

# Hepatic uroporphyrin accumulation and uroporphyrinogen decarboxylase activity in cultured chick-embryo hepatocytes and in Japanese quail (*Coturnix coturnix japonica*) and mice treated with polyhalogenated aromatic compounds

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The relationship between hepatic uroporphyrin accumulation and uroporphyrinogen decarboxylase (EC 4.1.1.37) activity was investigated in cultured chick-embryo hepatocytes, Japanese quail (*Coturnix coturnix japonica*) and mice that had been treated with polyhalogenated aromatic compounds. Chick-embryo hepatocytes treated with 3,3',4,4'-tetrachlorobiphenyl accumulated uroporphyrin in a dose-dependent fashion without a detectable decrease in uroporphyrinogen decarboxylase activity when either pentacarboxyporphyrinogen III or uroporphyrinogen III were used as substrates in the assay. Other compounds, such as hexachlorobenzene, parathion, carbamazepine and nifedipine, which have been shown previously to cause uroporphyrin accumulation in these cells, did not decrease uroporphyrinogen decarboxylase activity. Japanese quail treated with hexachlorobenzene for 7–10 days also accumulated hepatic uroporphyrin without any decrease in uroporphyrinogen decarboxylase activity. In contrast, hepatic uroporphyrin accumulation in male C57BL/6 mice treated with iron and hexachlorobenzene was accompanied by a 20–80% decrease in uroporphyrinogen decarboxylase activity, demonstrating that the assay used for uroporphyrinogen decarboxylase, using pentacarboxyporphyrinogen III as substrate, could detect decreased enzyme activity. Our results with chick hepatocytes and quail, showing uroporphyrin accumulation without a decrease in uroporphyrinogen decarboxylase activity, are consistent with a new two-stage model of the uroporphyrin: initially uroporphyrinogen is oxidized by a cytochrome *P*-450-mediated reaction, followed in rodents by a progressive decrease in uroporphyrinogen decarboxylase activity.

## INTRODUCTION

Polyhalogenated aromatic compounds (PHAs) have been shown to cause a porphyria, characterized by marked increases in hepatic and urinary levels of uroporphyrin (URO), in a variety of animals, including humans (Schmid, 1960), rodents (De Matteis *et al.*, 1961; Elder *et al.*, 1976; Jones & Sweeney, 1980) and birds (Vos *et al.*, 1971). Hexachlorobenzene (HCB) precipitated a severe outbreak of this uroporphyrin in humans in south-eastern Turkey (Gocmen *et al.*, 1986). Experiments in rodents have shown that there is a delay of several weeks before hepatic URO accumulates in rats (Goldstein *et al.*, 1976) and mice (Goldstein *et al.*, 1973). In contrast, Japanese quail (*Coturnix coturnix japonica*) accumulate hepatic URO within a few days of exposure to PHAs (Carpenter *et al.*, 1985). Cultured chick-embryo hepatocytes treated with PHAs have been shown to accumulate URO after only a few hours (Sinclair & Granick, 1974; Sinclair *et al.*, 1984) and have been used to study the mechanism by which PHAs cause hepatic URO accumulation (see the review by Marks, 1985). These cultures appear to be the only hepatocytes tested that accumulate URO in response to these compounds,

in contrast with cultures of rat hepatocytes, which do not accumulate URO under these conditions (Ferioli *et al.*, 1986; P. Sinclair, W. Bement & P. Guzelian, unpublished work).

Several authors have reported conditions under which treatment of rats (Elder *et al.*, 1976), mice (Jones & Sweeney, 1980; Smith *et al.*, 1986) or cultured chick-embryo hepatocytes (Sinclair *et al.*, 1983; Swain *et al.*, 1983; de Verneuil *et al.*, 1983) with PHAs caused both URO accumulation and decreased the activity of uroporphyrinogen decarboxylase. Involvement of cytochrome *P*-450 in the URO accumulation caused by PHAs has also been suggested (Sinclair & Granick, 1974; Jones & Sweeney, 1980; Sinclair *et al.*, 1984). A mechanism has been proposed in which cytochrome *P*-450 metabolizes the PHA to a reactive intermediate which binds to uroporphyrinogen decarboxylase, leading to irreversible inactivation of the enzyme (Sinclair & Granick, 1974; Debets & Strik, 1979). This inhibition then causes accumulation of substrate, uroporphyrinogen III, with subsequent oxidation of the porphyrinogen to URO, which accumulates in the liver and is excreted in the urine.

There is evidence which appears to be contradictory to

Abbreviations used: URO, uroporphyrin; TCB, 3,3',4,4'-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HCB, hexachlorobenzene; PHA(s), polyhalogenated aromatic compound(s).

this proposed mechanism. For instance, Smith *et al.* (1986) were unable to find evidence for covalent bond formation between uroporphyrinogen decarboxylase and any metabolites of HCB. Another PHA, 3,3',4,4',5,5'-hexachlorobiphenyl, was found to cause URO accumulation in cultured chick-embryo hepatocytes, but without any measurable metabolism of the biphenyl, suggesting that metabolism of this PHA is not required to cause URO accumulation (Sinclair *et al.*, 1986). It was also shown that URO accumulation in cells treated with PHAs is rapidly reversed by addition of the cytochrome *P*-450 inhibitor piperonyl butoxide (Sinclair *et al.*, 1986). These results are inconsistent with irreversible inhibition of uroporphyrinogen decarboxylase by reactive metabolites of PHAs.

Hence we examined the relationship between URO accumulation and uroporphyrinogen decarboxylase activity in cultured chick-embryo hepatocytes, Japanese quail and mice treated with PHAs. We found conditions under which chick-embryo hepatocytes and livers of Japanese quail accumulated massive amounts of URO without decreased enzyme activity.

A preliminary report of this work has been presented in abstract form (Lambrecht *et al.*, 1987).

## EXPERIMENTAL

### Chemicals

Sources for most of the chemicals were given previously (Sinclair *et al.*, 1984). HCB (Organic Analytical Standard grade) was from BDH, Poole, Dorset, U.K. Carbamazepine and nifedipine were from Sigma, St. Louis, MO, U.S.A. Parathion was from Ultra-Science, Hope, RI, U.S.A. Ferric nitriloacetate was prepared as described previously (Shedlofsky *et al.*, 1983). Synthetic pentacarboxyporphyrin III was from Professor A. Jackson, Department of Chemistry, University College Cardiff, Wales, U.K. Imferon was from Merrell-Dow, Cincinnati, OH, U.S.A.

### Hepatocyte cultures

Primary cultures from livers of 16-day-old White Leghorn chicken embryos were prepared and maintained in Williams E medium as previously described (Sinclair *et al.*, 1982). Cells were cultured in 10 cm-diameter plastic dishes, yielding 4–5 mg of protein per dish. On the second day of culture, cells were rinsed twice with Williams E medium containing dexamethasone and 3,3',5-tri-iodothyronine, but not insulin. Chemicals to be tested were added in dimethyl sulphoxide (not exceeding 4  $\mu$ l/ml) to Williams E medium containing dexamethasone and 3,3',5-tri-iodothyronine. Appropriate amounts of this vehicle were added to control dishes. Cells were typically exposed to test chemicals overnight (18–24 h), except for the 48 h experiments, where the medium was changed and chemicals re-added after 24 h. At harvest, cells were rinsed with 2 ml of 0.15 M-saline (0.9% NaCl), scraped into 0.4 ml of 0.25 M-sucrose, and homogenized (10 up-and-down strokes at 500 rev./min) in a 1.0 ml Potter-Elvehjem homogenizer (Thomas Scientific Co., Philadelphia, PA, U.S.A.). Portions of this homogenate were saved for porphyrin analysis, and the remainder was centrifuged at 10000 *g* for 10 min. The supernatant was assayed immediately for uroporphyrinogen decarboxylase activity or was stored at  $-70^{\circ}\text{C}$ .

### Mice

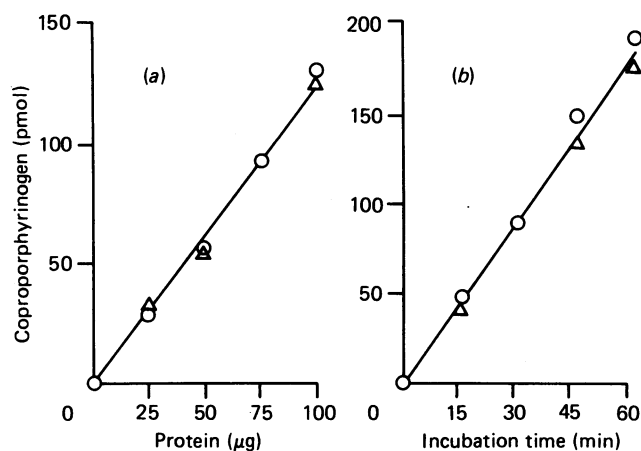
Male C57BL/6 mice ( $n = 14$ ) weighing 23–25 g were given intraperitoneal (i.p.) injections of Imferon (12.5 mg of iron per mouse), followed after 3 days by the first of three weekly i.p. injections of HCB in corn oil (125 mg/kg per injection). Control mice ( $n = 14$ ) were untreated. All mice received standard laboratory chow and tap water *ad libitum*. They were killed by cervical dislocation 70 days after the first HCB injection. Livers were removed and homogenized [20% (w/v) in 0.25 M-sucrose]. Portions of these homogenates were centrifuged at 10000 *g* for 15 min, and the supernatants were stored at  $-70^{\circ}\text{C}$ .

### Quail

Female Japanese quail were obtained from an inbred colony (Department of Poultry Science, Oregon State University, Corvallis, OR, U.S.A.) and were maintained as previously described (Carpenter *et al.*, 1985). Birds received standard layer feed and water *ad libitum*. In experiment 1, HCB (500 mg/day per kg for 7 days) was administered to four birds orally via gelatin capsules, using lactose as a filler. Controls ( $n = 3$ ) received lactose alone. In experiment 2, birds received either corn oil or  $\beta$ -naphthoflavone in corn oil (150 mg/day per kg) for 4 days. On day 5 each pretreatment group was divided into two; half received lactose and half received lactose plus HCB (500 mg/day per kg) for 10 days. The four treatment groups ( $n = 4$  birds/group) were: lactose plus corn oil, HCB plus corn oil, lactose plus  $\beta$ -naphthoflavone, and HCB plus  $\beta$ -naphthoflavone. Birds were killed by cervical dislocation, and liver samples were processed as for mice above.

### Assay of uroporphyrinogen decarboxylase activity

This assay measured the decarboxylation of pentacarboxyporphyrinogen III (or uroporphyrinogen III) by 10000 *g* supernatants prepared from liver homogenates and is a modification of previously published procedures (de Verneuil *et al.*, 1980; Elder & Wyvill, 1982; Cantoni *et al.*, 1982; Francis & Smith, 1984). The incubation mixture contained 195  $\mu$ l of assay buffer, 50  $\mu$ l of 0.25 M-sucrose (containing 75  $\mu$ g of supernatant protein) and 5  $\mu$ l of freshly prepared porphyrinogen (final concn. approx. 5  $\mu$ M). The assay buffer was 0.1 M-potassium phosphate/10 mM-dithiothreitol/1 mM-EDTA adjusted to pH 6.8 with concentrated  $\text{H}_3\text{PO}_4$ . Substrate was prepared by mixing 80  $\mu$ l of pentacarboxyporphyrin III solution (0.7 mM in 5 mM-NaOH) with 0.16 ml of assay buffer containing 0.1 M-dithiothreitol, chemically reducing this solution with sodium amalgam (4%), with shaking, until all red fluorescence was gone. The tubes were shaken at  $37^{\circ}\text{C}$  for 30 min in the dark, and the reaction was stopped in an ice/water bath, and 25  $\mu$ l of 6 M-HCl was added. The samples were irradiated under a fluorescent lamp (15 W) for 7 min to oxidize porphyrinogens to porphyrins, and were centrifuged (10000 *g* for 3 min). The supernatant was diluted with 0.1 M-HCl (1:7, v/v) and 50  $\mu$ l was injected on to a 5  $\mu$ m Microsorb  $\text{C}_{18}$  h.p.l.c. column (Rainin Instrument Co., Woburn, MA, U.S.A.; 25 cm). Porphyrins were eluted with a gradient of 0.1 M-ammonium phosphate and methanol, using a modification of the method of Bonkovsky *et al.* (1986). The initial mobile phase was 72% methanol/28% ammonium phosphate (pH 3.4).



**Fig. 1.** Effect of protein concentration and incubation time on formation of coproporphyrinogen III from pentacarboxyporphyrinogen III by supernatants from cultured chick-embryo hepatocytes

Cells on 10-cm-diameter dishes were treated with vehicle alone ( $\Delta$ ) or with  $3.4 \mu\text{M}$ -TCB ( $\circ$ ) for 22 h, and 10000 g supernatants from pooled samples were prepared as described in the Experimental section. Coproporphyrinogen formation was measured (a) for increasing amounts of protein and a 30 min incubation time, and (b) for increasing incubation time and 75  $\mu\text{g}$  of protein, as described in the Experimental section. Values are means for duplicate samples and varied by less than 10%.

The methanol concentration was increased linearly to 86% methanol over 12 min, and then to 100% by 12.2 min. This procedure completely separated pentacarboxyporphyrin and coproporphyrin, with retention times of 9.7 and 13.0 min respectively at a flow rate of 1.5 ml/min. Detection of the porphyrins was by fluorescence at 398 nm (excitation) and 620 nm (emission). Porphyrin standards were from Porphyrin Products, Logan, UT, U.S.A. Inter-assay variation in enzyme activity was assessed by the inclusion of 10000 g supernatants from control chick-embryo liver, assayed in triplicate with 75  $\mu\text{g}$  of protein per assay. These samples were from a single preparation from 19-day-old chick-embryo livers. Enzyme activity remained stable for at least 6 months when supernatants were stored at  $-70^\circ\text{C}$ . Protein was assayed by the Lowry procedure (Lowry *et al.*, 1951), with bovine serum albumin as standard.

#### Porphyrin measurements

Porphyrins were determined spectrofluorimetrically (Sinclair *et al.*, 1984) or by h.p.l.c. (Bonkovsky *et al.*, 1986; Sinclair *et al.*, 1986), as described previously.

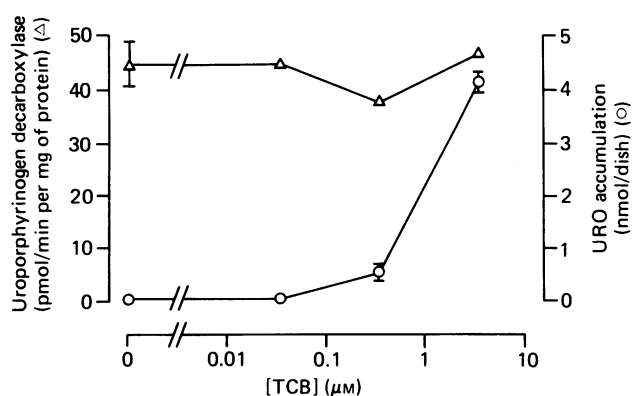
#### Statistics

The significance of difference between groups was determined using Student's *t* test.

## RESULTS

### Chick-embryo hepatocytes

Fig. 1(a) shows that TCB treatment did not decrease uroporphyrinogen decarboxylase activity. The assay was linear up to 400  $\mu\text{g}$  of protein/ml of incubation volume,



**Fig. 2.** Effect of TCB concentration on URO accumulation and uroporphyrinogen decarboxylase activity in cultured chick-embryo hepatocytes

Cells were treated with vehicle alone or with the indicated concentration of TCB for 22 h. URO accumulation ( $\circ$ ) from cells plus medium and uroporphyrinogen decarboxylase activity ( $\Delta$ ) in 10000 g supernatants were determined as described in the Experimental section. Values are means for three dishes. The s.d. fell within the symbol unless indicated otherwise by the vertical bars.

and 300  $\mu\text{g}/\text{ml}$  was used routinely in subsequent assays. Product formation was also shown to be linear with respect to incubation time for up to 60 min (Fig. 1b), and an incubation time of 30 min was used routinely in subsequent assays.  $K_m$  and  $V_{max}$  (derived from a Lineweaver-Burk plot) were 1.3  $\mu\text{M}$  and 46 pmol/min per mg of protein respectively.

The effect of preparation and storage of culture samples on uroporphyrinogen decarboxylase activity was also investigated. Activities in homogenates prepared from pelleted whole cells (after storage of cell pellets for up to 3 months at  $-70^\circ\text{C}$ ) were less than 25% of those in supernatants prepared at harvesting from the same cells and also stored at  $-70^\circ\text{C}$ . Activities in homogenized cell pellets varied considerably between experiments. Nevertheless, in most of the experiments in which homogenized cell pellets were assayed, decreases in enzyme activity caused by TCB were not observed. Samples that had been prepared as supernatants and stored at  $-70^\circ\text{C}$  maintained constant uroporphyrinogen decarboxylase activity for at least 6 months. These supernatants could also be thawed and refrozen up to five times without loss of enzyme activity (results not shown).

Fig. 2 shows the effect of TCB concentration on URO accumulation and uroporphyrinogen decarboxylase activity in chick-embryo hepatocytes. URO accumulation in these cells depended on the concentration of TCB with the highest accumulation of URO at 3.4  $\mu\text{M}$ -TCB (approximate limit of solubility in media). Uroporphyrinogen decarboxylase activity was not decreased by any of the concentrations of TCB tested. Table 1 shows URO accumulation and uroporphyrinogen decarboxylase activity for both control cells and cells treated with 3.4  $\mu\text{M}$ -TCB from six separate cultures where treatment of the cells with TCB caused significant accumulation of URO, with no significant decrease in enzyme activity. These data indicate that we have found highly reproducible conditions under which chick-

**Table 1. Effect of TCB on URO accumulation and uroporphyrinogen decarboxylase activity in cultured chick-embryo hepatocytes**

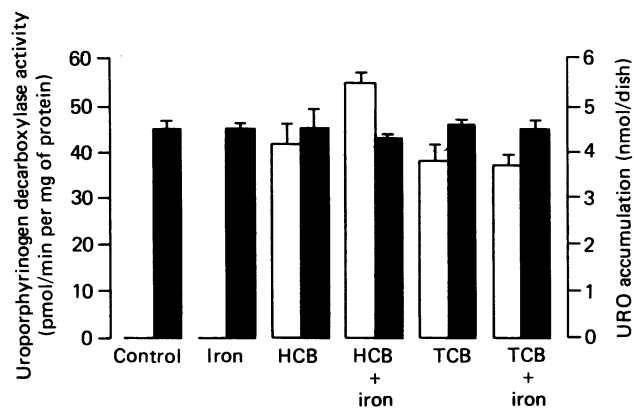
Cells on 10 cm-diameter dishes were treated with either vehicle alone or 3.4  $\mu\text{M}$  TCB for 18–24 h. URO accumulation from cells plus medium was determined by the spectrofluorimetric method, and uroporphyrinogen decarboxylase activity was determined in 10000 g supernatants using pentacarboxyporphyrinogen III as substrate, as described in the Experimental section. Values are means  $\pm$  s.d. for three dishes.

Expt.	URO accumulation (nmol/dish)		Uroporphyrinogen decarboxylase activity (pmol/min per mg of protein)	
	Control	TCB	Control	TCB
1	0.02 $\pm$ 0.01	5.62 $\pm$ 0.87	55 $\pm$ 3.0	57 $\pm$ 4.7
2	0.05 $\pm$ 0.02	8.62 $\pm$ 1.06	47 $\pm$ 3.5	52 $\pm$ 4.2
3	0.06 $\pm$ 0.03	3.12 $\pm$ 0.12	43 $\pm$ 6.0	41 $\pm$ 2.3
4	0.01 $\pm$ 0.02	2.22 $\pm$ 0.27	52 $\pm$ 3.9	49 $\pm$ 7.6
5	0.03 $\pm$ 0.01	4.15 $\pm$ 0.18	45 $\pm$ 4.1	47 $\pm$ 0.8
6	0.00 $\pm$ 0.00	3.81 $\pm$ 0.34	45 $\pm$ 1.6	46 $\pm$ 0.7

embryo hepatocytes treated with TCB accumulate URO without a decrease in uroporphyrinogen decarboxylase activity. Inter-assay variation of enzyme activity was determined by using 10000 g supernatants from livers of untreated 19-day-old chick embryos, and these samples had a mean activity of 45 pmol/min per mg of protein, with a coefficient of variation of 11% ( $n = 18$ ).

Iron and HCB have been shown to cause accumulation of hepatic URO in mice in association with decreased uroporphyrinogen decarboxylase activity (Smith *et al.*, 1986). When chick-embryo hepatocytes were treated with 0.1 mM-ferric nitriloacetate (a treatment previously shown to increase iron content of these cells; Shedlofsky *et al.*, 1983) and either HCB or TCB for 48 h, the cells accumulated large amounts of URO, in both the presence and absence of excess iron, but none of these treatments caused any significant decrease in uroporphyrinogen decarboxylase activity (Fig. 3). Treatment with PHAs for 48 h was toxic to these cells, as evidenced by their rounded appearance, and the presence of an increased number of floating cells.

Previous experiments with chick-embryo hepatocytes had indicated that treatment with TCB plus 2-propyl-2-isopropylacetamide would cause maximal URO accumulation from endogenously generated 5-aminolaevulinic acid (Sinclair *et al.*, 1984). To determine if this combination of treatments could cause decreased uroporphyrinogen decarboxylase activity, cultures were treated with vehicle alone, 3.4  $\mu\text{M}$ -TCB alone, or with TCB plus 0.14 mM-2-propyl-2-isopropylacetamide for 24 h. These treatments resulted in URO accumulations (mean  $\pm$  s.d.) of 0.05  $\pm$  0.02, 8.6  $\pm$  1.1 and 12.8  $\pm$  0.64 nmol/dish for control, TCB and TCB plus 2-propyl-2-isopropylacetamide respectively. Uroporphyrinogen decarboxylase activity, however, was not significantly affected by any of the treatments (mean  $\pm$  s.d. of 47  $\pm$  3.5, 52  $\pm$  4.2 and 50  $\pm$  4.6 pmol/min per mg of protein for control, TCB, and TCB plus 2-propyl-2-isopropylacetamide respectively).



**Fig. 3. Effect of iron, HCB and TCB on URO accumulation ( $\square$ ) and uroporphyrinogen decarboxylase activity ( $\blacksquare$ ) in cultured chick-embryo hepatocytes**

Cells were treated with 100  $\mu\text{M}$ -ferric nitriloacetate, 3.5  $\mu\text{M}$ -HCB or 3.4  $\mu\text{M}$ -TCB for 26 h, then the medium was changed and the cells were treated with the same chemicals for an additional 21 h. URO accumulation and uroporphyrinogen decarboxylase activity were determined as described in the Experimental section. Control cells and cells treated with iron alone accumulated less than 0.01 nmol of URO per dish. Values are means  $\pm$  s.d. for three dishes.

**Table 2. Effect of TCB and other compounds on URO accumulation and uroporphyrinogen decarboxylase activity in cultured chick-embryo hepatocytes**

Cells were treated as indicated with 43  $\mu\text{M}$ -nifedipine, 85  $\mu\text{M}$ -carbamazepine, 69  $\mu\text{M}$ -parathion or 3.4  $\mu\text{M}$ -TCB for 19 h. URO accumulation and uroporphyrinogen decarboxylase activity were determined as described in the Experimental section. Values represent means  $\pm$  s.d. for triplicate dishes, except as indicated for carbamazepine in Expt. 1, where the enzyme assay was performed on duplicate dishes (individual values are given in parentheses).

Expt.	Treatment	URO accumulation (nmol/dish)	Uroporphyrinogen decarboxylase activity (pmol/min per mg of protein)
1	Control	0.06 $\pm$ 0.03	43 $\pm$ 6.0
	Parathion	0.43 $\pm$ 0.04	42 $\pm$ 7.1
	Carbamazepine	1.02 $\pm$ 0.36	39 (39,39)
	Nifedipine	1.43 $\pm$ 0.55	44 $\pm$ 5.4
	TCB	3.12 $\pm$ 0.12	41 $\pm$ 2.3
2	Control	0.01 $\pm$ 0.02	52 $\pm$ 3.9
	Nifedipine	0.12 $\pm$ 0.07	52 $\pm$ 2.5
	Carbamazepine	0.36 $\pm$ 0.03	55 $\pm$ 10.8
	Parathion	0.77 $\pm$ 0.02	46 $\pm$ 1.4
	TCB	2.22 $\pm$ 0.27	49 $\pm$ 7.6

In addition to TCB and HCB, other chemicals have been reported to cause URO accumulation and decreased uroporphyrinogen decarboxylase activity in chick-embryo hepatocytes. These compounds include carbamazepine (Schoenfeld *et al.*, 1985a) and nifedipine (Schoenfeld *et al.*, 1985b). Table 2 shows the effect of these compounds as well as parathion on URO accumulation and uroporphyrinogen decarboxylase activity, and

**Table 3. Pentacarboxyporphyrinogen III or uroporphyrinogen III as substrates in uroporphyrinogen decarboxylase assays in control or TCB-treated cultured chick-embryo hepatocytes**

Cells were treated with vehicle alone or 3.4  $\mu\text{M}$ -TCB for 22 h. URO accumulation and uroporphyrinogen decarboxylase activity, using either 5.8  $\mu\text{M}$ -pentacarboxyporphyrinogen III or 7.9  $\mu\text{M}$ -uroporphyrinogen III as substrate, were determined on pooled samples as described in the Experimental section. Values for uroporphyrinogen decarboxylase activity are given as means (and individual values) for coproporphyrinogen III formation from pentacarboxyporphyrinogen III or as mean  $\pm$  s.d. for triplicate determinations for heptacarboxyporphyrinogen III formation from uroporphyrinogen III.

Treatment	URO accumulation (nmol/dish)	Uroporphyrinogen decarboxylase activity (pmol/min per mg of protein) of protein)	
		Pentacarboxyporphyrinogen	Uroporphyrinogen
Control	0.02	57 (55, 59)	22 $\pm$ 1.5
TCB	4.14	52 (49, 54)	25 $\pm$ 1.7

indicates that all of these compounds, under these culture conditions, caused a significant accumulation of URO without any significant decrease in uroporphyrinogen decarboxylase activity.

Table 3 shows that TCB-treated cells accumulated significantly increased amounts of URO, but uroporphyrinogen decarboxylase activity was not significantly decreased with either uroporphyrinogen III or pentacarboxyporphyrinogen III as substrates. These results indicate that URO present in these samples (approx. 0.3  $\mu\text{M}$ ) did not inhibit enzyme activity. Pentacarboxy-

porphyrinogen III was decarboxylated at least twice as quickly as uroporphyrinogen III. Under these assay conditions (0.3 mg of protein/ml of incubation volume; 30 min incubation time), the only significant amount of product accumulated from uroporphyrinogen was heptacarboxyporphyrinogen.

#### Japanese quail

Compared with rodent models, Japanese quail treated with PHAs accumulate hepatic URO relatively rapidly, that is, in days rather than in weeks or months (Carpenter *et al.*, 1985). Table 4 shows the results from two separate experiments where quail were treated with HCB (500 mg/day per kg) and hepatic URO accumulation and uroporphyrinogen decarboxylase activity were measured. These quail developed porphyria by 7 or 10 days, but the response varied widely from individual to individual. The three most porphyric quail in these two experiments had hepatic URO concentrations of 0.5, 1.3 and 5.2 nmol/mg of protein and uroporphyrinogen decarboxylase activities of 97, 84 and 111 pmol/min per mg of protein respectively. These values for uroporphyrinogen decarboxylase activities are not decreased relative to control values (Table 4).

#### Mice

URO accumulation and uroporphyrinogen decarboxylase activity were measured in mice treated with iron and HCB, as shown in Fig. 4. All of the treated mice had a lower enzyme activity than the controls, a result that is in substantial agreement with previous reports (Smith & Francis, 1983; Smith *et al.*, 1986; Elder *et al.*, 1986). In control mice, the values for  $K_m$  and  $V_{max}$ , derived from a Lineweaver-Burk plot, were 2.3  $\mu\text{M}$  and 100 pmol/min per mg of protein, respectively for pentacarboxyporphyrinogen III (results not shown). These values are similar to those previously reported (Francis & Smith, 1984).

#### DISCUSSION

In the present paper we have found conditions under

**Table 4. Effect of HCB and  $\beta$ -naphthoflavone (BNF) on hepatic porphyrin accumulation and uroporphyrinogen decarboxylase activity in Japanese quail**

Japanese quail were treated orally with HCB (500 mg/day per kg) for 7 days (Expt. 1) or with four doses of BNF (150 mg/day per kg) by i.p. injection before HCB (500 mg/day per kg) for 10 days (Expt. 2) as described in the Experimental section. Porphyrin accumulation was determined by h.p.l.c., and uroporphyrinogen decarboxylase activity, using pentacarboxyporphyrinogen III as substrate, was determined in 10000 g supernatants as described in the Experimental section. Values are means (with range in parentheses) for porphyrin accumulation and means  $\pm$  s.d. for enzyme activity.

Expt.	Treatment	n	Porphyrin accumulation (pmol/mg of protein)		Uroporphyrinogen decarboxylase activity (pmol/min per mg of protein)
			URO	Heptacarboxyporphyrin	
1	Control	3	< 1	< 1	83 $\pm$ 23
	HCB	4	397 (< 1-1320)	51 (< 1-121)	77 $\pm$ 25
2	Control	4	< 1	< 1	69 $\pm$ 11
	BNF	4	< 1	< 1	75 $\pm$ 5
	HCB	4	24 (17-31)	8 (4-11)	81 $\pm$ 13
	BNF plus HCB	4	1412 (71-5172)	155 (16-496)	96 $\pm$ 10

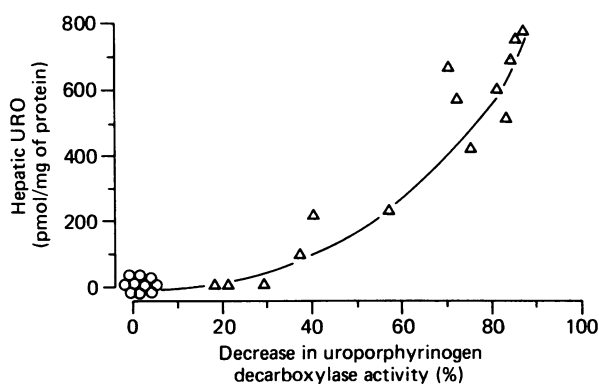


Fig. 4. Hepatic URO accumulation versus inhibition of uroporphyrinogen decarboxylase activity from individual mice treated with iron and HCB.

Male C57BL/6 mice were treated with iron and HCB and killed after 70 days as described in the Experimental section. Hepatic URO accumulation was determined spectrofluorimetrically, and uroporphyrinogen decarboxylase activity, using pentacarboxyporphyrinogen III as substrate, was determined in 10000 g supernatants as described in the Experimental section. Untreated control mice had hepatic URO concentrations of less than 10 pmol/mg of protein, and a mean enzyme activity of 110 pmol/min per mg of protein, a value that was used to calculate inhibition of enzyme activity. Values are the results for individual mice. Symbols: ○, control; △, treated.

which URO accumulated in cultured chick-embryo hepatocytes and in livers of Japanese quail after treatment with PHAs and other compounds with no detectable decrease in uroporphyrinogen decarboxylase activity (Tables 1, 2 and 4). These results and our previous observation that piperonyl butoxide rapidly and totally reverses URO accumulation (Sinclair *et al.*, 1986) indicate that PHA-induced URO accumulation in chick-embryo hepatocytes and in Japanese-quail liver occurred by a mechanism which was independent of decreased uroporphyrinogen decarboxylase activity.

Since there are several reports of conditions under which chick-embryo hepatocytes treated with PHAs, and other compounds, accumulate URO with an associated decrease in uroporphyrinogen decarboxylase activity (Sinclair *et al.*, 1983; Swain *et al.*, 1983; de Verneuil *et al.*, 1983; Nichol & Angel, 1984; Schoenfeld *et al.*, 1985a,b), we paid particular attention to the validation of the uroporphyrinogen decarboxylase assay. The assay was shown to be linear with respect to protein concentration and incubation time for both control and TCB-treated cells (Fig. 1) and was highly reproducible (Table 1). Two different substrates, pentacarboxyporphyrinogen III and uroporphyrinogen III, were used, and neither indicated decreased enzyme activity in TCB-treated cells (Table 3). The uroporphyrinogen decarboxylase activities of untreated chick-embryo hepatocytes were similar to, or exceeded, those previously reported (Sinclair *et al.*, 1983; Swain *et al.*, 1983; de Verneuil *et al.*, 1983). Finally, this assay detected decreased uroporphyrinogen decarboxylase activity in livers of mice treated with iron and HCB (Fig. 4), indicating that the assay is capable of detecting a decrease in enzyme activity.

Other workers have reported decreases (10–15%) in uroporphyrinogen decarboxylase activity in chick-

embryo hepatocytes treated with PHAs and other compounds. Swain *et al.* (1983) used TCB and reported a small decrease in uroporphyrinogen decarboxylase activity when uroporphyrinogen III was used as substrate. Under our conditions, the TCB-treated cells accumulated URO with no significant decrease in enzyme activity using either pentacarboxyporphyrinogen III or uroporphyrinogen III as substrate (Table 3). de Verneuil *et al.* (1983) reported that Aroclor 1254 and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) caused decreases in enzyme activity. We found no decrease in uroporphyrinogen decarboxylase activity caused by TCDD in experiments similar to those described here (results not shown). Other compounds, including organophosphorous pesticides (Nichol & Angel, 1984) and calcium-channel-blocking drugs (Schoenfeld *et al.*, 1985a,b), have been reported to cause both URO accumulation and decreased uroporphyrinogen decarboxylase activity. Under our culture and assay conditions, parathion, carbamazepine or nifedipine caused URO accumulation; however, no decrease in enzyme activity was observed after treatment with these compounds (Table 2). We found that cells treated with paraoxon, another organophosphorous pesticide reported to cause both URO accumulation and decreased uroporphyrinogen decarboxylase activity, gave variable results (R. Lambrecht and P. Sinclair, unpublished work). Differences between our current results and those previously reported may be due to different culture conditions or different methods of preparation and storage of samples.

It is not clear that the amount of decrease of uroporphyrinogen decarboxylase activity reported previously would cause URO to accumulate in these cells. This enzyme is not normally rate-limiting in haem biosynthesis; this raises the question of how great a decrease is required before uroporphyrinogen would accumulate. If inhibition of uroporphyrinogen decarboxylase were the sole cause of URO accumulation, then we would expect that the enzyme activity would have to be decreased by more than 50% before appreciable amounts of uroporphyrinogen would accumulate. Furthermore, the decrease in uroporphyrinogen decarboxylase activity would have to precede URO accumulation in cultured chick-embryo hepatocytes treated with TCB.

Japanese quail treated with HCB were also found to accumulate hepatic URO without decreased uroporphyrinogen decarboxylase activity (Table 4). This is the first report of an intact-animal model accumulating hepatic URO after treatment with PHAs with no decrease in enzyme activity and is consistent with our results with cultured chick-embryo hepatocytes.

If, as we suggest, the URO accumulation caused by PHAs is independent of uroporphyrinogen decarboxylase, what then is the mechanism by which PHAs cause URO to accumulate? The process of URO accumulation appears to involve a specific isoenzyme of cytochrome *P*-450 that is induced by 3-methylcholanthrene (Sinclair *et al.*, 1984). Several authors have suggested that oxidation of uroporphyrinogen is a possible cause of URO accumulation (Heikel *et al.*, 1958; Ferioli *et al.*, 1984; Mukerji *et al.*, 1984). We suggest that URO accumulation in these cells is caused by oxidation of uroporphyrinogen to URO by the PHA-induced cytochrome *P*-450. This proposed mechanism is sup-

ported by recent observations that supernatants or microsomal fractions from chick embryos treated with 3-methylcholanthrene convert uroporphyrinogen into URO by an NADPH-dependent reaction which is inhibited by CO or other appropriate inhibitors of cytochrome *P*-450 and by antibody to the appropriate cytochrome *P*-450 isoenzyme (Sinclair *et al.*, 1987). For reasons yet to be determined, this oxidation requires the presence of TCB, perhaps owing to the binding of PHAs to cytochrome *P*-450 *d* (Voorman & Aust, 1987). The oxidation of uroporphyrinogen is also catalysed by microsomal fractions from rodents treated with 3-methylcholanthrene (J. Jacobs & P. Sinclair, unpublished work).

It has been clearly established that rodents treated with PHAs accumulate URO and have decreased hepatic uroporphyrinogen decarboxylase activity (Elder *et al.*, 1976; Jones & Sweeney, 1980; Smith *et al.*, 1986). We have confirmed these observations in C57BL/6 male mice treated with iron and HCB, and we have used these samples to demonstrate that our enzyme assay could detect decreased activity (Fig. 4). We propose a two-stage mechanism by which PHAs can cause URO accumulation. In the first stage, the role of the PHA is to induce a specific isoenzyme of cytochrome *P*-450, which causes the oxidation of uroporphyrinogen to URO in a manner analogous to that described above for chick-embryo hepatocytes and for Japanese quail. In rodents there is a second stage, in which uroporphyrinogen decarboxylase becomes inhibited, uroporphyrinogen accumulates and is oxidized to URO, which accumulates to even greater levels than in the first stage. The relationship of these two stages is not yet clear; however, Cantoni *et al.* (1984) and Smith & Francis (1987) have provided evidence that a heat-stable compound is responsible for the irreversible inactivation of uroporphyrinogen decarboxylase.

The results reported here suggest that avian uroporphyrinogen decarboxylase is less susceptible to inactivation by PHAs than is the rodent or human enzyme. Further work is required to establish the nature of this resistance to inactivation.

#### Note added in proof (received 3 May 1988)

Since this paper was accepted for publication, Lyon *et al.* (1988) have reported a 39% inhibition of uroporphyrinogen decarboxylase activity in cultured chick-embryo hepatocytes treated with 34  $\mu$ M-TCB, and De Matteis *et al.* (1988) have reported that microsomal fractions from the livers of  $\beta$ NF-treated rats cause oxidation of uroporphyrinogen to URO.

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