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Experimental Hepatic Porphyria Induced by Polychlorinated Biphenyls[1]

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Experimental Hepatic Porphyria Induced by Polychlorinated Biphenyls. GOLDSTEIN, J. A., HICKMAN, P. AND JUE, D. L. (1974). *Toxicol. Appl. Pharmacol.* 27, 437–448. Aroclor 1254, which consists of a mixture of polychlorinated biphenyls (PCBs) containing 54% chlorine, produced an experimental hepatic porphyria in rats resembling hexachlorobenzene poisoning and human porphyria cutanea tarda. The PCB-induced porphyria is characterized by delayed development, increased excretion of urinary uroporphyrins, accumulation of 8- and 7-carboxyporphyrins in the liver and increased drug-metabolizing capacity of the liver. Cytochrome P-450 and microsomal heme were increased maximally at 1 week, in the absence of an increase in the rate-limiting enzyme in heme synthesis, δ-aminolevulinic acid (ALA) synthetase. Induction of ALA synthetase and porphyria occurred later, after 2–7 months' exposure to PCBs. No induction of ALA synthetase could be demonstrated prior to the onset of porphyria. Marked induction of ALA synthetase occurred 5 hr after large single doses of Aroclor 1254; however, the doses required were larger than those used to produce porphyria when administered chronically, and induction appeared to be related to the marked increase in cytochrome P-450 seen 24 hr after administration of the drug.

Previously, only hexachlorobenzene, other chlorinated benzenes and chlorinated phenols were known to produce a delayed type of hepatic porphyria resembling human porphyria cutanea tarda. Hexachlorobenzene-induced porphyria is characterized by a delayed onset of action, massive increases in the excretion of 8- and 7-carboxyporphyrins in the urine and accumulation of uroporphyrins in the liver (Ockner and Schmid, 1961; San Martin de Viale *et al.*, 1970). In contrast, porphyrogenic compounds such as allylisopropylacetamide (AIA) produce increased excretion of porphyrins and their precursors within a few hours (De Matteis and Prior, 1962).

Many porphyrogenic chemicals such as AIA apparently cause porphyria by inducing hepatic δ-aminolevulinic acid (ALA) synthetase, the rate-limiting enzyme in heme synthesis (Granick and Urata, 1963; Granick, 1966). The increase is accompanied by a decrease in cytochrome P-450, the terminal cytochrome involved in drug oxidations,

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15

MONS 081910

pellet was stored overnight at 0°C and used for determination of cytochrome P-450 and protoheme.

Assay of ALA synthetase. ALA synthetase was assayed in liver homogenates by the method of Marver *et al.* (1966a). The incubation mixture contained 0.5 ml of homogenate, 200 μmol of glycine, 12.5 μmol of EDTA and 150 μmol of tris-HCl buffer, pH 7.2 in a final volume of 2 ml. After incubation for 30 min at 37° with shaking, the reaction was stopped with 1 ml of 0.3 M trichloroacetic acid. The protein-free supernatants from 7 to 9 flasks were pooled, and ALA was estimated after column chromatography as described below (Marver *et al.*, 1966b).

Microsomal cytochromes and protein. Microsomal cytochrome P-450 and protoheme were measured by the methods of Omura and Sato (1964a,b) using a Cary 14 recording spectrophotometer. Microsomal protein was measured by the method of Lowry *et al.* (1951) using crystalline albumin as the standard.

Assay of ALA, porphobilinogen, and porphyrins.[4] Urinary ALA and porphobilinogen (PBG) were separated by column chromatography and the eluates were assayed after mixing with an equal volume of Ehrlich reagent (Marver *et al.*, 1966b) by the absorbance measured at 556 nm (Mauzerall and Granick, 1956). Urinary porphyrins were routinely estimated in 1- or 2-ml aliquots of urine by a slight modification of the method of Schwartz *et al.* (1951). Briefly, porphyrins were extracted into ethyl acetate at pH 3.1, the uroporphyrin fraction was extracted 4 times with 10% sodium acetate, adsorbed on a 2.5 × 1 cm column of aluminum oxide, washed with 10% sodium acetate and 3% acetic acid, and eluted with 1.5 N HCl containing 1% methanol. The coproporphyrin was then extracted from the ethyl acetate with 1.5 N HCl. Values were estimated with an Aminco fluorometer, with respect to standards of uroporphyrin I and coproporphyrin I. Under these conditions, daily recoveries of coproporphyrin I averaged 100% with less than 1% found in the uroporphyrin fraction, and recoveries of uroporphyrin I averaged 66%, less than 0.2% being found in the coproporphyrin fraction. Values were corrected for recoveries. The only modifications were the substitution of 10% sodium acetate washes for 3% sodium acetate which gave much better separation of uroporphyrin without affecting coproporphyrin recoveries, the addition of 1% methanol to the 1.5 N HCl used to elute uroporphyrin from the aluminum oxide column and pre-elution of the aluminum oxide column with 1 ml of the eluent.

Liver porphyrins and some samples of urine were methylated, extracted into chloroform, separated by thin-layer chromatography and eluted with chloroform (Doss, 1967). The porphyrin methyl esters in the eluate were estimated by spectrophotometry with a Cary 14 spectrophotometer (Dresel and Falk, 1956) or fluorometry when the amount of porphyrin was too small for spectrophotometric determination.

Results were analyzed by the Student *t* test.

RESULTS

Urinary Porphyrins and Porphyrin Precursors

Figure 1 shows that 4 rats fed 100 ppm Aroclor 1254 became porphyric after a delay of 2–7 months. After this time, urinary porphyrin excretion increased rapidly. The most

[4] δ-Aminolevulinic acid, porphobilinogen, coproporphyrin I and uroporphyrin I were obtained from Sigma Chemical Co., St. Louis, Missouri.

MONS 081911

and the accumulation of breakdown products of heme in the liver (Abritti and De Matteis, 1971). Abritti and De Matteis (1971) and Levin *et al.* (1972) have proposed that destruction of cytochrome P-450 may be related to the porphyrogenic action of these chemicals and have shown that only allyl-containing analogs of AIA and barbiturates induce ALA synthetase and reduce cytochrome P-450. Conversely, a number of xenobiotics including phenobarbital increase liver content of cytochrome P-450, resulting in increased ability to metabolize drugs (Conney, 1967). It has been suggested that ALA synthetase induction may be necessary for the increase in cytochrome P-450. However, most xenobiotics produce large increases in cytochrome P-450 and negligible or moderate increases in ALA synthetase (Wada *et al.*, 1968; Marver, 1967; De Matteis and Gibbs, 1972).

Polychlorinated biphenyls (PCBs) are widespread contaminants of the environment and have been detected in samples of human tissue as well as a variety of wildlife (Risebrough *et al.*, 1968; Yobbs, 1972). Several commercial PCBs have been reported to increase tissue fluorescence in rats, chicks and rabbits and to increase excretion of a porphyrin fraction in the feces of chicks and rabbits (Vos *et al.*, 1970, 1971, Vos and Notenboom-Ram, 1972). However, no detailed study of the porphyria induced by PCBs has been reported.

In the present study, porphyrins and their precursors were identified and quantitated in liver and urine of rats fed a commercial mixture of PCBs. The time course of the porphyria was determined, and the relationship between ALA synthetase, cytochrome P-450 and porphyria was studied to evaluate the role of ALA synthetase in PCB-induced porphyria.

METHODS

Animals. One-month-old female Sherman rats were fed ad libitum ground Purina laboratory chow containing 0 or 100 ppm Aroclor 1254[3] (approximately 7.9 mg kg/day), a commercial mixture of PCBs containing 54% chlorine by weight. Ninety-six rats were divided into 20 groups of 4–6 each, fed 0 or 100 ppm Aroclor 1254, and sacrificed by decapitation after 1, 2, 3, and 7 days and 1, 2, 3, 4, 7 and 13 months. Livers were removed for assay of ALA synthetase, cytochrome P-450, microsomal protein and liver porphyrin content as described below. Urine was collected in metabolic cages for the 24-hr period preceding sacrifice in groups sacrificed at 7 days, 1, 2, 3, 4, 7 and 13 months.

For single dose studies, 36 weanling female rats were starved 24 hr before administration by gastric intubation of a single dose of 0, 5, 10, 100 or 500 mg/kg Aroclor 1254 dissolved in peanut oil, and food was withheld until sacrifice 5 or 24 hr later. Additional rats were dosed with 0 (6 rats) or 1000 mg/kg Aroclor 1254 (6 rats), and urine was collected 48–76 hr and 4 months after dosing.

Liver. Livers were minced, one portion was homogenized with 0.9% NaCl containing 0.5 mM EDTA and 10 mM Tris-HCl buffer for the assay of ALA synthetase. The remainder of the liver was homogenized in 1.15% KCl, a portion utilized for estimation of porphyrins, and the rest was centrifuged at 9000 *g* for 20 min. The 9000 *g* supernatant was then centrifuged for 1 hr at 100,000 *g* in a Beckman model L-2 ultracentrifuge, the microsomal pellet was washed once in 1.15% KCl and resuspended in 1.15% KCl containing 0.1 M K_2HPO_4–KH_2PO_4 buffer, pH 7.4, and 25% glycerol. The resuspended

[3] Aroclor 1254 was a gift from Monsanto, St. Louis, Missouri.

MONS 081912

dramatic increase was in the uroporphyrin fraction. Uroporphyrin excretion was elevated a maximum of 540-fold (588 μg/day vs 1.1 ± 0.2 μg/day for controls), coproporphyrin 27-fold (86 μg/day vs 3.2 ± 1.4 for controls, PBG 50-fold (1266 μg/day vs 24.3 ± 5.5 for controls) and ALA 18-fold (334 μg/day vs 18.3 ± 4.8 for controls)

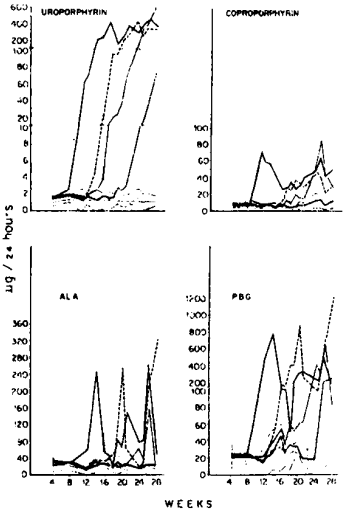

FIG. 1. Effect of 100 ppm Aroclor 1254 on urinary excretion of uroporphyrin, coproporphyrin, ALA and PBG in the female rat. The shaded area represents the 99% confidence limits of the mean for control rats. Each line represents urinary excretion in a single treated rat with time.

The increase in uroporphyrin was the first and most consistent sign of porphyria; variable elevation in coproporphyrin and ALA occurred several weeks after the onset of uroporphyrinuria. An increase in urinary PBG occurred with or slightly later than the increase in uroporphyrin.

More detailed analysis of urinary porphyrins by thin-layer chromatography confirmed the major porphyrin in control rat urine as coproporphyrin (81%), with lesser amounts of 8- and 7-carboxyporphyrins (14%) (Table 1). In contrast, the major porphyrins found in urine from Aroclor-treated rats were 8-carboxyporphyrin (73%

MONS 081913

TABLE 1

COMPOSITION OF URINARY PORPHYRINS IN RATS FED 100 PPM AROCLOR 1254 FOR 1 YEAR*

Porphyrin	Control	Aroclor	Control	Aroclor
	μg/24 hr		% of total	
4-COOH	0.87 ± 0.14	10.5 ± 1.4	81%	5%
5-COOH	0.06 ± 0.01	3.0 ± 0.6	6%	1%
6-COOH	ND	9.6 ± 2.6	0%	4%
7-COOH	0.13 ± 0.03	11.7 ± 13.8	14%	16%
8-COOH		151.1 ± 17.7		73%

* Urinary porphyrins were methylated and analyzed by thin-layer chromatography. Results are given as the mean ± SE.

and 7-carboxyporphyrin (16%). There was a 10-fold increase in the excretion of coproporphyrin, but the increase in uroporphyrin excretion was 1400-fold. There were also substantial increases in the amounts of 5- and 6-carboxylic porphyrins. Doss *et al.* (1971) found a similar reversal of the coproporphyrin/uroporphyrin ratio in urine of patients with porphyria cutanea tarda.

Liver ALA synthetase, Cytochrome P-450 and Porphyrins

ALA synthetase, cytochrome P-450, microsomal heme and liver porphyrin content were determined in rats sacrificed periodically between 1 day and 13 months of treatment. Urinary porphyrins were determined 24 hr prior to sacrifice. There was a slight (30–60%) increase in the excretion of urinary porphyrins after only 1 week of treatment; however, the increase was slight during the first month (Table 2). Rats became grossly porphyric between 2 and 4 months. There was good correlation between urinary porphyrins and liver porphyrins (Table 2 versus Fig. 2). Only a trace of porphyrins was found in liver samples from control rats (<0.1 μg/liver) after 1 month (0.3 μg/liver) of treatment. However, the increase was appreciable only after 2 months feeding.

ALA synthetase was not increased during the first month of exposure (Fig. 2). After this time, ALA synthetase was elevated 3- to 6-fold in porphyric rats, but was normal in nonporphyric Aroclor-treated rats. Since some porphyrin accumulation occurred in the liver prior to a demonstrable increase in ALA synthetase activity, it is probable that ALA synthetase induction was secondary to the porphyria. There was no correlation between ALA synthetase activity and cytochrome P-450 or microsomal heme. Cytochrome P-450 and microsomal heme were increased throughout the study, but the increase was maximal at 1 week, despite the absence of an increase in the rate-limiting enzyme in heme synthesis, ALA synthetase. Liver weight and microsomal protein were also increased maximally at 1 week (Table 3).

At 4 months, the porphyrin concentration in the livers of rats fed 100 ppm Aroclor 1254 was 1,410 ± 150 μg/g of liver vs <0.1 μg/g for control rats. Only uroporphyrins (75% 8-carboxy and 25% 7-carboxyporphyrins) could be detected in the livers of PCB-treated rats. No fluorescence was observed in the bone marrow. All rats appeared healthy throughout the feeding period; there were no deaths in the rats fed Aroclor 1254 and weight gain was normal.

MONS 081914

TABLE 2

EFFECT OF 100 PPM AROCLOR 1254 ON URINARY PORPHYRIN EXCRETION (μg/24 HR)[a]

Treatment		ALA	PBG	Coproporphyrin	Uroporphyrin
1 Week	Control (4)	22.7 ± 1.3	15.6 ± 0.8	4.5 ± 0.3	1.37 ± 0.07
	Treated (4)	28.0 ± 1.7	15.5 ± 0.9	8.3 ± 1.1[b]	1.86 ± 0.13[b]
1 Month	Control (4)	21.6 ± 1.2	20.6 ± 0.9	3.7 ± 0.3	1.02 ± 0.5
	Treated (4)	32.6 ± 4.6	23.0 ± 0.7	5.8 ± 0.4[b]	1.35 ± 0.04[b]
2 Months	Control (4)	19.7 ± 1.5	18.2 ± 1.5	2.9 ± 0.3	1.08 ± 0.05
	Treated (4)	29.1 ± 4.4 (3)	19.5 ± 2.1 (3)	7.7 ± 2.2 (3)[b]	1.59 ± 0.09 (3)[b]
		43.7 (1)[c]	94.5 (1)[c]	29.5 (1)[c]	11.3(1)[c]
3 Months	Control (4)	20.0 ± 1.4	21.8 ± 1.8	5.9 ± 1.1	2.97 ± 0.61
	Treated (4)	24.1 ± 2.5 (2)	28.0 ± 2.8 (2)	5.7 0 (2)	3.75 ± 1.1 (2)
		268 (1)[c]	650 (1)[c]	167 (1)[c]	337(1)[c]
		30 (1)[c]	41 (1)[c]	47 (1)[c]	5.4 (1)[c]
4 Months	Control (4)	29.6 ± 0.3	25.2 ± 1.7	2.1 ± 0.3	0.85 ±0.04
	Treated (4)[c]	37.5 ± 6.8	38.2 ± 13.7	10.2 ± 1.7[b]	264 ± 121[b]
7 Months	Control (6)	16.8 ± 0.8	21.1 ± 1.1	3.5 ± 0.9	1.13 ± 0.11
	Treated (6)[c]	41.6 ± 7.5[b]	105 ± 33.8[b]	27.6 ± 6.9	262 ± 58[b]
13 Months	Control (6)	16.6 ± 1.7	18.4 ± 1.1	4.5 ± 1.0	0.89 ± 0.16
	Treated (6)[c]	55.1 ± 10.3[b]	220 ± 45[b]	29.6 ± 8.8[b]	306 ± 38[b]

[a] Rats were fed 100 ppm Aroclor 1254 and sacrificed at the times indicated. Urine was collected for a 24-hr period prior to sacrifice for determination of urinary porphyrins. The number of animals per group are shown in parentheses. Values represent the mean ± SE.

[b] $p < 0.05$.

[c] Rats were considered porphyric (total urinary porphyrins > 40 μg/24 hr). At 2 and 3 months, only some of the animals were porphyric and individual values for porphyric rats are given below the mean for the remainder of the group.

MONS 0819515

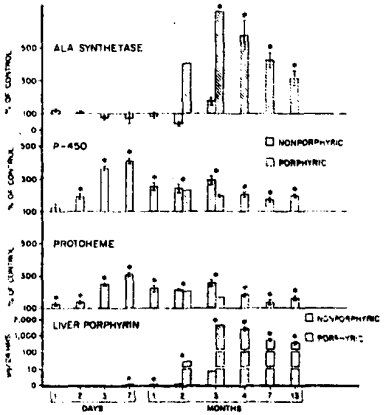

FIG. 2. Effect of 100 ppm Aroclor 1254 on liver porphyrins, ALA synthetase, microsomal P-450 and protoheme in the liver of female rats. Groups of 4–6 rats were killed periodically for 13 months. Rats were designated porphyric when total urinary porphyrin was increased at least 5-fold (all porphyric rats excreted at least 40 μg porphyrin/24 hr). Vertical bars represent means ± SE. Control values for ALA synthetase (23 nmol/g), Cytochrome P-450 (16 nmol/g) and microsomal heme (30 nmol/g) did not vary with age.
* $p < 0.05$.

TABLE 3
EFFECT OF 100 PPM AROCLOR 1254 ON LIVER WEIGHT AND MICROSOMAL PROTEIN*

Treatment	Liver/body weight ratio (%)		Microsomal protein (mg/g liver)	
	Control	Treated	Control	Treated
1 Day	4.36 ± 0.11	4.45 ± 0.14	16.6 ± 0.7	17.0 ± 0.9
2 Days	4.15 ± 0.06	4.60 ± 0.09†	16.6 ± 0.7	16.4 ± 0.6
3 Days	4.16 ± 0.08	5.13 ± 0.12†	18.6 ± 1.5	24.9 ± 0.8†
7 Days	4.50 ± 0.07	5.64 ± 0.21†	15.6 ± 0.5	22.5 ± 0.5†
1 Month	3.34 ± 0.06	4.34 ± 0.06†	17.4 ± 0.2	20.8 ± 1.4†
2 Months	2.79 ± 0.10	3.50 ± 0.13†	18.0 ± 0.5	23.8 ± 1.6†
3 Months	3.01 ± 0.12	3.65 ± 0.06†	14.3 ± 0.8	17.4 ± 0.3†
4 Months	2.71 ± 0.09	3.46 ± 0.17†	20.4 ± 1.2	16.2 ± 1.4†
7 Months	2.56 ± 0.05	3.18 ± 0.10†	15.1 ± 1.1	16.3 ± 0.6
13 Months	2.39 ± 0.07	3.36 ± 0.12†	14.8 ± 1.4	17.1 ± 0.2

* See Fig. 2 for treatment of animals. The results are expressed as mean ± SE.
† $p < 0.05$.

MONS 081916

TABLE 4

EFFECT OF A SINGLE DOSE OF AROCLOR 1254 ON RAT LIVER δ-AMINOLEVULINIC ACID SYNTHETASE ACTIVITY AND CYTOCHROME P-450[a]

Dose mg/kg	ALA synthetase activity (nmol/g/hr)		Cytochrome P-450 (nmol/g)		Liver/body weight (% of control)	Microsomal protein (% of control)
	5 Hr	24 Hr	5 Hr	24 Hr	24 Hr	24 Hr
Control (oil)	26.1 ± 2.2	48.9 ± 7.2[b]	11.0 ± 0.6	12.5 ± 1.5	100 ± 3	100 ± 5
5	33.3 ± 5.9	67.2 ± 16.5	9.7 ± 1.3	13.1 ± 2.7	105 ± 5	102 ± 5
10	37.5 ± 7.2	113.7 ± 28.6	9.8 ± 1.3	17.3 ± 0.7[c]	108 ± 4	112 ± 7
50	70.1 ± 17.1[c]	97.1 ± 8.3[c]	10.4 ± 0.7	37.8 ± 4.8[c]	125 ± 5[c]	125 ± 12
100	76.3 ± 6.0[c]	91.0 ± 12.3	11.1 ± 0.4	52.8 ± 8.9[c]	128 ± 5[c]	143 ± 8[c]
500	99.9 ± 19.2[c]	128.9 ± 13.8[c]	11.5 ± 1.4	63.7 ± 5.5[c]	141 ± 4[c]	161 ± 16[c]

[a] One-month-old female rats were starved 24 hr and Aroclor 1254 was administered in peanut oil by gastric intubation (0.005 ml/g body weight). Each value represents the mean ± SE of 6 animals.

[b] Starvation for 48 hr produced a slight increase in δ-aminolevulinic acid synthetase activity.

[c] Significantly different from controls at $p < 0.05$.

MONS 081917

Effect of a Single Dose of PCBs

Table 4 shows the acute effects of single doses of Aroclor 1254 on ALA synthetase and cytochrome P-450 in starved female rats. Fourfold induction of ALA synthetase could be demonstrated 5 hr after large doses of Aroclor 1254 (500 mg/kg). The minimal effective dose was 50 mg/kg, 5-times larger than the dose which produced porphyria when fed chronically in this study. Aroclor 1254 did not affect microsomal P-450 at 5 hr at any dosage level, but at 24 hr, P-450 was increased 5-fold, liver weight 40% and microsomal protein 60%. Administration of a large single oral dose of Aroclor 1254 (1000 mg/kg) increased ALA synthetase 5-fold at 24 hr, but did not result in any increase in urinary porphyrins, ALA or PBG 2 to 3 days after administration or in the surviving rats (2) 4 months after administration.

DISCUSSION

Porphyria occurred in all female rats fed 100 ppm Aroclor 1254 for at least 7 months (16 rats) and was characterized by delayed onset, excretion of large amounts of 7- and 8-carboxyporphyrins in the urine and accumulation of uroporphyrins in the liver. The delayed onset and excretion of large amounts of urinary uroporphyrins is similar to hexachlorobenzene-induced porphyria (Ockner and Schmid, 1961; San Martin de Viale *et al.*, 1970). However, rats fed PCBs exhibited none of the nervous or cutaneous signs associated with hexachlorobenzene poisoning (De Matteis *et al.*, 1961; Pearson and Malkinson, 1965). Induction of hepatic ALA synthetase could be demonstrated only after the onset of PCB-induced porphyria. Some accumulation of uroporphyrins occurred in the liver prior to a demonstrable increase in ALA synthetase. Our results suggest that induction of ALA synthetase is not the primary defect in PCB-induced porphyria in the rat.

The tremendous accumulation of uroporphyrins in the liver and urine of rats fed Aroclor 1254 suggests that PCBs may affect uroporphyrin formation or utilization. Hemosiderin has been reported in the liver of rats treated with Aroclor 1254 and in the liver of patients with porphyria cutanea tarda, indicating an increase in liver iron content (Kimbrough *et al.*, 1972; Uys and Eales, 1963). Kushner *et al.* (1972) recently reported that iron decreases uroporphyrin III cosynthetase activity and suggested iron may also affect uroporphyrin decarboxylase. If a block in heme synthesis is involved in PCB-induced porphyria, heme synthesis is apparently sufficient for synthesis of cytochrome P-450, since microsomal heme and cytochrome P-450 were elevated in porphyric rats.

Many xenobiotics increase cytochrome P-450 and activity of the liver microsomal drug-metabolizing enzymes (Conney, 1967). It has been suggested that increased ALA synthetase activity may be necessary for increased synthesis of P-450 (Marver, 1966a). We found no correlation between changes in ALA synthetase activity and cytochrome P-450 or microsomal heme after feeding 100 ppm PCBs. Cytochrome P-450 and microsomal heme were increased maximally at 1 week, in the absence of an increase in ALA synthetase. Phenylbutazone also increases cytochrome P-450 without affecting ALA synthetase activity (De Matteis and Gibbs, 1972). Administration of large doses of exogenous ALA or heme to rats does not increase cytochrome P-450 levels (De Matteis, 1971), suggesting that synthesis of cytochrome P-450 is regulated by factors other than availability of heme.

MONS 081918

The porphyrogenic compound AIA induces ALA synthetase as early as 5 hr after administration, perhaps as a result of destruction of cytochrome P-450 and accumulation of breakdown products of heme in the liver (Abritti and De Matteis, 1971). Acutely, large doses of Aroclor 1254 produced a 4-fold increase in ALA synthetase. However, Aroclor 1254 did not decrease cytochrome P-450; instead, Aroclor acted as a powerful inducer of drug metabolism, increasing cytochrome P-450 5-fold by 24 hr. The dose of PCBs required to induce ALA synthetase was larger than the dose which produced porphyria when fed chronically, and induction of ALA synthetase by a single large dose of PCBs was probably related to large, rapid increases in cytochrome P-450.

Two European commercial preparations of PCBs have been reported to be contaminated with small amounts of tetrachlorodibenzofuran (Vos *et al.*, 1970). The chlorinated dibenzodioxins and dibenzofurans are very potent chloracneogenic and hepatotoxic agents. (Bauer *et al.*, 1961; Linn Jones and Krizek, 1962). Recently, Poland and Glover (1973) have shown that chlorinated dibenzodioxins induce ALA synthetase in the chick embryo at doses as low as 3 ng/kg. Possibly, contamination with chlorinated dibenzofurans could be responsible for the porphyria produced by PCBs. However, Vos and Notenboom-Ram (1972) have found 2,4,5,2',4',5'-hexachlorobiphenyl to be porphyrogenic in the rabbit.

The dietary level of Aroclor 1254 used to produce porphyria in rats (100 ppm) is at least 20 times higher than the 0.1 to 5 ppm temporary tolerances set by FDA for PCBs in food in the United States. The concentration of PCBs in fat of rats fed 100 ppm Aroclor 1254 for 6 months is 1000 ppm in our laboratory (Curley *et al.*, 1971). Although a similar level of PCBs has been detected in the fat of some wildlife (Risebrough *et al.*, 1968), only 5% of human fat samples tested contain more than 2 ppm PCBs (Yubbs, 1972). It is unlikely that present environmental levels of PCBs will result in porphyria in humans. However, an episode of human poisoning due to contamination of rice oil with 2000 ppm Kanechlor 400 occurred recently in Japan, resulting in chloracne and pigmentation of the skin and nails (Kuratsune *et al.*, 1972). Unfortunately, the concentration of porphyrin in the urine was apparently not determined.

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MONS 081921

UROPORPHYRIN FORMATION INDUCED BY CHLORINATED HYDROCARBONS (LINDANE, POLYCHLORINATED BIPHENYLS, TETRACHLORODIBENZO-p-DIOXIN). REQUIREMENTS FOR ENDOGENOUS IRON, PROTEIN SYNTHESIS AND DRUG-METABOLIZING ACTIVITY

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Summary: The chlorinated hydrocarbons, lindane (gamma-hexachlorocyclohexane), polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin, induce chick embryo liver cells in culture to rapidly accumulate uroporphyrin III. This is in marked contrast to the delayed effect in animals. The induction is dependent on protein synthesis (cycloheximide inhibited), endogenous iron (chelators inhibited) and perhaps cytochrome P-450 (SKF-525A inhibited). Evidence was obtained for an unstable intermediate that is generated in the liver from chlorinated hydrocarbons which inhibits uroporphyrinogen decarboxylase thus causing uroporphyrin to accumulate. The data suggest that the intermediate may be a hydroxylated derivative of the chlorinated hydrocarbon.

Many chemicals, including barbiturates, steroids and chlorinated hydrocarbons will induce an increase in porphyrin accumulation by chick embryo liver cells in culture (1,2). This increase in accumulation is preceded by an increase in the activity of the enzyme, delta-aminolevulinic acid synthetase.

Humans, other mammals and some birds accumulate large amounts of uroporphyrin in their livers after treatment with chlorinated hydrocarbons (3,4,5,6,7). However, the effect is delayed, varying from several days in the case of birds, to several weeks for mammals. In this paper we will show that the cultured cells respond rapidly to the chemicals to specifically accumulate uroporphyrin. This accumulation is dependent on both protein synthesis and endogenous iron. Our results suggest that these cells may metabolize the chlorinated hydrocarbons to active metabolites which inhibit the enzyme, UROgen decarboxylase

Materials and methods: The chlorinated hydrocarbons used were: lindane (gamma-hexachlorocyclohexane) 99.5% from Shell Company; Aroclor 1254, a mixture of

Abbreviations: URO, uroporphyrin; COPRO, coproporphyrin; PROTO, protoporphyrin; ALA, delta-aminolevulinic acid.

MONS 081922

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chlorinated biphenyls predominantly 4,5 and 6 chlorines per molecule, from Monsanto; 2,4,5,2',4',5'-hexachlorobiphenyl from Analabs; and 2,3,7,8-totrachlorodibenzo-p-dioxin from Dow Chemical Company. All except the dioxin were dissolved in ethanol. The dioxin was dissolved in acetone. One to two microliters of solvent were added per ml of culture medium. Desferrioxamine was Desferal from Ciba and was freshly prepared in saline from the ampoule; the activity decreases when the powder is exposed to moist air but less so in a dessicator at 4°. Rhodotorulic acid was a gift from Dr. H. Akers of this university. SKF-525A (2-diethylaminoethyl 2,2-diphenylvalerate HCl) was a gift from Smith Kline and French laboratories. Piperonyl butoxide was 96% and was a gift from FMC Corporation.

The composition and quality of porphyrins extracted from cells or homogenates in 1N perchloric acid (aqueous):methanol (1:1) were determined fluorometrically on the Hitachi-Perkin Elmer spectrofluorometer using the filters and phototube described previously (8). This procedure yields a 2-banded spectrum with peaks at about 605 and 660nm. The ratios of the peak heights at these wavelengths permit PROTO to be simply distinguished from URO and/or COPRO. The actual identification of URO was confirmed by thin layer chromatography on silica gel by the kerosene/chloroform (2:8) system. After determining the ratios of the peak heights, and the fluorescence yield at 660nm of standard solutions, this method can be used to easily give the % composition of PROTO vs. URO and/or COPRO and the quantity of porphyrins in mixtures. The method will be described in detail in a subsequent publication.

To determine the isomer composition of the URO's they were decarboxylated to COPRO (9) and chromatographed on Eastman Chromagrams (silica gel) (10).

RESULTS

The chlorinated hydrocarbons cause the cells to accumulate uroporphyrin. Iron is involved. Table I shows that 2 of the tested chlorinated hydrocarbons, lindane and 2,4,5,2',4',5'-hexachlorobiphenyl, cause the accumulation of URO. This is in contrast to allylisopropylacetamide and other inducers which cause the accumulation of mainly PROTO. Incubation of the cells with any tested inducer together with a chelator of iron (e.g. desferrioxemine) caused the accumulation of PROTO rather than URO.

MONS 081923

TABLE I

Effect of chlorinated hydrocarbons on chick embryo liver cells in culture

Treatment	Porphyrin pmole/mg protein	Type of porphyrin
None	12	PROTO
Desferrioxamine (1.5mM)	18	PROTO
Allylisopropylacetamide (200µg/ml)	900	80% PROTO*
Allylisopropylacetamide + Desferrioxamine	1700	PROTO
Lindane (3µg/ml)	280	URO
Lindane + Desferrioxamine	1100	PROTO
Hexachlorobiphenyl (2µg/ml)	90	URO
Hexachlorobiphenyl + Desferrioxamine	250	PROTO

* and 20% URO + COPRO

Primary chick embryo liver cells were cultured as described (11). The last change of medium was to the completely defined Ham-F12 medium containing insulin. Chemicals were added and porphyrin assayed in cells and medium after a further 16 hr incubation.

In other words, addition of the chelator removed the block in the biosynthetic chain so that PROTO accumulated. The other chlorinated hydrocarbons described in this paper also block porphyrin synthesis at the URO step. Of the chelators tested, effective and non-toxic ones in appropriate concentrations were: desferrioxamine (1.5mM), CaMg EDTA (equimolar Ca^{2+}, Mg^{2+}, EDTA) (10mM), 8-hydroxyquinoline sulfonate (0.1mM), and rhodotorulic acid (1.5mM).

Another effect of the chelator of iron, independent of the effect on the kind of porphyrin accumulated, is to increase the total porphyrin, i.e. to cause a potentiation. This is due to an increase in the total activity of the enzyme, delta-aminolevulinic acid synthetase (11).

Active metabolism of the cells is required for URO-production. In these experiments

MONS 081924

TABLE II

Requirements for URO accumulation in the presence of different chlorinated hydrocarbons* in chick embryo liver cells in culture

Expt.	Addition	Porphyrin	Required for URO accumulation
1	ALA**	PROTO	
2	ALA + Chlorinated HC	URO	Chlorinated HC
3	ALA + Chlorinated HC + Chelator	PROTO	Endogenous iron
4	ALA + Chlorinated HC + Cycloheximide	PROTO	Protein synthesis
5	ALA + Chlorinated HC + SKF-525A	PROTO	Cytochrome P-450 dependent metabolism
6	ALA + Chlorinated HC + Piperonyl butoxide	PROTO	Cytochrome P-450 dependent metabolism
7	ALA + Chlorinated HC + Heme	URO	Induction of ALA-synthetase not required

* The individual chlorinated hydrocarbons in μg/ml that were used were: lindane (3); 2,4,5,2',4',5'-hexachlorobiphenyl (2); Aroclor 1254 (3); tetrachlorodibenzo-p-dioxin (0.03).

** The concentrations in μg/ml used were: for ALA, 25; for cycloheximide, 0.1; for SKF-525A, 5; for piperonyl butoxide, 5; for heme, 5. Culture conditions and chelator concentrations were as described in legend to Table 1.

(Table II) exogenous ALA was given so that porphyrin production was not dependent on the activity of the cellular ALA-synthetase. The total porphyrin from ALA whether URO or PROTO was about the same regardless of the treatment. 1. The accumulation of URO by the cells was dependent on protein synthesis because cycloheximide (and emetine) blocked URO formation (expt 4). 2. Endogenous iron is required because in the presence of chelators of iron, PROTO, rather than URO is formed (expt 3). 3. The inhibition by SKF-525A and piperonyl butoxide (expts 5,6) suggests that the compounds act via metabolism possibly involving cytochrome P-450 since these are compounds known to inhibit drug metabolism (12). 4. The chlorinated hydrocarbons

MONS 081925

have 2 effects: they cause induction of synthesis of ALA-synthetase, an effect which is blocked by heme (11); and they cause URO accumulation from added ALA, an effect which is not blocked by heme (expt 7).

Evidence for a labile intermediate generated in vivo, detected in liver homogenates, and required for URO formation. Liver homogenates from untreated chick embryos when incubated with ALA accumulated PROTO (Table III). Addition of chlorinated hydrocarbons plus ALA to the homogenate from untreated embryos did not affect the porphyrin produced. However, as seen in Table III, when chick embryos were previously treated with Aroclor 1254 and their livers were homogenized and incubated with ALA, a short-lived component (or activity) appeared to be present in the homogenate which caused URO to accumulate. The short life of this component was shown by preincubating the fresh liver homogenate for 1 hr. before addition of ALA. This preincubation caused the loss of the ability of the homogenate to form URO from ALA, i.e.

TABLE III

Evidence for labile compound that causes URO accumulation by liver homogenates from chick embryos pretreated with Aroclor

Pretreatment of embryo	Preincubation of homogenate before addition of ALA	Porphyrin accumulated after addition of ALA
-	-	PROTO
-	+	PROTO
AROCLOR	-	URO
AROCLOR	+	PROTO

16-day chick embryos were injected with 15mg Aroclor 1254 in 0.2ml dimethyl sulfoxide. After 1.5 days homogenates were prepared in 0.25M sucrose, 30mM tris chloride (pH 7.4) and preincubated or not for 1hr, 37°. Incubation volume was 0.2ml containing 125µg wet weight of liver. ALA (3µg) was added to all tubes after preincubation period and tubes were incubated for another 1hr, 37°.

MUNS 081926

when ALA was added 1 hr. after preincubation, the porphyrin that was formed was PROTO. Thus once the blocking effect caused by the short-lived component has been removed, synthesis to PROTO proceeds. The fact that PROTO is formed indicates in addition that no damage of enzymes from ALA to PROTO has been caused by the chlorinated hydrocarbon or its metabolites.

The liver homogenates from control and Aroclor-treated embryos both contained very little porphyrin and it was PROTO. In the embryos, excess ALA is evidently required to observe the effect of the short-lived component in blocking at the URO step.

Effect of chlorinated phenols on URO production in liver homogenates. Rats and mice completely metabolize lindane to a mixture of di-, tri-, tetra- and penta- chlorophenols (13,14,15) and in part metabolize chlorinated biphenyls to the hydroxylated derivatives (16,17). The possibility occurred to us that a phenol metabolite might be

TABLE IV

Effect of phenols on URO production from ALA

by liver homogenates

Addition	Porphyrin accumulated
ALA	PROTO
ALA + 2,4,6-trichlorophenol	PROTO
ALA + tetrachlorophenol	45% PROTO*
ALA + pentachlorophenol	URO
ALA + 2,4-dinitrophenol	URO
ALA + pentachlorophenol + chelator	URO
ALA + pentachlorophenol + Mn^{2+}	URO
ALA + pentachlorophenol + diphenylamine	URO

* and 55% URO

Liver homogenates from untreated embryos were prepared as in legend to Table III. Phenols were 1mM added in 1µl of acetone. Chelators were desferrioxamine (1.5mM); EDTA (10mM); and α,α-dipyridyl (0.5mM). Diphenylamine and Mn^{2+} were 1mM.

MONS 081927

the actual blocking compound or related to it. We therefore tested certain phenols (1mM) plus ALA (0.15mM), added directly to liver homogenates from untreated chick embryos prepared as described in the legend to Table III. The 2,4,6-trichlorophenol caused PHOTO accumulation. In contrast, the tetrachlorophenol caused 45% PHOTO + 55% URO formation. Pentachlorophenol and 2,4-dinitrophenol caused only URO accumulation. Perhaps the electron-withdrawing groups such as chloro- or nitro- on the aromatic ring function to stabilize some intermediate required for blocking at the URO step.

URO formation in the homogenate containing ALA and pentachlorophenol is not affected by the addition of a number of chelators of iron (1.5mM desferrioxamine, 10mM EDTA, 0.5mM α,α-dipyridyl). Therefore iron is not necessary for this particular reaction. This reaction is different from the reaction that occurs in the cultured cells in which the chlorinated hydrocarbons require the presence of iron to produce URO (Table II, expt 3). Attempts to seek for the presence of a radical generated by the chlorinated phenols were unsuccessful. Manganese ions and diphenylamine have been used to block lipid peroxidation that is generated by free radicals in rat liver microsomes (18). When Mn^{2+} (1mM) or diphenylamine (1mM) were incubated together with the chlorophenols in a liver homogenate they did not prevent URO accumulation from ALA.

Homogenates of embryonic heart treated directly with pentachlorophenol accumulated just as much URO in the presence of ALA as did the liver homogenates. This shows that the pentachlorophenol reaction is not specific to the liver. Cytochrome P-450 is probably very low in heart homogenate. If cytochrome P-450 is low it would suggest that cytochrome P-450 may not be involved in the pentachlorophenol reaction to block the synthesis of heme at the URO step.

DISCUSSION

Figure 1 summarizes the possible actions of the chlorinated hydrocarbons in the blocking of porphyrin formation at the URO step: 1. There is an induction of a metabolizing system - this requires protein synthesis since it is blocked by cycloheximide. 2. The chlorinated hydrocarbon is converted to a metabolite by a metabolizing system containing cytochrome P-450, since known inhibitors of cytochrome P-450 action (e.g. SKF-525A) prevent URO formation. 3. Iron is required at some reaction

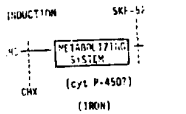

Fig. 1. Summary of ac

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Three ways may b
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MONS 081928

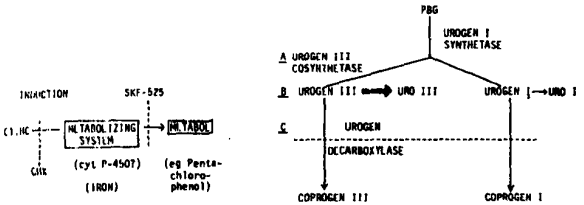

Fig. 1. Summary of actions of chlorinated hydrocarbons to cause URO accumulation.

step for the formation of the metabolite that causes URO accumulation, and 4. The metabolite may be a hydroxylated derivative of the chlorinated hydrocarbons since pentachlorophenol caused URO accumulation in the homogenate.

Three ways may be considered by which the chlorinated hydrocarbons can act probably via a metabolite (Fig. 1):

A) Inhibition of UROgen III cosynthetase. If this were the case then mainly the URO I isomer would have accumulated which is reported to occur when supernatants of homogenates of pig or human liver are incubated with ALA plus ferrous iron (19). However, the URO isomer accumulated by the cultured cells or the liver homogenates we found to be mainly the III isomer as well as some I. Therefore inhibition of cosynthetase as a mechanism of URO formation may be rejected. This conclusion is consistent with the fact that rats treated with hexachlorobenzene excrete URO III in the urine (20).

B) There may be a very rapid direct oxidation of the reduced UROgen III to URO III. In the case where pentachlorophenol was added to liver homogenates in the presence of ALA, the product formed was UROgen as indicated by the fact that no fluorescent porphyrin appeared, except after extraction and oxidation by iodine or in the presence of light. This result does not support the idea that the metabolite of chlorinated hydrocarbons generates a mechanism for rapid autoxidation of UROgen to URO.

C) Only a third possibility remains: the inhibition of the

MUNS 081929

enzyme, UROgen decarboxylase. We have not as yet directly demonstrated that this enzyme is inhibited. Inhibition of this enzyme would be consistent with 1) the findings of Taljaard et al (21) that the enzyme is completely deficient in rats treated for several weeks with both hexachlorobenzene and iron and 2) that lower levels of the enzyme have been found in rats treated with hexachlorobenzene alone (M. Tomio and S. San Martin de Viale, personal communication).

These findings support the conclusion that the chlorinated hydrocarbons are converted by liver metabolism to products that inhibit the enzyme, UROgen decarboxylase. Such products have tentatively been identified as the hydroxyl derivatives of the parent compounds which are known metabolites. However no metabolites have yet been reported for the very chemically inert dioxin. It has therefore been assumed that dioxin acts directly. The rapid effect on chick embryo liver cells seen with dioxin suggests that this may be a very good system for investigating whether it acts directly or via some metabolite.

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MONS 081931