Increased oxidation of uroporphyrinogen by an inducible liver microsomal system

Possible relevance to drug-induced uroporphyria

Francesco DE MATTEIS,* Carolyn HARVEY, Celia REED and Rixta HEMPENIUS

M.R.C. Toxicology Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

1. The hypothesis that uroporphyria-inducing drugs stimulate the oxidation of uroporphyrinogen by a microsomal NADPH-dependent mechanism was tested. 2. 3,4,3',4'-Tetrachlorobiphenyl, a very effective inducer of uroporphyria in chick-embryo hepatocyte cultures, stimulates the NADPH-dependent oxidation of uroporphyrinogen by chick-embryo microsomal fraction *in vitro*. 3. Two different actions of 3,4,3',4'-tetrachlorobiphenyl are apparently required for this effect: (a) induction of a microsomal system by treatment *in vivo* and (b) interaction with the induced microsomal fraction *in vitro*, producing an oxidizing species. 4. The analogue 2,4,2',4'-tetrachlorobiphenyl is relatively ineffective in both the production of porphyria in culture and the stimulation of porphyrinogen oxidation *in vitro*. 5. Rat hepatocytes do not develop uroporphyria when treated with polychlorinated biphenyls in culture, yet they respond to these drugs with typical induction of cytochrome *P*-448-dependent drug metabolism. 6. These data provide support for the hypothesis of an increased oxidation of uroporphyrinogen in drug-induced uroporphyria, but also suggest that induction of cytochrome *P*-448 is not the only factor involved. 7. Both I and III isomers of uroporphyrin and heptacarboxylate porphyrin accumulate when chicken hepatocytes are made uroporphyrinic by drugs; treatment with desferrioxamine causes a marked decrease in both isomers, suggesting that iron may be involved in the accumulation of both.

INTRODUCTION

Previous work (Sinclair & Granick, 1974; Swain *et al.*, 1983; De Verneuil *et al.*, 1983) has shown that chick-embryo hepatocytes in culture are a very sensitive cell system for drug-dependent induction of uroporphyria, a metabolic disorder that closely resembles porphyria cutanea tarda, the most common type of human hepatic porphyria. Because of the potentiation of the disorder by excess iron and also on account of the variety of precipitating drugs that are effective in this cell-culture system, the hypothesis has been put forward (De Matteis & Stonard, 1977; Ferioli *et al.*, 1984) that the drugs responsible may interact with the NADPH-cytochrome *P*-450 reductase/cytochrome *P*-450 system of the hepatocyte leading to mobilization of stored iron and also to production of reduced oxygen species, such as H_2O_2 . Accumulation of uroporphyrin may then result from inhibition of uroporphyrinogen decarboxylase by some mechanism requiring non-haem iron. A contributing factor may be direct oxidation of uroporphyrinogen III to uroporphyrin by peroxides (Ferioli *et al.*, 1984), since the oxidized porphyrin cannot be further metabolized but can still bind the decarboxylase (Elder *et al.*, 1983) and thus inhibit the decarboxylation reaction (Smith & Francis, 1981). Evidence is now presented that 3,4,3',4'-tetrachlorobiphenyl (3,4-TCB), one of the most powerful uroporphyria-inducing drugs, can in fact stimulate the NADPH-dependent oxidation of uroporphyrinogen by hepatic chick-embryo microsomal fraction (referred to below simply as microsomes) *in vitro*, whereas the

relatively ineffective analogue 2,4,2',4'-tetrachlorobiphenyl (2,4-TCB) cannot. A similar oxidation of uroporphyrinogen is also shown *in vitro* with a typical H_2O_2 -producing system, that involving the enzyme glucose oxidase. Sinclair *et al.* (1987) have also reported stimulation by 3,4-TCB of oxidation of uroporphyrinogen by microsomes of 3-methylcholanthrene-induced chick embryos that they found to be inhibited by selective inhibitors of the 3-methylcholanthrene-inducible cytochrome *P*-450.

In contrast with the chick-embryo hepatocytes, hepatocytes from adult rats do not develop uroporphyria when treated with polychlorinated biphenyls in culture, yet they respond to these drugs with typical induction of cytochrome *P*-448-dependent drug metabolism. The lack of uroporphyria response of the rat in this short-term test system appears to correlate with the inability of 3,4-TCB to stimulate the oxidation of uroporphyrinogen by induced rat liver microsomes *in vitro*. These studies therefore provide support for the hypothesis of an increased oxidation of uroporphyrinogen in drug-induced uroporphyria, but also suggest that, although induction of cytochrome *P*-448 by these drugs is probably required, it cannot be the only factor involved.

MATERIALS AND METHODS

Treatment of animals

Chick embryos, 16 days of age, of the Rhode Island Red × White Rock strain, were obtained from Orchard

Abbreviations used: 3,4-TCB, 3,4,3',4'-tetrachlorobiphenyl; 2,4-TCB, 2,4,2',4'-tetrachlorobiphenyl; α -NF, α -naphthoflavone; β -NF, β -naphthoflavone; copro, coproporphyrin; uro, uroporphyrin; proto, protoporphyrin; hepta, heptacarboxylate porphyrin; hexa, hexacarboxylate porphyrin; penta, pentacarboxylate porphyrin; ERDE, 7-ethoxyresorufin *O*-de-ethylase.

* To whom correspondence should be addressed.

Farm, Pinner, Middx., U.K. Liver microsomes were prepared 24 h after a single dose of 3,4-TCB (145 µg/embryo), 2,4-TCB (3 mg/embryo) or β -naphthoflavone (β -NF) (3 mg/embryo), the first two drugs dissolved in 10 µl of 1,4-dioxan and the last in 100 µl of dimethyl sulphoxide, given by injection through the air sac into the fluids surrounding the embryo. Liver microsomes were also obtained from control embryos, which had received no treatment. 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 by differential centrifugation, as described by Unsel & De Matteis (1978), washed with 1.15% (w/v) KCl and stored frozen (at -70 °C) as a pellet overlaid with 0.5 ml of the KCl solution, until needed.

Locally bred fed adult male rats of the Porton Wistar-derived strain (body wt. 180–200 g) were also used. Treatment of rats with β -NF involved two intraperitoneal injections of a solution in arachis oil, with a total dose of either 80 or 120 mg/kg, over 2 days. The animals were killed 24 h after the last injection, and their liver microsomes were obtained and stored as described above for the chick.

Enzymic assays

The cytochrome P-450 content of isolated microsomes was determined by the method of Omura & Sato (1964). The 7-ethoxyresorufin O-de-ethylase (ERDE) activity of the microsomes isolated from the liver of intact animals or from isolated hepatocytes was assayed by the method of Lake & Paine (1983) as modified by Reed *et al.* (1986).

Preparation and treatment of cultures

Chick-embryo hepatocytes (viability usually > 90%) were obtained and cultured (0.9×10^6 cells/ml of culture medium) as described previously (Feroli *et al.*, 1984), in either 100 mm- or 150 mm-diameter dishes (10 ml or 20 ml/dish) or eight-well multi-plates (2 ml/well). A change to serum-free medium was carried out at 20 h and again at 22 h, drugs and/or 5-aminolaevulinate were added at 24 h after seeding and the cultures were examined 19 h later. Rat hepatocytes were obtained by a collagenase perfusion technique (Paine & Legg, 1978), and viability was 81–94% at time of seeding. The method followed for culturing rat hepatocytes and the composition of the medium were identical with those used for chick-embryo hepatocytes, but the number of cells was lowered to 0.5×10^6 /ml of culture.

In order to obtain microsomes, freshly obtained hepatocytes or the combined cultured cells of six to eight 150 mm-diameter dishes were first washed with excess phosphate-buffered saline at room temperature, then resuspended in ice-cold 0.25 M-sucrose, and homogenized by three 5 s bursts in an Ultra-Turrax homogenizer at 0 °C; the homogenate was then subjected to differential centrifugation, and the microsomal fraction was tested for ERDE activity without delay.

Analytical techniques

Porphyrins were routinely assayed in cell extracts and in media as described previously (Feroli *et al.*, 1984) by employing the fluorimetric technique of Grandchamp *et al.* (1980) and a program containing a matrix constructed with standard solutions of coproporphyrin,

uroporphyrin and protoporphyrin (copro, uro and proto). Cellular proteins were determined by the method of Lowry *et al.* (1951), after solubilization in alkali of the acid-extracted cell monolayers. A reverse-phase gradient-elution h.p.l.c. technique was also employed to characterize the composition and isomeric type of the accumulating porphyrins, as described elsewhere (De Matteis *et al.*, 1987): the absorbance of the eluate was monitored at 400 nm and the areas under the elution profile corresponding to the porphyrin peaks (given by a Shimadzu model C-RIA integrator) were converted into pmol of the various porphyrins by using appropriate standards.

Oxidation of porphyrinogens *in vitro*

Uroporphyrin III, heptacarboxylate porphyrin I and coproporphyrin III (all free carboxylate) were fully reduced to the corresponding porphyrinogens by treatment with sodium amalgam under N₂. The oxidation of porphyrinogens *in vitro* was monitored in disposable plastic cuvettes [incubated in the presence of air in the thermostatically controlled (28 °C) compartment of a Cary 2200 spectrophotometer], by following the increase in absorption at the Soret maximum (390 nm for copro, 396 nm for hepta and 396.5 nm for uro), against a blank of identical composition but containing no porphyrinogen. In most experiments the samples were scanned at the end of the incubation to verify that the recorded increase in absorbance was in fact due to the Soret maximum, and in some experiments the spectrum was also scanned between 600 and 350 nm during the progress of the re-oxidation reaction. When the effect of glucose oxidase on the rate of oxidation of the porphyrinogens was studied, the incubation mixture contained, in a total volume of 3.4 ml, the following components (with final concentrations in parentheses): sodium/potassium phosphate buffer, pH 7.4 (0.1 M), EDTA (1 mM), glucose (9.5 mg/ml) and glucose oxidase (35.3 µg/ml); after a preliminary incubation of 10 min, during which a baseline absorbance was recorded, uro- or copro-porphyrinogen was added and the rate of oxidation monitored.

In the experiments with liver microsomes the total volume of the incubation mixture was 3.3 ml and the following components were present (with final concentrations in parentheses): sodium/potassium phosphate buffer, pH 7.4 (0.1 M), EDTA (1 mM), NADPH or NADH (73 µM), microsomes from control or induced livers (corresponding to a concentration of cytochrome P-450 or P-448 of 60–70 pmol/ml) and dimethyl sulphoxide (3 µl/ml) with or without the drug to be tested. After a preliminary incubation of 10 min, uroporphyrinogen or heptacarboxylate porphyrinogen was added to the sample cuvette and the rate of oxidation was monitored. In experiments with induced chick-embryo microsomes, the rate of oxidation of uroporphyrinogen in presence of NADPH (alone or together with 3,4-TCB) could not be saturated by increasing the concentration of the porphyrinogen from 1.6 to 12.3 µM; instead, it increased linearly with amount of porphyrinogen added. Because of this a 2.4 µM concentration of porphyrinogen was arbitrarily chosen for all experiments with liver microsomes. With both liver microsomes and glucose oxidase a lag phase in the oxidation of the porphyrinogen was observed, and this was followed by a period in which the rate of oxidation became gradually greater, until the maximum and approximately

Table 1. Effect of several drugs on the concentration and composition of porphyrins accumulating in chick-embryo hepatocyte cultures in the absence (a) and in the presence (b) of exogenous 5-aminolaevulinic acid

Cultures of hepatocytes were prepared in multi-well plates as described in the Materials and methods section, exposed to different drugs in the presence and in the absence of exogenous 5-aminolaevulinic acid (ALA) (25 µg/ml of culture) at 24 h, and 19 h later the concentration and composition of the porphyrin accumulating in cells and media were determined by the fluorimetric assay. Results are means ± S.E.M. for four observations obtained in one experiment, which was confirmed once for α-NF and β-NF and twice for the biphenyls. They are given as pmol of total porphyrins/mg of cellular protein and as percentage of total porphyrin, which is represented by either uro or proto (copro representing the porphyrins unaccounted for in all cases). The sign – indicates that the corresponding porphyrin could not be detected.

Drug added (µg/ml of medium)	(a) Exogenous ALA absent			(b) Exogenous ALA present		
	Total porphyrins (pmol)	Percentage of total		Total porphyrins (pmol)	Percentage of total	
		Uro	Proto		Uro	Proto
None	104 ± 3	20 ± 0.3	33 ± 3	2360 ± 90	17 ± 1.5	62 ± 2.5
3,4-TCB (0.025)	860 ± 75	87 ± 1.0	3 ± 0.5	2500 ± 40	91 ± 0.5	2 ± 0.05
3,4-TCB (0.25)	1870 ± 80	96 ± 0.5	–	2460 ± 42	96 ± 0.25	–
3,4-TCB (1.00)	2360 ± 50	97 ± 0.2	–	2650 ± 40	97 ± 0.25	–
2,4-TCB (0.25)	147 ± 4	21 ± 1.0	23 ± 2.5	2220 ± 40	40 ± 2.5	38 ± 3
2,4-TCB (1.00)	200 ± 17	23 ± 0.5	23 ± 1	2160 ± 35	55 ± 3.0	25 ± 3
2,4-TCB (10.0)	646 ± 45	55 ± 3.0	5 ± 1	2100 ± 75	73 ± 2.0	9 ± 1.5
α-NF (2.5)	360 ± 20	45 ± 2.0	14 ± 0.5	2270 ± 65	53 ± 1.5	28 ± 1
β-NF (2.5)	296 ± 20	43 ± 0.5	16 ± 1.6	1940 ± 37	75 ± 0.5	7 ± 1

linear rate was obtained. Rates given refer to the maximum rates observed.

Sources of special chemicals

The source of the culture medium and of its components has been given previously (Ferioli *et al.*, 1984). NADPH (type III) and catalase (11 000 units/mg) were obtained from Sigma Chemical Co., Poole, Dorset, U.K.; NADH (grade II) and glucose oxidase (grade I, 280 units/mg) were from Boehringer, Lewes, East Sussex, U.K.; desferrioxamine mesylate was from Ciba Laboratories, Horsham, West Sussex, U.K.; 7-ethoxyresorufin was from Pierce Chemical Co., Rockford, IL, U.S.A.; uroporphyrin III, heptacarboxylate porphyrin I and coproporphyrin III were from Porphyrin Products, Logan, UT, U.S.A. Aroclor 1254 (a mixture of polychlorinated biphenyls) was from Monsanto Co., St. Louis, MO, U.S.A., 2,4-TCB was from Analabs, New Haven, CT, U.S.A., and 3,4-TCB was from Ultra Scientific, Hope, RI, U.S.A. Tetrabutylammonium hydroxide (0.1 M solution containing phosphate buffer) was obtained from Fisons, Loughborough, Leics., U.K.

RESULTS AND DISCUSSION

Induction of uroporphyrin by drugs in cultures of chick-embryo hepatocytes

Previous work (Goldstein *et al.*, 1977, and references cited therein; Kawanishi *et al.*, 1978; Sinclair *et al.*, 1984; Sassa *et al.*, 1986) has suggested that the ability of polychlorinated biphenyls to inhibit uroporphyrinogen metabolism and to induce uroporphyrin depends on their planar configuration, a property also known to be important for the induction of cytochrome P-448 by this class of compounds. In agreement with this we now find

that, although both 3,4-TCB and 2,4-TCB (a planar and a non-planar isomer respectively) induced accumulation of uroporphyrin in chick-embryo hepatocyte cultures, the former was markedly more active (Table 1). Two other compounds that are known to induce and/or interact with cytochrome P-448, β-NF and α-NF, also induced uroporphyrin in this system at relatively low concentrations [Table 1, and see also Debets *et al.* (1980) for similar findings on β-NF]. It will be noticed that, when these drugs were added together with exogenous 5-aminolaevulinic acid, in amounts saturating the porphyrin-producing capacity of the hepatocytes (Sinclair *et al.*, 1983), the accumulation of uro was increased at the expense of proto (Table 1), a finding compatible with a block in uroporphyrinogen metabolism in all cases.

Isomeric type of the accumulating porphyrins

The type of porphyrin accumulating in control and treated cultures was further analysed by a reverse-phase gradient elution h.p.l.c. technique that allows simultaneous separation of type I and type III isomers of octa-, hepta-, hexa-, penta- and tetra-carboxylated porphyrins (referred to below as uro, hepta, hexa, penta and copro respectively), and also of protoporphyrin IX (proto). After incubation with excess 5-aminolaevulinic acid for 19 h, the hepatocytes accumulated mostly proto, but uro and hepta of both isomeric types were also found in the cell extract (Table 2), and penta and copro, both I and III isomers, accumulated in the medium (Table 2). When 3,4-TCB was added together with 5-aminolaevulinic acid, proto and both isomers of penta and copro decreased, whereas uro and hepta of both isomeric types accumulated in excess (Table 2). Under these conditions traces of hexa (retention time 16–18 min) also appeared in the cell extracts. Essentially the same picture was obtained with other uroporphyrin-inducing drugs, whether halogenated (2,4-TCB) or not (2,2-diethyl-2-phenylacet-

Table 2. Amount and isomeric type of porphyrins accumulating in chick-embryo hepatocyte cultures in the presence of exogenous 5-aminolaevulinic acid: effects of 3,4-TCB and desferrioxamine

Cultures of hepatocytes were prepared in 100 mm-diameter dishes; 5-aminolaevulinic acid (ALA), desferrioxamine (DES, 1.5 mM) and a tetrachlorobiphenyl (3,4-TCB) were added at 24 h. Then 19 h later porphyrins were extracted from both cells and media (or from cells alone in last two experiments), concentrated on Sep-Pak C₁₈ cartridges and separated as the free carboxylic acids on h.p.l.c. Results are means $\pm$ S.E.M. for the numbers of observations indicated in parentheses each obtained with a different cell preparation, or averages with individual results in parentheses. Abbreviation: N.D. indicates that these two porphyrins could not be determined as their retention time coincided with the elution of the Phenol Red present in the culture medium. The sign - indicates that the corresponding porphyrin could not be detected.

Treatment and dose (μ g/ml of culture)	Porphyrins recovered from culture (pmol/mg of protein)								
	Uro I	Uro III	Hepta I	Hepta III	Penta I	Penta III	Copro I	Copro III	Proto
ALA (25) Cells	90 $\pm$ 22 (4)	67 $\pm$ 18 (4)	30 $\pm$ 4 (4)	75 $\pm$ 19 (4)	—	—	—	—	460 $\pm$ 40 (4)
Medium	16 $\pm$ 3 (4)	Traces	N.D.	N.D.	65 $\pm$ 25 (4)	105 $\pm$ 17 (4)	77 $\pm$ 20 (4)	120 $\pm$ 42 (4)	280 $\pm$ 60 (4)
ALA (25)+3,4-TCB (0.25) Cells	350 $\pm$ 48 (3)	680 $\pm$ 25 (3)	110 $\pm$ 17 (3)	280 $\pm$ 30 (3)	—	—	—	—	14 (traces-20)
Medium	40 $\pm$ 5 (3)	56 $\pm$ 9 (3)	N.D.	N.D.	—	Traces	9 $\pm$ 5 (3)	4 (traces-6)	—
ALA (25) + DES Cells	20 (21, 18)	14 (15, 13)	13 (13, 13)	17 (18, 16)	—	—	—	—	1330 (1440, 1220)
Medium	Traces	Traces	N.D.	N.D.	10 (20, traces)	20 (40, traces)	83 (98, 68)	87 (80, 95)	670 (830, 510)
ALA (25) + 3,4-TCB (0.0025) Cells	90 (85, 95)	130 (130, 130)	27 (24, 30)	130 (115, 145)	—	—	—	—	540 (490, 590)
ALA (25) + 3,4-TCB (0.0025) +DES Cells	12 (11, 12)	14 (13, 16)	12 (13, 11)	16 (13, 20)	—	—	—	—	1180 (1150, 1210)

amide and 3,5-diethoxycarbonylcollidine; results not shown).

In contrast, the iron chelator, desferrioxamine, which limits the supply of iron for incorporation into proto, caused marked accumulation of proto from exogenous 5-aminolaevulinate, as expected (Sinclair & Granick, 1975), but also markedly decreased both isomers of uro and hepta (Table 2). A similar effect of desferrioxamine was found when the chelator was added together with 3,4-TCB and exogenous 5-aminolaevulinate (Table 2).

The results of these experiments are in line with previous findings (Doss *et al.*, 1976; Meyer & Schmid, 1977; Sinclair *et al.*, 1983; Smith & Francis, 1986) that both isomers of hepta and/or uro accumulate when the activity of uroporphyrinogen decarboxylase is inhibited, both in human porphyria cutanea tarda and in experimental uroporphyrin. As the iron chelator desferrioxamine is now found to diminish the accumulation of both isomers of uro and hepta, it is possible that iron may be involved in causing the accumulation of both isomers of these porphyrins seen in uroporphyrin, for example by depressing the activity of uroporphyrinogen decarboxylase (Smith & Francis, 1983). It may be noticed from Table 2 that cultured chick-embryo cells showed accumulation of uro and hepta of both isomeric types from exogenous 5-aminolaevulinate also in the absence of a porphyria-inducing drug, and this too was markedly diminished by treatment with desferrioxamine. This suggests that a mechanism for accumulation of highly carboxylated porphyrins may operate to a small extent also under 'basal' metabolic conditions in these cells, and that this may also be dependent on iron. These aspects will require further study.

Differences between rat and chick-embryo hepatocytes in their responses to polychlorinated biphenyls in culture

In contrast with the great sensitivity of chick-embryo hepatocytes to the uroporphyrin-inducing properties of

drugs, hepatocytes from adult rats were completely unresponsive (Table 3, and see also Ferioli *et al.*, 1986). Aroclor 1254, a powerful uroporphyrin-inducing agent in the chick-embryo system, proved to be completely inactive in elevating the total porphyrin content of the rat cultures when added on its own, nor could it alter the profile of porphyrins accumulating from exogenous 5-aminolaevulinate in favour of uro (Table 3). In spite of the lack of porphyria, rat hepatocytes treated with Aroclor 1254 showed a marked induction of ERDE activity, a specific marker for cytochrome *P*-448 (Burke *et al.*, 1977); in fact, the stimulation of the ERDE activity seen in rat hepatocytes was far greater than in chick-embryo hepatocytes, possibly because the development of porphyria in the latter may have limited the supply of haem and therefore the synthesis of cytochrome *P*-448. This interpretation is supported by the finding (see below) that, when chick embryos were treated with either β -NF or 3,4-TCB *in ovo*, then the extent of induction of ERDE activity was similar to that seen in the intact rat. Ferioli *et al.* (1984) have shown that these drugs cause uroporphyrin only in the chick-embryo hepatocytes in culture, not in the chick embryo treated *in ovo*, and so a limitation in haem supply would only be expected in the former experimental model. The data of Table 3 show clearly that no correlation was found in culture between concentrations of cytochrome *P*-448 (as judged by the ERDE activity) and degree of porphyria. So, if cytochrome *P*-448 is involved in the mechanism of porphyria (see below), it must only be one of several factors.

Oxidation of uroporphyrinogens by liver microsomes *in vitro*

The hypothesis has been put forward by several authors (Heikel *et al.*, 1958; Ferioli *et al.*, 1984; Mukerji *et al.*, 1984; Sinclair *et al.*, 1986) that in uroporphyrin the block in uroporphyrinogen metabolism may arise, at

Table 3. Differences in porphyrin accumulation and induction of ERDE activity in rat and chick-embryo hepatocytes treated with Aroclor 1254 in culture

Cultures were prepared in multi-well plates as described in the Materials and methods section and exposed to Aroclor 1254 (5 μ g/ml of culture) in the presence and in the absence of exogenous 5-aminolaevulinate (ALA) (25 μ g/ml), and the porphyrins accumulating in cells and media were determined as indicated in the legend to Table 1. In parallel experiments hepatocytes from either rat or chick embryos were similarly cultured in 150 mm-diameter plates and exposed to Aroclor 1254 for 19 h. Their microsomal fraction was then isolated to determine the ERDE activity. The ERDE activity of microsomes from freshly isolated (non-cultured) hepatocytes was found to be 30.3 pmol of resorufin/min per mg of protein and 5.3 pmol/min per mg in the case of rat and chick-embryo hepatocytes respectively. Results are means $\pm$ S.E.M. for the numbers of observations in parentheses. *P < 0.001, when compared with corresponding values obtained without any treatment.

Treatment and dose (μ g/ml of medium)	Porphyrins in cells and media						ERDE activity (pmol of resorufin produced/min per mg of microsomal protein)
	(a) Exogenous ALA absent			(b) Exogenous ALA present			
	Total porphyrins (pmol/mg of protein)	Percentage of total		Total porphyrins (pmol/mg of protein)	Percentage of total		
		Uro	Proto		Uro	Proto	
Rat hepatocytes							
None	62 $\pm$ 4 (5)	15 $\pm$ 1 (5)	66 $\pm$ 0.6 (5)	405 $\pm$ 20 (5)	21 $\pm$ 3 (5)	70 $\pm$ 1 (5)	30.5 $\pm$ 6.0 (3)
Aroclor 1254 (5)	59 $\pm$ 8 (5)	17 $\pm$ 4 (5)	61 $\pm$ 0.7 (5)	378 $\pm$ 9 (5)	23 $\pm$ 4 (5)	64 $\pm$ 3 (5)	2034 $\pm$ 480 (3)*
Chick-embryo hepatocytes							
None	83 $\pm$ 7 (4)	38 $\pm$ 3 (4)	46 $\pm$ 2 (4)	3240 $\pm$ 250 (4)	12 $\pm$ 0.4 (4)	86 $\pm$ 0.4 (4)	7.5 $\pm$ 1.4 (4)
Aroclor 1254 (5)	3240 $\pm$ 250 (4)*	89 $\pm$ 1 (4)*	10 $\pm$ 1 (4)*	3010 $\pm$ 60 (4)	86 $\pm$ 0.3 (4)*	12 $\pm$ 0.4 (4)*	32.5 $\pm$ 2.6 (3)*

least in part, from its oxidation to the corresponding porphyrin. Oxidizing species, such as H_2O_2 , produced by the cytochrome *P*-450 system on interaction with a uroporphyrin-inducing drug have been postulated to be involved (Ferioli *et al.*, 1984; Sinclair *et al.*, 1986). Accordingly the ability of various microsomal preparations (obtained from either control or drug-induced chick embryos and rats) to catalyse conversion of uroporphyrinogen and heptacarboxylate porphyrinogen into the corresponding porphyrins was examined *in vitro*. The ERDE activity of these microsomal preparations (expressed as pmol/min per mg of microsomal protein) was as follows: chick embryos: control, 36; 2,4-TCB-induced, 140; 3,4-TCB-induced, 1600; β -NF-induced, 5030; rats: control, 55; β -NF-induced (80 mg/kg), 5100; β -NF-induced (120 mg/kg), 14800.

In a first series of experiments it was shown that a typical H_2O_2 -producing enzyme system, glucose oxidase, could rapidly convert both uroporphyrinogen (Fig. 1a) and coproporphyrinogen (results not shown) into the corresponding porphyrins, yields of porphyrins in excess of 90% being obtained after 3 h incubation. In agreement with a role of H_2O_2 in the oxidative conversion, catalase almost completely prevented the effect of glucose oxidase when added at the beginning of the incubation, although it was only partially effective when added after the oxidation rate had become established. Repeated scanning of the spectrum during the re-oxidation process confirmed that the increased absorbance at 396.5 nm (Fig. 1a) was in fact due to the Soret band of the porphyrin neutral spectrum; it also demonstrated the early appearance of a peak of absorbance at about 500 nm, a peak, that is, approximately corresponding to band IV of the porphyrin spectrum, but of greater intensity than expected from the amount of porphyrin present, particularly at early time points. In one experiment the absorbances at the Soret maximum and at 497 nm were both monitored during the re-oxidation of uroporphyrinogen by the glucose oxidase system (Fig. 1b): the 497 nm absorbance was found to increase first, preceding the increase in Soret absorbance, most of which took place at a time when the 497 nm absorbance did not show any further increase and was in fact declining. This may suggest the presence of an intermediary in the re-oxidation process or of a transitory reaction product between a H_2O_2 -derived species and the porphyrin(ogen), although this aspect will require further study. A similar spectral species was detected with porphyrinogens undergoing re-oxidation in presence of microsomes (results not shown).

Microsomes isolated from the liver of untreated chick embryos or from embryos treated with either 3,4-TCB or 2,4-TCB were tested *in vitro* for their ability to oxidize uroporphyrinogen III. When microsomes from control and induced chick embryos were supplemented with NADPH, a significant stimulation of uroporphyrinogen oxidation was seen [as compared with either the 'spontaneous' rate of oxidation in the absence of microsomes ($P < 0.001$) or with the rate seen with the same microsomal preparation in the absence of NADPH (Table 4)]. In addition, a pronounced stimulation of the rate of oxidation over that observed with NADPH alone was seen when 3,4-TCB was also added to the microsomal suspension, but this drug-dependent stimulation was only seen with microsomes from 3,4-TCB-induced embryos (Table 4). Dimethyl sulphoxide, the solvent

Fig. 1. Oxidation of uroporphyrinogen by a glucose oxidase system

(a) Rate of increase of Soret (396.5 nm) absorbance with time and effect of catalase added either (curve A) 10 min before uroporphyrinogen or (curve B) 22 min thereafter. (b) Increase in absorbance at 396.5 nm (—) and 497 nm (---). In both panels times of addition of uroporphyrinogen [7.4 nmol in (a) and 3.7 nmol in (b)] and catalase [400 μ g added at 26 min in panel (a)] are indicated by arrows. Note different time scales in panels (a) and (b).

used to add the biphenyl to the microsomal incubation, did not itself influence the rate of oxidation due to NADPH. Catalase inhibited the rate of 3,4-TCB-induced oxidation, but only slightly. The weakly porphyrinogenic 2,4-TCB was almost completely inactive at stimulating uroporphyrinogen oxidation with both control and induced microsomes. Finally, in contrast with NADPH, NADH would not support the 3,4-TCB-dependent oxidation of uroporphyrinogen (Table 4). These results therefore indicate that, of two tetrachlorobiphenyl isomers tested, only the strongly uroporphyrin-inducing (and ERDE-inducing) isomer, 3,4-TCB, can stimulate an NADPH-dependent oxidation of uroporphyrinogen, and two different actions are apparently involved: (a) induction of cytochrome *P*-448 by treatment with 3,4-TCB *in vivo*, and (b) interaction of 3,4-TCB with the induced cytochrome *P*-448 *in vitro*.

This interpretation is supported by the finding that, when chick embryos were treated with β -NF, another inducer of cytochrome *P*-448 (and related ERDE activity), their microsomes not only stimulated uroporphyrinogen oxidation in the presence of NADPH, but again responded to addition of 3,4-TCB with a further increase in rate of oxidation (Table 5). The behaviour of β -NF-induced rat microsomes was different. They showed the NADPH-dependent stimulation of uroporphyrinogen oxidation to an extent even greater than shown by the chick microsomes. However, they failed to elicit any drug-dependent stimulation of uroporphyrino-

Table 4. Microsome-dependent oxidation of uroporphyrinogen III to uroporphyrin *in vitro*: effects of treatment of chick embryos *in vivo* with either 3,4-TCB or 2,4-TCB and of addition of these biphenyls to microsomes from control and pretreatment embryos

Liver microsomes obtained from either control chick embryos or embryos pretreated with a biphenyl *in vivo* were tested *in vitro* for their ability to catalyse oxidation of uroporphyrinogen III in the presence of a cofactor and/or a drug, as indicated in the Materials and methods section. The cytochrome *P*-450 contents (nmol/mg of protein) of the microsomal preparations tested were as follows: 3,4-TCB-induced, 0.44; 2,4-TCB-induced, 0.18; no pretreatment, 0.25; the corresponding ERDE activities are given in the text. Results are expressed as pmol of porphyrinogen oxidized/min per nmol of cytochrome *P*-450 and are means $\pm$ s.e.m. for the numbers of observations in parentheses or averages with individual observations in parentheses. * $P < 0.001$ when compared with corresponding value without NADPH; † $P < 0.001$ when compared with corresponding value without 3,4-TCB; ** $P < 0.001$ when compared with corresponding value obtained when NADPH was the cofactor (rather than NADH). ††These two rates [calculated as pmol of uroporphyrinogen oxidized/min per total incubation, 7.2 ± 2.7 (6)] were not significantly different from the rate of 'spontaneous' oxidation, 7.4 ± 0.2 (3) pmol/min per total incubation, seen in the absence of microsomes, drugs and cofactors.

Pretreatment of chick embryos <i>in vivo</i>	Cofactor	Drug and amount added (μ g)	Other addition (μ g)	Rate of uroporphyrinogen oxidation (pmol/min per nmol of cytochrome <i>P</i> -450)
3,4-TCB	None	None	—	28 ± 1.3 (3)††
	None	3,4-TCB (10)	—	30 ± 2.0 (3)††
	NADPH	None	—	46 ± 1.2 (4)*
	NADPH	3,4-TCB (0.01)	—	57 (57, 57)
	NADPH	3,4-TCB (0.1)	—	75 (72, 78)
	NADPH	3,4-TCB (1.0)	—	94 (91, 97)
	NADPH	3,4-TCB (10.0)	—	94 ± 3.4 (4)††
	NADPH	3,4-TCB (10.0)	Catalase (400)	84 (83, 85)
	NADPH	3,4-TCB (10.0)	Catalase (1000)	77.5 (77, 78)
3,4-TCB	NADH	None	—	34 ± 7 (3)
	NADH	3,4-TCB (10)	—	28 ± 5 (4)**
3,4-TCB	NADPH	2,4-TCB (10)	—	46 ± 0.9 (3)
	NADPH	2,4-TCB (50)	—	42 ± 3.8 (3)
2,4-TCB	NADPH	None	—	52.7 ± 2.0 (3)
	NADPH	3,4-TCB (10)	—	66.5 (64, 69)
	NADPH	2,4-TCB (10)	—	53.5 (53, 54)
None	NADPH	None	—	40.7 ± 1.0 (4)
	NADPH	3,4-TCB (10)	—	42.7 ± 0.7 (4)
	NADPH	2,4-TCB (50)	—	46.9 ± 3.4 (3)

Table 5. Oxidation of uroporphyrinogen III to uroporphyrin *in vitro* by liver microsomes obtained from β -NF-induced rat or chick embryos: differences between rat and chick-embryo microsomes in their responses to addition of 3,4-TCB

Rat and chick embryos were treated with β -NF and their microsomes were tested *in vitro* for oxidation of porphyrinogen, as indicated in the Materials and methods section. Results (averages of the two observations in parentheses or means $\pm$ s.e.m. for the numbers of observations in parentheses) are given as pmol of uroporphyrinogen oxidized/min per nmol of cytochrome *P*-450. The cytochrome *P*-450 contents (nmol/mg of protein) of the microsomal preparations used were as follows: chick, 0.47; rat (80 mg of β -NF/kg), 0.77; rat (120 mg of β -NF/kg), 0.95. The corresponding ERDE activities are given in the text. Rates of uroporphyrinogen oxidation obtained in the presence of NADPH alone are also given as pmol/min per total incubation, to allow comparison with rate of 'spontaneous' re-oxidation of uroporphyrinogen [7.4 ± 0.2 (3)] seen under identical conditions, but in the absence of microsomes, drugs and cofactors. * $P < 0.01$, ** $P < 0.001$, when compared with rate of 'spontaneous' re-oxidation.

Species	Cofactor	Drug and amount added (μ g)	Rate of uroporphyrinogen oxidation	
			(pmol/min per nmol of cytochrome <i>P</i> -450)	(pmol/min per total incubation)
Chick	NADPH	—	45 (44, 46)	91 (89, 93)
	NADPH	3,4-TCB (10)	81 (80.5, 81.5)	—
Rat	NADPH	—	60 ± 5 (6)	127 ± 10 (6)*
	NADPH	3,4-TCB (10)	61 ± 4.5 (7)	—
	NADPH	—	82.5 ± 2.7 (6)	184 ± 4 (6)**
	NADPH	3,4-TCB (10)	73 ± 3 (6)	—

gen oxidation, whether they were added under standard incubation conditions (Table 5) or 10 min after uroporphyrinogen and NADPH or, finally, when 5-fold greater amounts of NADPH were employed (results not shown).

The dependence of the rate of oxidation of the heptacarboxylate porphyrinogen on the concentration of microsomal cytochrome *P*-448 (varying from 30 to 120 pmol/ml) was also studied, with microsomes from β -NF-induced chicks or rats. The results of this experiment (not shown) again confirmed the difference between the microsomes obtained from the two species in their ability to respond to 3,4-TCB *in vitro*, only the chick microsomes showing a drug-dependent stimulation of porphyrinogen oxidation, which was linearly related to the concentration of the cytochrome. These findings therefore indicate that of the two species tested only the chick, which develops porphyria after treatment with polychlorinated biphenyls in culture, will also exhibit a biphenyl-dependent oxidation of either uroporphyrinogen or heptacarboxylate porphyrinogen *in vitro*.

GENERAL DISCUSSION

Heikel *et al.* (1958) first suggested that in uroporphyria the metabolic block in uroporphyrinogen metabolism might be due to oxidative escape of the intermediate from the biosynthetic sequence, resulting in increased accumulation and excretion of the corresponding porphyrin. Polyhalogenated chemicals have long been known to induce uroporphyria in man and experimental animals (Elder, 1978), and the following two alternative hypotheses have been put forward to account for the accumulation of uroporphyrin and the associated defect of uroporphyrinogen decarboxylase. (1) The drug may require metabolic activation by the cytochrome *P*-450 system to a metabolite that inhibits the enzyme uroporphyrinogen decarboxylase (see, e.g., De Verneuil *et al.*, 1983; Carpenter *et al.*, 1984). (2) Alternatively, the drugs may produce the metabolic block indirectly, by interacting with the cytochrome *P*-450 system of the liver microsomes, acting as uncouplers of the mono-oxygenase function of the cytochrome and stimulating the production of reduced oxygen species, such as H_2O_2 (De Matteis & Stonard, 1977; Ferioli *et al.*, 1984; Sinclair *et al.*, 1986). This could either directly oxidize the uroporphyrinogen to uroporphyrin, causing its accumulation, or could lead to a block of uroporphyrinogen decarboxylase by producing a long-lived inhibitor of the enzyme, such as that detected in porphyric livers by Rios de Molina *et al.* (1980) and Cantoni *et al.* (1984).

The present work has now shown that the uroporphyria-inducing property of a biphenyl can be correlated with its ability to promote oxidation of uroporphyrinogen and heptacarboxylate porphyrinogen by liver microsomes *in vitro*. The correlation between induction of uroporphyria in cultured cells and oxidative potential *in vitro* applies not only when comparing a very active porphyria-inducing biphenyl with a relatively inactive isomer, but also when a very sensitive species (the chick embryo) is compared with an unresponsive species (the adult rat).

The drug-dependent oxidation requires NADPH (not NADH), an induced microsomal system and the presence of the drug itself. This is compatible with the proposed mechanism of uncoupling of the microsomal electron

transport, and suggests that two different actions of 3,4-TCB are required for the observed oxidation of porphyrinogens: (a) the drug first acts as an inducer of cytochrome *P*-448 *in vivo*, and (b) it can then interact with the induced cytochrome *in vitro* so as to produce an oxidizing species. Action (a) above is also shared by β -NF (Table 5) and, in similar experiments conducted by Sinclair *et al.* (1987), by 3-methylcholanthrene, possibly because these inducers all stimulate the synthesis of a form of cytochrome *P*-448 that can recognize 3,4-TCB and bind it effectively. Phenobarbitone and 2-isopropyl-2-propylacetamide (inducers of different forms of cytochrome *P*-450) have also been reported to induce uroporphyria in cultured chick-embryo hepatocytes (Marks, 1978; De Verneuil *et al.*, 1983; Ferioli *et al.*, 1984), and it is possible that a similar mechanism involving induction and subsequent uncoupling of a different cytochrome *P*-450 may be involved with these drugs. It must be stressed, however, that rat hepatocytes in culture as well as chick embryos *in ovo* responded to treatment with polychlorinated biphenyls with marked induction of ERDE activity without developing uroporphyria. Also, it will be noticed that the stimulation by 3,4-TCB of porphyrinogen oxidation seen with either chick-embryo or rat microsomes did not correlate with extent of ERDE induction. Perhaps different forms of cytochrome *P*-448 are induced under different experimental conditions or, alternatively, factors other than cytochrome *P*-448 may also be involved.

The relevance of these present findings to the long-lived inhibitor of uroporphyrinogen decarboxylase that has been isolated from the liver of animals with uroporphyria remains to be determined. It is possible that, during or accompanying the drug-induced oxidation of uroporphyrinogen, an oxidative derivative of the porphyrinogen is produced that may be sufficiently stable to survive isolation. This may conceivably act as a targeted inhibitor of uroporphyrinogen decarboxylase. The potentiation of the disorder by iron also requires elucidation.

REFERENCES

- Burke, M. D., Prough, R. A. & Mayer, R. T. (1977) Drug Metab. Dispos. **5**, 1-8
- Cantoni, L., Dal Fiume, D., Rizzardini, M. & Ruggieri, R. (1984) Toxicol. Lett. **20**, 211-217
- Carpenter, H. M., Williams, D. E., Henderson, M. C., Bender, R. C. & Buhler, D. R. (1984) Biochem. Pharmacol. **33**, 3875-3881
- Debets, F., Hamers, W. & Strik, J. (1980) Int. J. Biochem. **12**, 1019-1025
- De Matteis, F. & Stonard, M. D. (1977) Semin. Hematol. **14**, 187-192
- De Matteis, F., Manno, M., Harvey, C. & Ferioli, A. (1987) in Chemical Monitoring of Occupational Toxicity (Colombi, A., Emmett, E. A., Foà, V. & Maroni, M., eds.), pp. 284-286, Ellis Horwood, Chichester
- De Verneuil, H., Sassa, S. & Kappas, A. (1983) Biochem. J. **214**, 145-151
- Doss, M., Schermuly, E., Look, D. & Henning, H. (1976) in Porphyrins in Human Disease (Doss, M., ed.), pp. 286-298, S. Karger, Basel
- Elder, G. H. (1978) Handb. Exp. Pharmacol. **44**, 157-200
- Elder, G. H., Tovey, J. A. & Sheppard, D. M. (1983) Biochem. J. **215**, 45-55
- Ferioli, A., Harvey, C. & De Matteis, F. (1984) Biochem. J. **224**, 769-777

- Feroli, A., Harvey, C. & De Matteis, F. (1986) *IARC Sci. Publ.* **77**, 465–466
- Goldstein, J. A., Hickman, P., Bergman, H., McKinney, J. D. & Walker, M. P. (1977) *Chem.-Biol. Interact.* **17**, 69–87
- Grandchamp, B., Deybach, J. C., Grellet, M., De Verneuil, H. & Nordmann, Y. (1980) *Biochim. Biophys. Acta* **629**, 577–586
- Heikel, T., Lockwood, W. & Rimington, C. (1958) *Nature (London)* **182**, 313
- Kawanishi, S., Mizutani, T. & Sano, S. (1978) *Biochim. Biophys. Acta* **540**, 83–92
- Lake, B. G. & Paine, A. J. (1983) *Xenobiotica* **13**, 725–730
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marks, G. S. (1978) *Handb. Exp. Pharmacol.* **44**, 201–237
- Meyer, U. A. & Schmid, R. (1977) in *The Metabolic Basis of Inherited Diseases*, 4th edn. (Stanbury, J. B., ed.), pp. 1203–1205, McGraw-Hill, New York
- Mukerji, S. K., Pimstone, N. R. & Burns, M. (1984) *Gastroenterology* **87**, 1248–1254
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
- Paine, A. J. & Legg, R. F. (1978) *Biochem. Biophys. Res. Commun.* **81**, 672–679
- Reed, C. J., Lock, E. A. & De Matteis, F. (1986) *Biochem. J.* **240**, 585–592
- Rios de Molina, M. C., Wainstock de Calmanovici, R. & San Martin de Viale, L. C. (1980) *Int. J. Biochem.* **12**, 1027–1032
- Sassa, S., Sugita, O., Ohnuma, N., Imajo, S., Okumura, T., Noguchi, T. & Kappas, A. (1986) *Biochem. J.* **235**, 291–296
- Sinclair, P. R. & Granick, S. (1974) *Biochem. Biophys. Res. Commun.* **61**, 124–133
- Sinclair, P. R. & Granick, S. (1975) *Ann. N.Y. Acad. Sci.* **244**, 509–520
- Sinclair, P. R., Elder, G. H., Bement, W. J., Smith, S. G., Bonkovsky, H. L. & Sinclair, J. F. (1983) *FEBS Lett.* **152**, 217–221
- Sinclair, P. R., Bement, W. J., Bonkovsky, H. L. & Sinclair, J. F. (1984) *Biochem. J.* **222**, 737–748
- Sinclair, P. R., Bement, W. J., Bonkovsky, H. L., Lambrecht, R. W., Frezza, J. E., Sinclair, J. F., Urquhart, A. J. & Elder, G. H. (1986) *Biochem. J.* **237**, 63–71
- Sinclair, P. R., Lambrecht, R. & Sinclair, J. (1987) *Ann. N.Y. Acad. Sci.*, in the press
- Smith, A. G. & Francis, J. E. (1981) *Biochem. J.* **195**, 241–250
- Smith, A. G. & Francis, J. E. (1983) *Biochem. J.* **214**, 909–913
- Smith, A. G. & Francis, J. E. (1986) in *Porphyrias and Porphyrins* (Nordmann, Y., ed.), pp. 127–136, INSERM/John Libbey Eurotext, London
- Swain, M. G., Follows, S. B. & Marks, G. S. (1983) *Can. J. Physiol. Pharmacol.* **61**, 105–108
- Unsold, A. & De Matteis, F. (1978) *Int. J. Biochem.* **9**, 865–869

Received 30 April 1987/27 August 1987; accepted 22 October 1987