only catalyze the degradation of bilirubin in the presence of hydrogen peroxide but also possess bilirubin-degrading activity of their own. Because, on chelation by EDTA, copper loses its bilirubin-degrading potential, the ability of EDTA to depress the basal rates of bilirubin degradation both in the presence and in the absence of hydrogen peroxide (Table 1) may be explained by chelation of trace amounts of copper present in the reagents.

These experiments and those involving liver microsomes (see below) suggest that the degradation of bilirubin may be conveniently used to monitor production and utilization of oxidizing species, such as hydrogen peroxide, both under *in vitro* conditions and possibly also *in vivo*. These findings are in line with the recent proposal (25) that bilirubin possesses peroxide-quenching properties and may have an antioxidant role to play *in vivo*.

Degradation of bilirubin by liver microsomes *in vitro*. TCDD has been reported (10) to decrease the abnormally high plasma concentration of bilirubin in the Gunn rat by an unknown mechanism involving accelerated bilirubin breakdown to a number of polar products, several of which are hydroxylated. We have confirmed this effect of TCDD treatment on plasma bilirubin concentration in Gunn rats, by showing a marked loss of pigment 3 days after a single dose of TCDD to jaundiced rats of both sexes. Plasma bilirubin concentrations (μmol/liter of plasma; average ± SE of at least three observations) were, before treatment, 143 ± 11 and 172 ± 12 in male and female Gunn rats, respectively, compared with corresponding values of 76 ± 11 and 97 ± 13, 3 days after treatment.

Increased production of reduced oxygen species, such as H_2O_2, by an uncoupled cytochrome P-450 system (and subsequent toxic interaction of reduced oxygen with certain pools of liver iron) has been proposed to account for several aspects, among these uroporphyria, of the liver toxicity of TCDD and other poly-halogenated chemicals (26–29). Indirect support for this mechanism, possibly involving interaction of polyhalogenated chemicals with the cytochrome P-450/NADPH-dependent reductase system, has recently been reported (16, 30), by the demonstration that liver microsomes isolated from chick embryos treated with inducers of the TCDD class will rapidly oxidize a porphyrinogen (hexahydroporphyrin) *in vitro*, when a planar polychlorinated biphenyl is also present. Certain pools of iron may contribute to this reaction as catalysts (31).

We now show that liver microsomes from induced chick embryos, incubated with bilirubin in the presence of NADPH, will degrade bilirubin at a markedly increased rate when a planar biphenyl (3,4-TCB) is also added (Table 2). Under these conditions, NADH could not support bilirubin degradation; neither could microsomes from control uninduced chick embryos respond to addition of 3,4-TCB with an increased rate of bilirubin breakdown (Table 2).

Pretreatment of chick embryos with β-naphthoflavone, another inducer of the TCDD class, also increased the ability of liver microsomes to degrade bilirubin in the presence of 3,4-TCB (Table 3). With both types of induced chick liver microsomes, the rate of bilirubin breakdown could be stimulated by EDTA, both in the presence and in the absence of 3,4-TCB, a greater stimulation being obtained in the presence of 3,4-TCB (Table 3). The nonplanar biphenyl 2,4-TCB was also active in stimulating bilirubin degradation by induced microsomes in the presence of NADPH, but its potency was at least 100 times less than that of 3,4-TCB (Table 3), as shown by the much greater concentration of 2,4-TCB required to produce a similar stimulation.

These results suggest that two different actions of 3,4-TCB are involved in the observed degradation of bilirubin: 1) the drug first acts as an inducer of a cytochrome P-450 *in vivo* (an effect also shared by β-naphthoflavone and, in the rat experiments to be described below, by TCDD); and 2) 3,4-TCB can then interact with the induced cytochrome *in vitro* so as to produce a bilirubin-degrading species. The marked difference

TABLE 2

Degradation of bilirubin by liver microsomes obtained from chick embryos: effect of treatment of chick embryos in vivo with 3,4-TCB and of the addition of 3,4-TCB to microsomes from control or pretreated embryos in vitro

The incubation mixture contained, in a total volume of 3.5 ml, the following components (final concentration): Tris·HCl buffer, pH 8.2 (0.1 M), KCl (26 mM), microsomes delivering cytochrome P-450 to a final concentration of 65 pmol/ml, and DMSO (2.8 μl/ml) containing, where indicated, either 3,4-TCB or 2,4-TCB. (The cytochrome P-450 contents of the microsomal preparations are given in Table 5.) The reaction was started by addition of a cofactor, where appropriate, followed immediately by addition of bilirubin to a final concentration of 10 μM. Results are expressed as pmol of bilirubin oxidized/min per nmol of cytochrome P-450 (and per mg of microsomal protein) and are means ± standard errors of the number of observations in parentheses or averages with individual observations in parentheses.

Pretreatment of chick embryos *in vivo*	Cofactor	Drug added	Rate of bilirubin degradation	
			pmol/min/nmol of cytochrome P-450	*pmol/min/mg of protein*
3,4-TCB	None	3,4-TCB, 10 μg	220 ± 5.4 (3)	110 ± 3 (3)
	NADH	None	210 (224, 198)	136 (145, 128)
	NADH	3,4-TCB, 10 μg	270 ± 12.4 (3)	173 ± 8 (3)
3,4-TCB	NADPH	None	474 ± 23 (7)	274 ± 12 (7)[a]
	NADPH	3,4-TCB, 10 μg	2300 ± 140 (8)[b]	1280 ± 108 (8)[b]
	NADPH	2,4-TCB, 10 μg	646 ± 9 (3)[b]	420 ± 5 (3)[b]
None	NADPH	None	444 ± 79 (3)	92 ± 16 (3)
	NADPH	3,4-TCB, 10 μg	366 ± 48 (5)	76 ± 10 (5)

[a] $p < 0.001$, when compared with corresponding values obtained with microsomes from uninduced chick embryos.
[b] $p < 0.001$, when compared with corresponding values obtained without TCB.

TABLE 3

Degradation of bilirubin by liver microsomes obtained from chick embryos pretreated with 3,4-TCB or β-naphthoflavone in vivo: effect of addition of EDTA to the incubation mixture (Experiment 1) and comparison between the effects of adding 3,4-TCB and 2,4-TCB in vitro (Experiment 2)

In Experiment 1, the incubation mixture (3.5 ml) contained Tris-HCl buffer, pH 8.2 (0.1 M), KCl (26 mM), liver microsomes from chick embryos pretreated with 3,4-TCB or β-naphthoflavone (equivalent to a final concentration of cytochrome P-450 of 65 pmol/ml), and, where indicated, EDTA to a final concentration of 2 mM. (The cytochrome P-450 contents of the microsomal preparations are given in Table 5.) After preincubation, NADPH was added to all samples, immediately followed by bilirubin (35 nmol), and the rate of bilirubin degradation was then monitored. In Experiment 2, similar conditions were adopted, except that EDTA was present in all samples and the effects of different amounts of 3,4-TCB and 2,4-TCB were compared. Results given are averages ± standard errors of the number of observations in parentheses or averages with individual observations in parentheses.

Pretreatment of chick embryos *in vivo*	Drug added	Further addition	Rate of bilirubin degradation	Stimulation caused by	
				EDTA	TCB
			pmol/min/nmol of cytochrome P-450	%	
Experiment 1					
3,4-TCB	None	None	420 ± 20 (3)	74	
	None	EDTA	730 ± 40 (7)[a]		
	3,4-TCB, 10 μg	None	2360 ± 300 (3)[b]	84	
	3,4-TCB, 10 μg	EDTA	4350 ± 200 (4)[a,b]		
β-Naphthoflavone	None	None	760 ± 100 (3)	45	
	None	EDTA	1100 ± 140 (3)[a]		
	3,4-TCB, 1 μg	None	1820 ± 160 (3)[b]	145	
	3,4-TCB, 1 μg	EDTA	4480 ± 370 (3)[a,b]		
Experiment 2					
3,4-TCB	None	EDTA	930 (770, 1090)		
	3,4-TCB, 0.01 μg	EDTA	1600 (1640, 1560)		72
	3,4-TCB, 0.1 μg	EDTA	3040 (3080, 3000)		227
	3,4-TCB, 1.0 μg	EDTA	3920 (4040, 3800)		320
	3,4-TCB, 10.0 μg	EDTA	4150 (4120, 4180)		346
	2,4-TCB, 1.0 μg	EDTA	1400 (1550, 1250)		50
	2,4-TCB, 10.0 μg	EDTA	1600 (1700, 1500)		72

[a] $p < 0.01$, when compared with corresponding values obtained without EDTA.
[b] $p < 0.01$, when compared with corresponding values obtained without 3,4-TCB.

between the two biphenyls in their ability to stimulate the rate of bilirubin breakdown probably reflects a difference in their affinity for a binding site in the induced microsomes, with the planar analogue (3,4-TCB) exhibiting greater affinity. Further support for this view is offered by the results of the effect of two biphenyls on the activity of EROD (see below). The ability of EDTA to stimulate further the rate of bilirubin breakdown (shown in Table 3) is not understood. Previous work has shown that ferritin iron can be mobilized in a prooxidant form by EDTA (31), but there is as yet no evidence that a mobilized pool of iron is involved in the microsomal degradation of bilirubin. This aspect should be further explored.

We next turned to consider whether treatment of Gunn rats with TCDD, known to promote accelerated disposal of bilirubin, as shown by the marked loss of plasma bilirubin *in vivo*, could also stimulate bilirubin breakdown by isolated rat liver microsomes *in vitro*. Inclusion of EDTA in the incubation mixture was routinely carried out with rat microsomes to prevent inactivation of cytochrome P-450 due to NADPH-dependent lipid peroxidation (32), processes that do not appear to take place with chick microsomes incubated with NADPH (33). Table 4 (Experiment 1) shows that microsomes isolated from Gunn rats that had been treated with TCDD *in vivo* had a greater bilirubin-degrading potential than microsomes from

TABLE 4

Effect of treatment of female rats of the Gunn strain with TCDD in vivo on the bilirubin-degrading activity of their liver microsomes in vitro: comparison between Gunn rats and similarly treated LAC:P rats and effect of adding 3,4-TCB or 2,4-TCB to the microsomes in vitro

The incubation mixture contained, in a total volume of 3.5 ml, the following components (final concentrations): Tris·HCl buffer, pH 8.2 (0.1 M); KCl (26 mM); EDTA (2 mM); DMSO (2.8 μl/ml) that contained, where indicated, 3,4-TCB or 2,4-TCB; and microsomes from either Gunn or LAC:P female rats (pretreated with TCDD, 10 μg/kg, in some cases) equivalent to 65 pmol of cytochrome P-450/ml. (The cytochrome P-450 contents of the microsomal preparations are given in Table 5.) Values are given as rates per nmol of cytochrome P-450 or per mg of microsomal protein and are means ± standard errors of the number of observations in parentheses. The reaction was initiated by addition of NADPH and bilirubin (10 μM) in all cases.

Strain of rat	Pretreatment of rat *in vivo*	Drug added	Rate of bilirubin degradation	
			pmol/min/nmol of cytochrome P-450	*pmol/min/mg of protein*
Gunn	None	None	258 ± 19 (4)	148 ± 13 (4)
	None	3,4-TCB, 10 μg	270 ± 26 (4)	156 ± 20 (4)
	TCDD	None	473 ± 18 (8)[a]	500 ± 20 (8)[a]
	TCDD	3,4-TCB, 10 μg	890 ± 52 (8)[b]	938 ± 40 (8)[b]
LAC:P	TCDD	None	290 ± 20 (12)	390 ± 40 (12)
	TCDD	3,4-TCB, 0.1 μg	540 ± 38 (6)[b]	728 ± 60 (6)[b]
	TCDD	3,4-TCB, 1.0 μg	724 ± 28 (6)[b]	950 ± 65 (6)[b]
	TCDD	3,4-TCB, 10.0 μg	704 ± 50 (5)[b]	960 ± 100 (5)[b]
	TCDD	None	250 ± 13 (6)	313 ± 17 (6)
	TCDD	2,4-TCB, 1.0 μg	365 ± 30 (4)[b]	460 ± 40 (4)[b]
	TCDD	2,4-TCB, 10.0 μg	425 ± 30 (4)[b]	540 ± 40 (4)[b]

[a] $p < 0.001$, when compared with corresponding values from uninduced rats.
[b] $p < 0.01$, when compared with corresponding values obtained in the absence of TCB.

untreated control rats, whether this was expressed as rate of degradation/nmol of cytochrome P-450 or, more markedly, as rate/mg of microsomal protein; addition of saturating amounts of 3,4-TCB could double the rate of bilirubin degradation in the induced system but had no effect with the uninduced microsomes. Very similar results were obtained with microsomes isolated from TCDD-treated rats of the LAC:P (nonjaundiced) strain (Table 4, Experiment 2). The nonplanar biphenyl 2,4-TCB was also active in stimulating bilirubin degradation by induced microsomes from LAC:P rats, but its potency was approximately 100 times less than that of 3,4-TCB (Table 4). A 30-fold greater dose of TCDD to female LAC:P rats increased the bilirubin-degrading activity of their liver microsomes (as compared with rats induced with the smaller TCDD dose) but the percentage stimulation caused by addition of 10 μg of 3,4-TCB to the incubation mixture remained unchanged. Rates (obtained under the same conditions as in the experiments of Table 4 and again expressed as pmol of bilirubin metabolized/min/nmol of cytochrome P-450) were as follows: in the absence of 3,4-TCB, 433 ± 30 (6); in the presence of 3,4-TCB, 1020 ± 25 (6).

The requirement for oxygen in the NADPH-dependent degradation of bilirubin by microsomes was also investigated. When TCDD-induced microsomes from Gunn rats (equivalent to 260 pmol of cytochrome P-450/ml) were incubated in the presence of NADPH and 3,4-TCB in stoppered cuvettes that had been made hypoxic by flushing with nitrogen, a significant reduction in the rate of bilirubin degradation was found, compared with samples incubated in air-saturated buffer, and the reduction in rate was almost completely reversed by allowing air into the cuvette by vigorous mixing. Results obtained, expressed as pmol of bilirubin degraded/min [average ± SE (number of observations)], were as follows: (a) samples incubated in air, 590 ± 19 (5); (b) samples incubated under N_2, 290 ± 16 (3); (c) samples b, above, after mixing with air, 540 ± 26 (3). (Values in b differed from values in both a and c; $p < 0.01$). More rigorous anaerobic conditions would probably be required for greater reduction of the rate of bilirubin degradation, but this was not attempted. These findings suggest an oxidative mechanism for the microsomal degradation of bilirubin that is stimulated by TCDD pretreatment, in keeping with the finding (10) that a number of polar bilirubin derivatives, some hydroxylated, are excreted in excess by Gunn rats after TCDD treatment. In support of an oxidative mechanism are also the previous findings that liver microsomes from animals treated with polyhalogenated chemicals produce increased amounts of reduced oxygen metabolites (34) and will catalyze the oxidation of porphyrinogens (hexahydroporphyrins) in the presence of NADPH, (16, 30). However, we cannot provide a satisfactory explanation as to why 3,4-TCB stimulated bilirubin degradation with microsomes from both chick embryos and rats (Tables 3 and 4), whereas in previous work (16) oxidation of porphyrinogens to porphyrins was stimulated by 3,4-TCB only with chick microsomes. The nature of the active species involved in these microsomal reactions is still unknown. Hydrogen peroxide is produced by microsomes incubated aerobically with NADPH, especially in the presence of drugs that act as uncouplers of microsomal electron transport (35) and, in chemical systems, authentic hydrogen peroxide will readily cause a loss of bilirubin (Table 1) or oxidize porphyrinogens (31) in the presence of a suitable catalyst. However there is as yet no data to indicate the involvement of hydrogen peroxide in the bilirubin-degrading and porphyrinogen-oxidizing reactions of the induced microsomes.

Effect of tetrachlorobiphenyls on the EROD activity of isolated liver microsomes. In the last set of experiments, the effect of the two biphenyls on the EROD activity was investigated in an attempt to obtain more direct evidence that these compounds may modify the activity of the induced cytochrome P-450. The hypothesis that has been proposed to account for certain aspects of the toxicity of TCDD and similar inducers (26–29) envisages these compounds binding the active site of the induced cytochrome P-450 as poor substrates, so that electron flow from NADPH to oxygen is facilitated and partially reduced oxygen species are produced in excess. These could then be responsible for oxidation of important target

TABLE 5

Cytochrome P-450 content and EROD activity of the microsomal preparations used in this work

Results are given as averages ± standard errors of the number of observations in parentheses or averages with individual observations, each obtained with a different preparation of microsomes isolated from the liver of an individual rat or from the pooled livers of 6–12 chick embryos. The EROD activity was measured with a concentration of 7-ethoxyresorufin of 400 nM.

Species	Treatment *in vivo*	Cytochrome P-450	EROD activity	
		nmol/mg of microsomal protein	*pmol/min/nmol of cytochrome P-450*	*pmol/min/mg of microsomal protein*
Chick embryo	None	0.25 ± 0.01 (4)	1.94	8.8
Chick embryo	3,4-TCB	0.59 ± 0.05 (3)	612 (610, 614)	392 (385, 400)
Chick embryo	β-Naphthoflavone	0.42	705	296
Gunn rat	None	0.64 ± 0.05 (4)	40.0	56.5
Gunn rat	TCDD, 10 μg/kg	1.20 ± 0.06 (5)	1550 (1460, 1650)	1790 (1680, 1900)
LAC:P rat	TCDD, 10 μg/kg	1.27 ± 0.13 (3)	1130 ± 25 (3)	1410 ± 42 (3)
LAC:P rat	TCDD, 300 μg/kg	1.30 (1.23, 1.38)	2020 (2016, 2028)	2640 (2480, 2800)

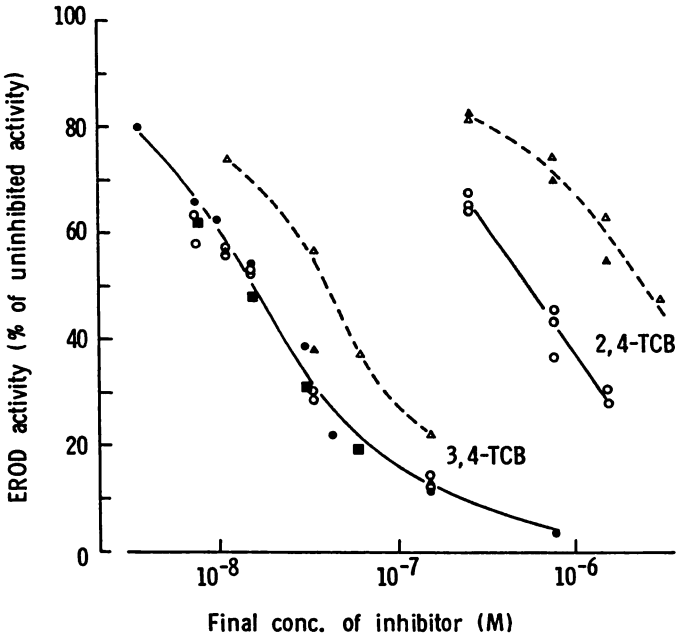

Fig. 1. Log dose-response curves for EROD activity obtained with microsomes from induced rats and chick embryos, in the presence of various concentrations of either 3,4-TCB or 2,4-TCB. The rate of EROD was determined as indicated in Materials and Methods, using a concentration of ethoxyresorufin of 0.125 μM and microsomes corresponding to 7.8 pmol of cytochrome P-448/ml of incubation mixture, except with chick microsomes induced with β-naphthoflavone (18.0 pmol/ml). The enzyme was preincubated with DMSO (2.2 μl/ml) containing various concentrations of either 3,4- or 2,4-TCB for 1 min, before addition of 7-ethoxyresorufin; NADPH was added 1 min thereafter. The source/treatments of the microsomes tested were Gunn/TCDD (●); LAC:P/TCDD, 10 μg/kg (○); LAC:P/TCDD, 300 μg/kg (■); chick/3,4-TCB (▲); and chick/β-naphthoflavone (△).

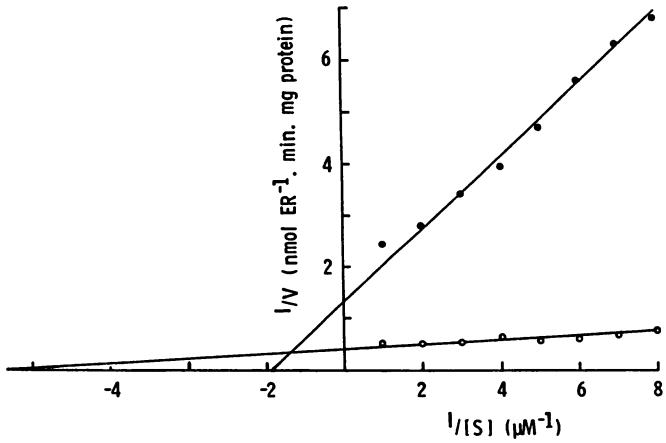

Fig. 2. Double-reciprocal plot of the EROD (*ER*) activity of microsomes from TCDD-induced Gunn rats in the absence (○) and the presence (●) of 3,4-TCB, at a final concentration of 150 nM. The EROD activity was determined as described in the legend to Fig. 1, using the concentrations of 7-ethoxyresorufin indicated. Values of V_{max} and K_m were 2.7 min^{-1} and 120 nM for the uninhibited enzyme and 0.76 min^{-1} and 530 nM in presence of 3,4-TCB.

molecules, among these porphyrinogens and as suggested by this present work also bilirubin.

If degradation of bilirubin was in fact related to occupancy of the cytochrome P-450 active site by the polyhalogenated chemicals, one might expect these drugs to inhibit the metabolism of 7-ethoxyresorufin, a selective substrate of the induced cytochrome P-450; in addition, the extent of EROD inhibition might be expected to show some correlation with the bilirubin degradation reaction. Both these predictions are to a large extent borne out experimentally, as shown below.

3,4-TCB and 2,4-TCB were found to be inhibitory with respect to EROD activity of induced microsomes from chick embryos and rats of both strains studied. The log dose-response curve of the inhibition obtained with 3,4-TCB and microsomes from Gunn rats induced with TCDD is shown in Fig. 1, together with the parallel line segments of the dose-response curves obtained for both 3,4-TCB and 2,4-TCB with microsomes from chick embryos and LAC:P rats given two different doses of TCDD. The results indicate that 3,4-TCB is between 35- and 160-fold more potent than 2,4-TCB as an inhibitor of EROD, potency ratios similar to those found for the stimulation of bilirubin degradation by the two biphenyls (see Tables 3 and 4) and, in a previous study (16), also for the stimulation of porphyrinogen oxidation by the two biphenyls. For example, doses producing 50% inhibition of EROD activity were as follows: LAC:P rats—3,4-TCB, 15.4 nM or 4.5 ng/ml; 2,4-TCB, 540 nM or 158 ng/ml; chick induced with 3,4-TCB—3,4-TCB, 15.4 nM; 2,4-TCB, 2.46 μM. The results of Fig. 1 also show that the biphenyls, while varying considerably in potency as inhibitors of EROD activity, displayed similar efficacy, a point of some interest in view of the suggestion (16, 27, 30) that, on interaction with the microsomal system, these compounds may lead to oxidation of uroporphyrinogen and induction of uroporphyria. Marks (36) has previously commented on the fact that the nonplanar biphenyls (such as 2,4-TCB) have less potency than, but similar efficacy to, the planar congeners (like 3,4-TCB) in inducing uroporphyria in chicken embryo hepatocytes in culture.

In one experiment, an attempt was made to correlate the extent of EROD inhibition with the degree of stimulation of bilirubin degradation, using the same preparation of microsomes from chick embryos pretreated with β-naphthoflavone *in vivo* and different final concentrations of 3,4-TCB. The experimental conditions followed for the two assays were those described in the legends to Fig. 1 and Table 3 (EDTA omitted), respectively, except that a concentration of 7-ethoxyresorufin of 0.4 μM was used to measure the EROD activity and cytochrome P-450 was 32.4 pmol/ml in both assays. The following inhibitions of EROD activity and stimulations of bilirubin degradation (both expressed as percentages of maximum effects observed) were found, given in parentheses, in this order, for each 3,4-TCB concentration tested: 15.4 nM, 10%, 8%; 30.8 nM, 22%, 34%; 60 nM, 32%, 61%; 500 nM, 89%, 100%. Therefore, over the whole range examined, the two effects were observed concurrently and were affected similarly (although not identically) by variation in the concentration of the biphenyl.

A double-reciprocal plot of the EROD activity of microsomes from TCDD-induced Gunn rats against substrate concentration is given in Fig. 2, both for the uninhibited enzyme and for the enzyme inhibited by 3,4-TCB (final concentration, 44 ng/ml or 152 nM). The results cannot be interpreted in terms of a purely competitive or noncompetitive type of inhibition, but a competitive component is suggested by the increase in the apparent K_m value, seen in the presence of the inhibitor (from 120 to 530 nM). Taken as a whole, these findings would, therefore, suggest that inhibition of EROD activity and stimulation of bilirubin degradation by biphenyls may be related phenomena and, further, suggest [together with binding data (37) discussed below] that both these effects may stem from an interaction of the biphenyls with an induced form of cytochrome P-450.

β-Naphthoflavone, polyhalogenated biphenyls, and TCDD cause accumulation in rat liver microsomes of at least two distinct forms of cytochrome P-450, P-450c and P-450d,[2] the former representing usually 45–70% of the total cytochrome P-450 and the latter 18–24% (39, 40). Voorman and Aust (37) have reported that, when rats were induced with a single dose of either TCDD or a polybrominated biphenyl, substantial binding of these inducers to cytochrome P-450d, but not to cytochrome P-450c, could be detected 3 days later, after purification of the cytochromes or immunoprecipitation with the appropriate specific antibody. Voorman and Aust (37) also showed that several polybrominated biphenyls added *in vitro* to purified cytochrome P-450d were powerful inhibitors of its estradiol 2-hydroxylase activity; the inhibitory activity decreased with increasing *ortho*-substitution of the biphenyl, a finding reminiscent of the less marked activity of 2,4-TCB (an *ortho*-substituted biphenyl) in promoting degradation of bilirubin (as compared with 3,4-TCB; this work) and again suggesting that planar biphenyls bind to the active site of the induced cytochrome(s) with greater affinity. Therefore, cytochrome P-450d could be the site where 3,4-TCB interacts to produce a bilirubin-degrading species. However, we have now shown that 3,4-TCB is a very potent inhibitor of EROD activity, a reaction catalyzed by cytochrome P-450c in preference to cytochrome P-450d. Taking into account the EROD specific activity of the two purified cytochromes (39, 41, 42) and considering the relative abundance of the two cytochromes in induced liver microsomes, both favoring cytochrome P-450c in rats of both sexes (43), it can be calculated that most of the EROD activity of the TCDD-induced rat microsomes must have been accounted for, in our experiments, by cytochrome P-450c. This suggests strongly that the biphenyls can also bind the active site of cytochrome P-450c, a conclusion supported by the finding (44) that this cytochrome can metabolize 3,4-TCB. Therefore, the possibility that cytochrome P-450c may be involved in bilirubin degradation cannot be excluded. In a recent paper (12) an increase in the "constitutive" level of cytochrome P-450c has been reported in newborn jaundiced Gunn rats, homozygous for the recessive trait (compared with their nonjaundiced heterozygous controls). These authors have suggested a role for this cytochrome in the maintenance of normal bilirubin homeostasis in congenitally jaundiced rats, also in the absence of an exogenous inducer such as TCDD. However, the finding (12) that the difference in cytochrome P-450c levels between jaundiced and nonjaundiced Gunn rats became less prominent in adults is not understood. More direct evidence for the involvement of cytochrome P-450 in bilirubin degradation (both in the presence and the absence of an exogenous inducer) and the relative contributions of cytochromes P-450c and d should be obtained by employing antibodies selectively inhibitory to the cytochromes, as shown by Sinclair *et al.* (30) for drug-dependent porphyrinogen oxidation.

In conclusion, we have provided evidence in this work for a bilirubin-degrading system, which can be induced in the microsomal fraction of the liver homogenate by treatment with TCDD *in vivo* and further stimulated by addition of 3,4-TCB to the microsomes *in vitro*. The effect of 3,4-TCB probably involves binding to the induced microsomes leading to loss of monooxygenase activity and production of a bilirubin-degrading species. These observations provide a plausible mechanism for the alternative pathway of bilirubin metabolism that can be activated by TCDD treatment (10). More work is necessary, however, to establish whether an inducible cytochrome P-450 is involved and whether reactive oxygen species are responsible for bilirubin degradation, as has been suggested (16, 26–29) for oxidation of uroporphyrinogen and induction of uroporphyria by polyhalogenated chemicals.

[2] According to a recently proposed nomenclature (38), these forms are also known as cytochrome P-450 IA1 and IA2, respectively.

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