Biochem. J. (1984) **222**, 737–748
Printed in Great Britain

Inhibition of uroporphyrinogen decarboxylase by halogenated biphenyls in chick hepatocyte cultures

Essential role for induction of cytochrome *P*-448

Peter R. SINCLAIR,*†§ William J. BEMENT,* Herbert L. BONKOVSKY,*‡ and Jacqueline F. SINCLAIR*†
* *VA Medical Center, White River Junction, VT 05001, U.S.A., and the Departments of* †*Biochemistry and* ‡*Medicine, Dartmouth Medical School, Hanover, NH 03756, U.S.A.*

(*Received 16 April 1984/Accepted 23 May 1984*)

Uroporphyrinogen decarboxylase (EC 4.1.1.37) activity was assayed in cultures of chick-embryo hepatocytes by the changes in composition of porphyrins accumulated after addition of excess 5-aminolaevulinate. Control cells accumulated mainly protoporphyrin, whereas cells treated with 3,4,3',4'-tetrachlorobiphenyl or 2,4,5,3',4'-pentabromobiphenyl accumulated mainly uroporphyrin, indicating decreased activity of the decarboxylase. 3-Methylcholanthrene and other polycyclic-hydrocarbon inducers of the *P*-448 isoenzyme of cytochrome *P*-450, did not affect the decarboxylase in the absence of the biphenyls. Induction of *P*-448 was detected as an increase in ethoxyresorufin de-ethylase activity. Pretreatment of cells with methylcholanthrene decreased the time required for the halogenated biphenyls to inhibit the decarboxylase. The dose response of methylcholanthrene showed that less than 40% of the maximal induction of cytochrome *P*-448 was needed to produce the maximum biphenyl-mediated inhibition of the decarboxylase. In contrast, induction of the cytochrome *P*-450 isoenzyme by propylisopropylacetamide had no effect on the biphenyl-mediated decrease in decarboxylase activity. Use of inhibitors of the *P*-450 and *P*-448 isoenzymes (SKF-525A, piperonyl butoxide and ellipticine) supported the concept that only the *P*-448 isoenzyme is involved in the inhibition of the decarboxylase by the halogenated biphenyls. The effect of preinduction with methylcholanthrene to enhance inhibition of the decarboxylase was also shown by the increased rate at which porphyrin accumulated from endogenously synthesized 5-aminolaevulinate after treatment of cells with the combination of propylisopropylacetamide and the biphenyls. Antioxidants, chelators of iron, and chromate affected the decrease in decarboxylase activity only if they prevented the induced increase in cytochrome *P*-448. We conclude that the *P*-448 and not the *P*-450 isoenzyme of cytochrome *P*-450 plays an obligatory role in the inhibition of uroporphyrinogen decarboxylase caused by halogenated biphenyls.

Uroporphyrinogen decarboxylase (EC 4.1.1.37) is an enzyme of the haem biosynthetic pathway that catalyses the decarboxylation of the four acetate side chains of uroporphyrinogen to yield coproporphyrinogen (Mauzerall & Granick, 1958). A human disease, porphyria cutanea tarda, is associated with hepatic accumulation of the oxidized porphyrinogens, URO and heptacarboxyl porphyrins, and with decreased activity of the enzyme (for review, see Pimstone, 1982). Activity of this enzyme is also decreased in liver homogenates from animals treated with hexachlorobenzene (Elder *et al.*, 1976), TCDD (Jones & Sweeney,

Abbreviations used: ALA, 5-aminolaevulinic acid; MC, 3-methylcholanthrene; PBB, 2,4,5,3',4'-pentabromobiphenyl; PIA, 2-propyl-2-isopropylacetamide; PROTO, protoporphyrin; TCB, 3,4,3',4'-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; URO, uroporphyrin; h.p.l.c., high-pressure liquid chromatography.

§ To whom reprint requests should be sent at the VA Medical Center address.

1980) or with some congeners of polybrominated biphenyls (Voorman *et al.*, 1983). These halogenated compounds also cause hepatic accumulation of URO and heptacarboxyl-porphyrins. Evidence from immunological studies indicates that, in animals accumulating these porphyrins, the concentration of immunoreactive enzyme is equivalent to that in controls (Elder & Shepperd, 1982), suggesting that decreased enzyme activity is due to inactivation rather than a decrease in amount of the enzyme. Iron has been implicated in both these animal and human porphyrias, since iron overload exacerbates the decrease in decarboxylase (Taljaard *et al.*, 1971), iron deficiency prevents it (Jones *et al.*, 1981), and the symptoms of porphyria cutanea tarda are relieved when patients are bled to remove iron (Pimstone, 1982).

Sinclair & Granick (1974) showed that cultured chick-embryo hepatocytes accumulate URO when exposed to a number of halogenated cyclic compounds. Chelators of iron prevented the URO accumulation, suggesting that, in the culture as in the human disease and experimental porphyrias, iron is somehow involved. Effects of cycloheximide and inhibitors of cytochrome *P*-450-mediated activities implied that the mechanism of this URO accumulation also involves an induction and action of cytochrome *P*-450, possibly via metabolism of the halogenated compounds to inhibitors of uroporphyrinogen decarboxylase.

Initially, no particular isoenzyme of cytochrome *P*-450 was implicated in the mechanism of the decrease in uroporphyrinogen decarboxylase. Subsequently, phenobarbital and MC were shown to induce different isoenzymes of cytochrome *P*-450 in chick liver culture (Althaus *et al.*, 1979), similar to the well-known inductions by these chemicals in animals (for review, see Lu & West, 1980). Phenobarbital has been reported to cause synergistic decrease in uroporphyrinogen decarboxylase in chick liver culture when combined with TCDD (de Verneuil *et al.*, 1983*a*). This synergism was postulated to be due to involvement of a phenobarbital-inducible isoenzyme of cytochrome *P*-450. In contrast, others had reported that the rate of URO production after treatment of the culture with hexachlorobenzene or mixtures of polybrominated biphenyls was increased by pretreatment of cells with MC or β-naphthoflavone (Debets *et al.*, 1980). These latter two compounds induce an isoenzyme that will be referred to in the present paper as 'cytochrome *P*-448'. In contrast with these mechanisms involving isoenzymes of cytochrome *P*-450, Kawanishi *et al.* (1981, 1983) have reported that uroporphyrinogen decarboxylase is directly inhibited by chlorinated biphenyls. This direct inhibition was said to account for URO accumulation in chick liver cultures (Kawanishi *et al.*, 1981, 1983). Thus, at present, the roles of particular *P*-450 isoenzymes in the loss of enzyme activity are not clear.

We have reinvestigated the role of isoenzymes of cytochrome *P*-450 in the mechanism by which individual halogenated biphenyls (TCB and PBB) inhibit uroporphyrinogen decarboxylase. We found that induction of the MC-inducible isoenzyme was an obligatory requirement for inhibition of the enzyme. Furthermore, we found that some compounds previously used for investigating the mechanism of the inhibition, such as antioxidants and chelators of iron, act by preventing induction of the cytochrome *P*-448.

Part of this work has already appeared in abstract form (Sinclair *et al.*, 1984*a*).

Materials and methods

Materials

Benzanthracene, benz[*a*]pyrene, β-naphthoflavone, MC, metyrapone and 2-acetylaminofluorene were purchased from Aldrich (Milwaukee, WI, U.S.A.). ALA, butylated hydroxyanisole, cycloheximide, and α-tocopherol were purchased from Sigma (St. Louis, MO, U.S.A.). Ellipticine was from Sigma or was a gift from Dr. P. Lesca, CNRS, Pharmacology Laboratory, Toulouse, France. TCB and piperonyl butoxide were purchased from Ultra-science (Hope, RI, U.S.A.). Spectral-grade dimethyl sulphoxide was from Fisher (Boston, MA, U.S.A.). Williams E medium was purchased as powder from Flow Laboratories, (McLean, VA, U.S.A.). URO and PROTO standards were purchased from Porphyrin Products (Logan, UT, U.S.A.). Desferrioxamine mesylate was purchased from Ciba-Geigy (Summit, NJ, U.S.A.). 7-Ethoxyresorufin (ethoxyphenoxazone) was purchased from Pierce (Rockford, IL, U.S.A.). Aminopyrine was purchased from Merck (Darmstadt, Germany). PBB, purified from the crude polybrominated biphenyls (Firemaster) mixture, was a gift from Dr. S. Aust, Michigan State University (East Lansing, MI, U.S.A.). SKF-525A (2-dimethylaminoethyl-2,2-diphenylvalerate hydrochloride) and cimetidine hydrochloride were gifts from Smith, Kline and French (Philadelphia, PA, U.S.A.). Rhodotorulic acid was a gift from Dr. H. Akers of Rockefeller University, New York, U.S.A. PIA was a gift from Hoffmann-La Roche (Nutley, NJ, U.S.A.). *N*-[9-^{14}C]-2-Acetylaminofluorene (47.6mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Hepatocyte cultures

Primary cultures from livers of 16-day White Leghorn chicken embryos were prepared and maintained in Williams E medium as described

previously (Sinclair *et al.*, 1982). Cells were cultured in 3.5cm-diameter plastic dishes unless otherwise indicated. Dishes contained 0.5–0.6mg of protein per 3.5cm dish or 1.5–1.7mg of protein/6.0cm dish. Chemicals were added during day 2 of culture after rinsing cells twice with Williams E medium containing dexamethasone and tri-iodothyronine but not insulin. Compounds of poor water-solubility were added to cultures in dimethyl sulphoxide (not exceeding 4μl/ml). Controls always included an appropriate amount of solvent.

Assay of uroporphyrinogen decarboxylase by porphyrin composition

Inhibition of the enzyme in intact cells was ascertained from the composition of porphyrins accumulated after adding 0.15mM-ALA to the cultures. Total porphyrin was extracted from the culture by adding an equal volume of 1M-$HClO_4$/methanol (1/1, v/v) to the medium in the culture dish. The porphyrin composition was measured fluorimetrically with a Hitachi 512 spectrofluorimeter equipped with a Kodak Wratten filter no. 25 on the emission side. Excitation and emission slits were 10nm wide. To quantify porphyrins as URO, PROTO or coproporphyrin, the method of Grandchamp *et al.* (1980) was used in which the fluorescence at three excitation/emission settings (400/595, 405/595, and 410/605nm), is measured and the three components determined with a matrix established from standard solutions of the porphyrins. These wavelengths represent the maxima for emission and excitation for the three porphyrins. The accuracy of the method was tested with known mixtures of the porphyrins and was reliable to ±5%. In particular, the method detected small amounts of PROTO, even in the presence of large amounts of URO. H.p.l.c. and t.l.c. analyses of porphyrins extracted from the cells treated with TCB or PBB showed considerable amounts of heptacarboxylic porphyrin (Sinclair *et al.*, 1983), which are detected as URO by the method of Grandchamp *et al.* (1980). The presence of porphyrinogens in the extract was checked by adding chloranil to a final concentration of 40μM. No further fluorescence was detected so porphyrinogens, if present in the cells, must have been oxidized during the extraction.

Conversion of 2-acetylaminofluorene into water-soluble products

Cells on 6cm dishes containing 1.5ml of medium were incubated with 0.1mM-[^{14}C]acetylaminofluorene (0.15μCi) for 1h, after which medium was removed and extracted once with 4ml of dichloromethane. A 1ml-portion of the aqueous layer was mixed with 10ml of ACS scintillation fluid (Amersham, Arlington Heights, IL, U.S.A.) and radioactivity determined. The 1h incubation was chosen because of the relatively rapid rate at which cells metabolized acetylaminofluorene. The single extraction removed more than 90% of the extractable radioactivity. The organic-soluble metabolites were separated by t.l.c. (hexane/tetrahydrofuran, 1:1, v/v) on silica gel (EM Laboratories, Elmsford, NY, U.S.A.) and detected by autoradiography. Spots were eluted with ethyl acetate and radioactivity was determined by liquid-scintillation counting.

Other methods

Ethoxyresorufin de-ethylase was assayed in homogenates of cells from 6cm-diameter dishes as described previously (Sinclair *et al.*, 1981). Cytochrome *P*-450 was assayed in 8700*g* supernatants of cells from 6cm dishes as described previously (Sinclair *et al.*, 1981). Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, after dissolving cells in 0.2M-NaOH/0.2% (w/v) sodium dodecyl sulphate. 4-Aminoantipyrine was measured as described by Poland & Kappas (1971).

Expression of results

Results shown are usually those from one typical experiment. Each experiment was performed at least twice. Error bars in Figures are S.D. or ranges of duplicate values.

Results

Assay of decreased uroporphyrinogen decarboxylase in intact cells

In the present paper the decrease in enzyme activity was detected by incubating cells with ALA and determining to what extent the resultant porphyrin composition shifted from mainly PROTO in untreated cells, to mainly URO in cells treated with PBB or TCB. Recently, we have shown that this method can detect a decrease in enzyme activity that cannot be detected by direct enzymic assay of cell homogenates (Sinclair *et al.*, 1983).

Decrease in uroporphyrinogen decarboxylase and increase in ethoxyresorufin de-ethylase caused by halogenated biphenyls

Fig. 1 shows that control cells accumulated 80% of total porphyrin as PROTO, whereas, after addition of PBB, there was a gradual shift in composition of accumulated porphyrins to 80% URO. In parallel, ethoxyresorufin de-ethylase, an enzyme activity specific for the MC-induced isoenzyme of cytochrome *P*-450 (Sinclair *et al.*, 1981) (hereafter called 'cytochrome *P*-448'), increased. Similar, but more rapid, changes were

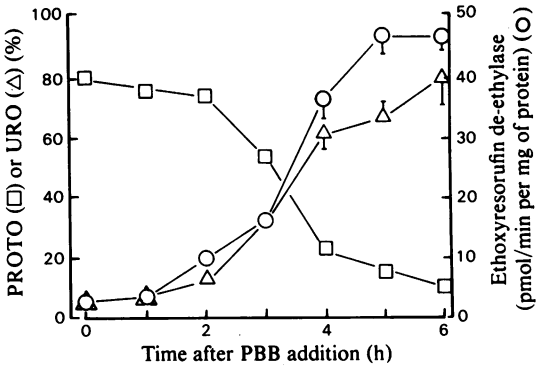

Fig. 1. *Time course of effects of PBB on uroporphyrinogen decarboxylase inhibition and ethoxyresorufin de-ethylase activity in chick-embryo liver cultures*

Cells on 6cm dishes were prepared as described in the Materials and methods section. All cells were harvested at the same time after the indicated time of exposure to PBB (1.8 μM). ALA (0.15mM) was added for the last 2h of incubation, except for the 1h point, for which ALA exposure was 1h. Porphyrin compositions [URO (△), PROTO (□)] and ethoxyresorufin de-ethylase (○) were determined as described in the Materials and methods section. Each point represent mean and S.D. for three determinations. S.D. values fall within symbol size unless otherwise shown. (In other experiments, it was shown that the treatment with ALA had no effect on induction of ethoxyresorufin de-ethylase induced by PBB.) Total porphyrin after 1h ALA exposure was 0.56nmol/dish and after 2h ranged from 0.8 to 1.0nmol/dish.

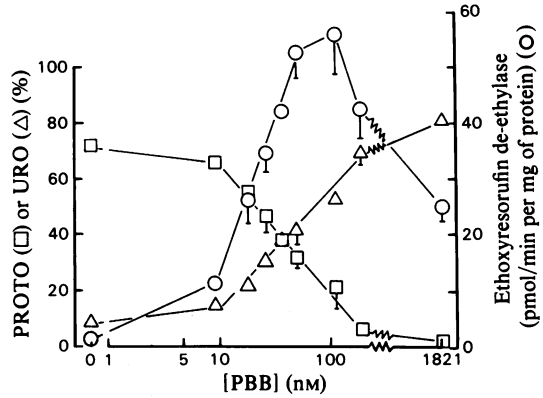

Fig. 2. *Effect of increasing concentrations of PBB on ethoxyresorufin de-ethylase and inhibition of uroporphyrinogen decarboxylase*

Cells on 6cm dishes were treated with PBB for 18h, then ALA (150 μM) was added for 2h further incubation. Ethoxyresorufin de-ethylase (○) was measured in homogenates and porphyrins in cells and medium [URO (△) or PROTO (□)]. For up to 180nM-PBB, porphyrin levels were 1.7±0.2nmol/plate (range 1.4–2.2). At 1.8 μM-PBB, total porphyrin was 2.8nmol, probably due to ALA synthase induction. β-Naphthoflavone (15 μM) induced de-ethylase activity in this experiment to 96pmol/min per mg of protein. Points show the mean and S.D. for triplicate dishes.

observed in cells treated with TCB (results not shown). Pretreatment with cycloheximide prevented TCB from inhibiting uroporphyrinogen decarboxylase (Table 1). In a separate experiment, we found identical dose responses for the actions of cycloheximide on the PBB induction of ethoxyresorufin de-ethylase activity and on the PBB-mediated shift of porphyrin composition to URO (results not shown). These results confirm our previous finding (Sinclair & Granick, 1974) and suggest that cycloheximide acted by inhibiting synthesis of cytochrome *P*-448.

Table 1 also shows that preinduction of cytochrome *P*-448 with MC prevented the inhibitory action of cycloheximide. The decrease in total porphyrin in cells in which uroporphyrinogen decarboxylase was inhibited was due, we believe, to conversion of PROTO into haem. In control cells exposed to ALA, PROTO accumulates in large amounts much of which leaks into the medium and thus is not converted into haem (Granick *et al.*, 1975).

To further examine the relationship between decrease in uroporphyrin decarboxylase and increase in cytochrome *P*-448, we measured the dose responses of the effects of PBB (Fig. 2) and TCB (Fig. 3) in the culture. In cells treated with PBB for 18h, the change in porphyrin composition occurred at the same doses that increased ethoxyresorufin de-ethylase. However, in cells treated with TCB for 22h, ethoxyresorufin de-ethylase had increased to 60–70% of the maximum before porphyrin composition began to change (Fig. 3). With both biphenyl treatments, there was a dramatic decrease in ethoxyresorufin de-ethylase activity at the higher concentrations (Figs. 2 and 3), corresponding to the maximal URO accumulation.

Effect of preinduction of cytochrome P-448 or P-450 on enzyme decrease

The results presented above strongly suggest that the increase in cytochrome *P*-448 and the decrease in uroporphyrinogen decarboxylase activity caused by the biphenyls were related. Pretreatment with MC, a known inducer of cytochrome *P*-448, caused the shift to URO accumulation in cells treated with TCB or PBB for 3.5h (Table 2). In this brief exposure, the biphenyls alone had little or no effect. Note that in the long-term exposures (Fig. 2), 100nM-PBB alone did inhibit the decarboxylase. These data (Fig. 2, Table 2) suggest that insufficient cytochrome *P*-448 had been induced

Table 1. *Effect of cycloheximide and MC on inhibition of uroporphyrinogen decarboxylase by TCB*
Cells on 3.5cm dishes were treated as indicated with 0.96 μM-MC for 21 h and with 1.8 μM-cycloheximide (CX) for 1 h before adding 3.4 μM-TCB and 0.15 mM-ALA. After 4 h further incubation, porphyrin composition was determined as described in the Materials and methods section.

Pretreatment	Treatment	Porphyrin composition (%) URO	PROTO	Total porphyrin (nmol/plate)
—	—	3	76	0.99
—	TCB	64	9	0.69
—	CX+TCB	11	77	1.03
MC	—	10	74	1.00
MC	TCB	86	0	0.73
MC	CX+TCB	78	0	0.63

Table 2. *Effect of MC pretreatment on porphyrin accumulated after exposure to PBB or TCB*
Cells on 3.5cm dishes were treated with 0.96 μM-MC for 19 h where indicated, and then TCB or PBB were added. After 1 h, ALA was added and the incubation continued for 2.5 h. Porphyrin composition was determined as described in the Materials and methods section. Data are means from duplicate dishes and varied less than 5% unless otherwise shown.

Pretreatment	[Halogenated biphenyl] (nM)	Porphyrin composition (%) URO	PROTO
MC	0	12	75
—	**TCB (10)**	9	78
MC	**TCB (10)**	38	55
—	**TCB (70)**	32 (27, 37)	52
MC	**TCB (70)**	88 (79, 97)	10 (2, 18)
—	**PBB (30)**	3	84
MC	**PBB (30)**	22	46
—	**PBB (100)**	3	84
MC	**PBB (100)**	67 (64, 71)	15

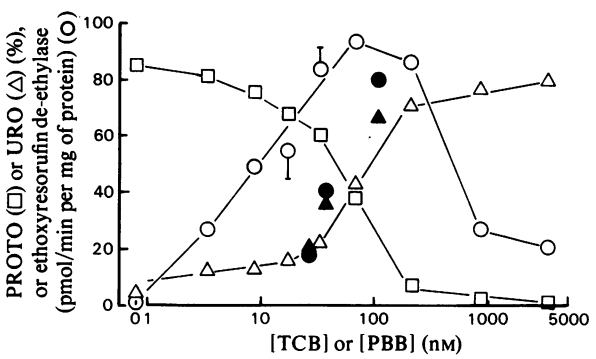

Fig. 3. *Effect of increasing concentrations of TCB on ethoxyresorufin de-ethylase and inhibition of uroporphyrinogen decarboxylase*
Cells in 6cm dishes were treated with TCB for 22 h, then ALA (150 μM) was added 1 h before harvesting. Porphyrin and ethoxyresorufin de-ethylase were assayed as described in the Materials and methods section. In this experiment PBB (1.8 μM) induced ethoxyresorufin de-ethylase to 144±19 pmol of resorufin/min per mg of protein. Open symbols for TCB are as in Fig. 2. For comparison, the closed symbols show values obtained at three PBB concentrations run in the same experiment.

by PBB in the short-term experiment to cause the inhibition of the decarboxylase. Fig. 4 shows that, in these short-term experiments, the more the ethoxyresorufin de-ethylase was increased by MC pretreatment, the more uroporphyrinogen decarboxylase was inhibited. However, it seemed that when ethoxyresorufin de-ethylase activity had been induced by 90 nM-MC to about 40% of the amount induced by 0.96 μM-MC, the decarboxylase was already maximally inhibited.

In the experiment shown in Table 2, 0.9 μM-MC treatment alone, followed by ALA, resulted in only 12% of the generated porphryin being URO. In similar experiments with other polycyclic aromatic hydrocarbons, benzanthracene (8.8 μM) and benz[*a*]pyrene (7.9 μM) were also ineffective in causing URO accumulation, though they induced ethoxyresorufin de-ethylase to high activities (44 and 52 pmol of resorufin/min per mg of protein respectively). β-Naphthoflavone (15 μM) induced this activity to 125 units, but unexpectedly caused a partial change in porphyrin composition (45% URO, 29% PROTO) as had been noted by Debets *et al.* (1981). None of these polycyclic hydrocarbons, at their maximally effective concentrations,

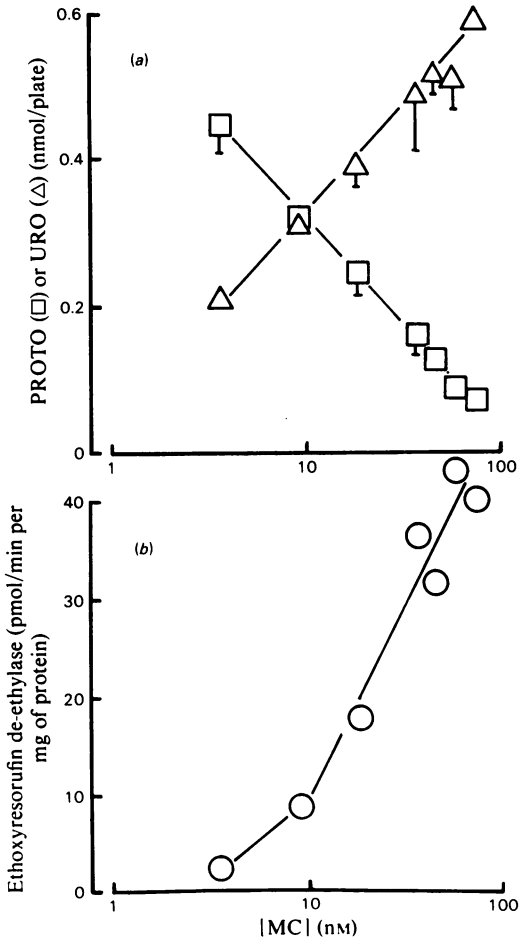

Fig. 4. *Dose–response relationship for effect of MC on (a) uroporphyrinogen decarboxylase and (b) ethoxyresorufin de-ethylase*

Cells cultured in 6cm dishes were treated with MC for 19h before addition of 1.8 μM-PBB and ALA (150 μM). After 2h further incubation, ethoxyresorufin de-ethylase and porphyrins [URO (△), PROTO (□)] in cells and medium were determined as described in the Materials and methods section. Enzyme activities in untreated cells, cells treated with PBB and ALA alone, or with 3.8 μM-MC and ALA were 4.0±0.8, 11.4±1.0, and 186.7±2.3 pmol of resorufin/min per mg of protein respectively. URO and PROTO in cultures treated with 0–70 nM-MC plus ALA were 0.08±0.03 and 0.77±0.08 nmol/dish respectively.

caused the decrease in ethoxyresorufin de-ethylase seen when cells were treated with the higher concentrations of the biphenyls (Figs. 2 and 3).

In contrast with the effects of pretreatment with MC, pretreatment of cells with PIA had no effect on the URO accumulation caused by 2h exposure to PBB (Table 3), even though PIA increased total cytochrome *P*-450, in particular, the isoenzyme that preferentially catalyses benzphetamine demethylase activity (Sinclair *et al.*, 1981).

Confirmation of the action of specific P-450 isoenzymes in inhibition of uroporphyrinogen decarboxylase

Specific inhibitors of *P*-450 isoenzymes (Testa & Jenner, 1981) were used to test the involvement of different isoenzymes in URO accumulation. Piperonyl butoxide is an inhibitor of several *P*-450 isoenzymes. SKF-525A is more specific in inhibiting the phenobarbital-induced isoenzyme. Ellipticine is highly specific in inhibiting one of the MC-inducible isoenzymes, in particular that catalysing ethoxyresorufin de-ethylase activity (Lesca *et al.*, 1979).

The inhibitors alone had no effect on the porphyrin patterns in control cells (results not shown). With MC-pretreated cells, piperonyl butoxide and ellipticine totally prevented the increase in URO mediated by PBB, whereas SKF-525A had only a partial effect. The selective action of inhibitors of the *P*-450 isoenzyme (SKF-525A, cimetidine, metyrapone and piperonyl butoxide) in the chick liver cells to inhibit *P*-450-catalysed reactions was tested on the demethylation of aminopyrine by intact cells preinduced with PIA. All the compounds inhibited the demethylation, but only piperonyl butoxide inhibited both the demethylation and the URO accumulation caused by PBB and TCB (results not shown).

The conversion of 2-acetylaminofluorene to water-soluble products by cells pretreated with PIA or MC was used to confirm the selectivity of the inhibitors used in Table 3. In cells pretreated with PIA, the inhibitors SKF-525A and piperonyl butoxide, but not ellipticine, strongly inhibited the conversion by over 70%. In cells pretreated with MC, piperonyl butoxide and ellipticine, but not SKF-525A, strongly inhibited the conversion by 60–70%. T.l.c. of dichloromethane extracts of the cultures incubated with acetylaminofluorene confirmed that conversion of acetylaminofluorene into water-soluble products was an accurate reflection of metabolism of the parent compound.

Thus the results with these inhibitors of *P*-450 isoenzymes strongly suggest that it is an MC-induced isoenzyme and not a PIA-induced isoenzyme of cytochrome *P*-450 that mediates the inhibition by halogenated biphenyls of uroporphyrinogen decarboxylase.

Inhibition of uroporphyrinogen decarboxylase detected by induction of ALA synthase

In the experiments described previously, the time course of inhibition of uroporphyrinogen

Table 3. *Effect of pretreating cells with inducers and inhibitors of different cytochrome P-450 isoenzymes on the rapidity of the decrease in uroporphyrinogen decarboxylase caused by exposure to PBB*

Cells were treated for 18 h in 3.5cm dishes with 0.96 μM-MC or 53 μM-PIA as indicated. Then, where indicated, piperonyl butoxide (30 μM), SKF-525A (22 μM) or ellipticine (10 μM) were added, and after 1 h incubation, 1.8 μM-PBB and ALA were added. After 2 h further incubation, total porphyrin composition was determined as described in the Materials and methods section. Porphyrin values are means for two plates whose values differed by less than ±5%. For cytochrome *P*-450 determinations in the same experiment, cells on 6cm dishes were treated as indicated, but without ALA. Cytochrome *P*-450 was determined as described in the Materials and methods section. Cytochrome *P*-450 concentrations in control cells and cells treated with 53 μM-PIA or 0.96 μM-MC were 36, 155 and 144 pmol/mg of protein respectively.

		Porphyrin composition (%)	
Pretreatment	Later treatment	URO	PROTO
None	—	2	88
	PBB	12	73
	PBB + piperonyl butoxide	6	86
	PBB + SKF-525A	10	83
	PBB + ellipticine	5	85
MC	—	6	82
	PBB	70	11
	PBB + piperonyl butoxide	6	87
	PBB + SKF-525A	43	41
	PBB + ellipticine	10	77
PIA	—	8	68
	PBB	12	61
	PBB + piperonyl butoxide	4	74
	PBB + SKF-525A	5	70
	PBB + ellipticine	9	74

decarboxylase was monitored by the change in the composition of porphyrins after adding ALA. This assay does not yield the extent of the inhibition of haem synthesis due to inhibition of the enzyme within intact cells. Previous work with this culture system has shown that combination of inhibitors of haem synthesis such as chelators or succinylacetone with inducers of cytochrome *P*-450 causes synergistic increases in ALA synthase (Granick *et al.*, 1975; Schoenfield *et al.*, 1982). Here we combined PIA with PBB or TCB and monitored porphyrin accumulation caused by induction of ALA synthase activity as a means to detect the rate at which the biphenyls inhibited haem synthesis (Fig. 5*a*). The effect of pre-inducing cytochrome *P*-448 with MC was to cause a more rapid and dramatic increase in porphyrin accumulation by cells treated with PIA and either TCB or PBB (Fig. 5*a*).

The time course of increase in ethoxyresorufin de-ethylase activity was also monitored; up to 6 h the activities were identical in cells treated with PIA and TCB or PBB (Fig. 5*b*). The differences between rates of the porphyrin accumulation after treatment with either of the biphenyls was probably not due to different amounts of cytochrome *P*-448 in the cells, but, rather, might have been due to different rates of conversion of the biphenyls into inhibitors of uroporphyrinogen decarboxylase. Thus the results of this experiment are consistent with previous experiments in showing that pretreatment with MC increased the rate at which TCB and PBB inhibited uroporphyrinogen decarboxylase. Ethoxyresorufin de-ethylase activity in the MC-pretreated cells was 163 pmol/min per mg of protein, i.e., 5-fold more than in cells treated with PIA and the biphenyls. This finding is consistent with the results shown in Fig. 4 that less than maximal de-ethylase activity was required to cause the decrease in uroporphyrinogen decarboxylase.

Effects of antioxidants and of chelators of iron

Pretreatment with the antioxidant butylated hydroxyanisole or with chelators of iron also prevented the inhibition of uroporphyrinogen decarboxylase. However, these effects seem to result from lack of synthesis of sufficient cytochrome *P*-448, since ethoxyresorufin de-ethylase activity in such cells was decreased. This was probably due to non-specific toxicity. The action of other chemicals can also be misinterpreted as specifically acting on uroporphyrinogen decarboxylase. Butylated hydroxyanisole at concentrations greater than 0.22 mM prevented any TCB-mediated decrease in PROTO accumulation from ALA or increase in ethoxyresorufin de-ethylase. In MC-preinduced cells, the antioxidant was slightly effective against the action of PBB, but not against TCB. Other antioxidants, α-tocopherol, Mn^{2+} or

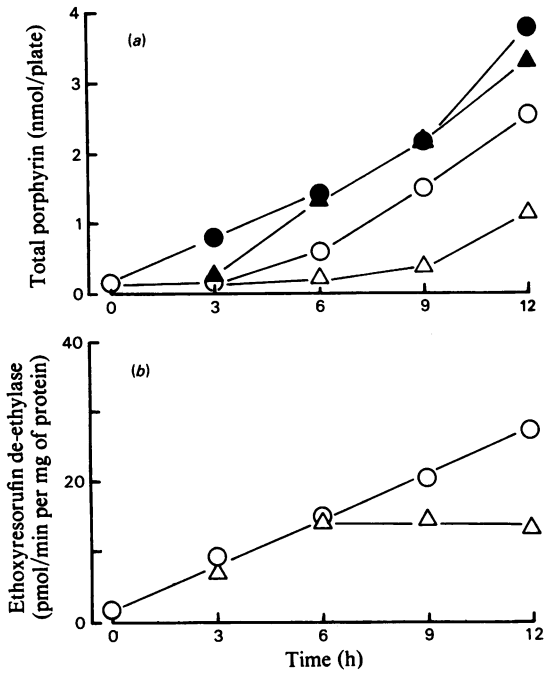

Fig. 5. *Effect of pretreatment with MC on increases in porphyrin accumulation (a) and ethoxyresorufin de-ethylase (b) caused by PIA combined with PBB and TCB*

Cells in 6cm dishes were treated with 0.14mM-PIA and with 3.4μM-TCB (○, ●) or PBB (△, ▲). Some cells were treated with 0.96μM-MC for 18h before addition of the other chemicals (closed symbols). Porphyrins were determined as described in the Materials and methods section. PIA, PBB, TCB or MC alone caused accumulation of less than 0.3nmol of porphyrin per plate over 12h. Ethoxyresorufin de-ethylase was measured only in plates not treated with MC. In cells treated with MC alone for 12h, this activity was 160pmol/min per mg of protein. In cells treated with PBB or TCB alone for 12h, this activity was 34 and 27pmol/min per mg of protein respectively.

mannitol, failed to prevent the PBB-mediated inhibition of the decarboxylase (results not shown). Ascorbate (0.5mM) partly prevented the decarboxylase inhibition caused by PBB, but not that caused by TCB, in agreement with Debets *et al.* (1980). Sodium chromate, an agent that causes DNA damage in these cells and prevents induction of ALA synthase (Tsapakos *et al.*, 1983) also prevented the inhibition of the decarboxylase by preventing induction of cytochrome *P*-448 as indicated by ethoxyresorufin de-ethylase activity (results not shown).

Table 4 shows that an 18h pretreatment with the chelators desferrioxamine and rhodotorulic acid prevented both the TCB-mediated decrease in decarboxylase and the induction of ethoxyresorufin de-ethylase activity. Previous experiments of this type, in which porphyrin only has been measured, have been interpreted to indicate a role for iron in the mechanism of inhibition of the decarboxylase (Sinclair & Granick, 1974; de Verneuil *et al.*, 1983*a*). However, Table 5 shows that a 3 or 6h treatment with the chelators after an MC-pretreatment was without effect on the decarboxylase inhibition caused by TCB. To indicate that the chelator could be effective in 6h, we determined its effect on PROTO accumulation in this experiment in cultures not treated with TCB. Regardless of the pretreatment with MC, 6h treatment with desferrioxamine doubled PROTO accumulation from ALA. Thus chelators of iron seem to affect TCB-mediated inhibition of uroporphyrinogen decarboxylase only when they prevented TCB-mediated induction of cytochrome *P*-448.

Discussion

Detection of decreased uroporphyrinogen decarboxylase in intact cells

In the present study we used chick-embryo hepatocytes to investigate the mechanism by

Table 4. *Effects of pretreatment with chelators of iron on the inhibition of uroporphyrinogen decarboxylase and on inducibility of ethoxyresorufin de-ethylase*

Cells on 3.5cm (Expt. 1) or 6cm dishes (Expt. 2) were treated with 30μM-desferrioxamine or 0.67mM-rhodotorulic acid for 18h, then with 3.5μM-TCB for 3h. During the last 2h, 0.15mM-ALA was included. Porphyrin composition and ethoxyresorufin de-ethylase were determined as described in the Materials and methods section. Enzyme activity induced by TCB alone was 27pmol/min per mg of protein. Data represent values from two dishes that did not differ more than 10%.

Expt.	Pretreatment	Porphyrin composition (%) URO	Porphyrin composition (%) PROTO	Ethoxyresorufin de-ethylase inducibility (% of control)
1	None	56	27	—
	Desferrioxamine	14	78	—
	Rhodotorulic acid	19	71	—
2	None	78	7	100
	Desferrioxamine	35	52	24
	Rhodotorulic acid	35	44	15

Table 5. *Effect of chelators of iron on inhibition of uroporphyrinogen decarboxylase caused by TCB after methylcholanthrene pretreatment*

Cells on 3.5cm dishes were pretreated for 18h with 0.9 μM-MC, then 30 μM-desferrioxamine or 0.67mM-rhodotorulic acid were added. After 3h or 6h incubation as indicated, 3.4 μM-TCB was added and after 1h, 0.15mM-ALA was added. Porphyrin composition was determined after 2h further incubation, as described in the Experimental procedures section. Total porphyrin ranged from 0.26 to 0.30nmol/dish. Values represent means of two determinations that varied less than ±5%.

Expt.	Chelator	Porphyrin composition (%) URO	PROTO
1	None	75	11
	Desferrioxamine (3h)	78	7
	Rhodotorulic acid (3h)	65	16
2	None	74	4
	Desferrioxamine (3h)	70	9
	Desferrioxamine (6h)	70	6

which halogenated biphenyls decrease activity of uroporphyrinogen decarboxylase. We detected decreased activity in the intact cells by adding excess ALA to the culture medium. ALA was converted into PROTO and presumably haem in untreated cells, but in cells treated with PBB or TCB, URO accumulated at the expense of PROTO (Figs. 2 and 3). The decrease in PROTO was more than the increase in URO, most probably because of the combination of continued synthesis of haem plus decreased synthesis of PROTO. The advantage of using ALA loading with intact cells to detect the decrease in enzyme activity is its simplicity, rapidity and sensitivity compared with the laborious homogenate assay (Elder & Wyvill, 1982; de Verneuil *et al.*, 1983*b*). Elsewhere, we have discussed the problem of determining the extent of decrease in enzyme activity in the cells by the ALA loading assay (Sinclair *et al.*, 1983).

Cytochrome P-448 and inhibition of uroporphyrinogen decarboxylase

Studies with inbred mice treated with TCDD have indicated there is an obligatory relationship between inducibility of the *P*-448 isoenzyme of cytochrome *P*-450 and the decrease in enzyme activity (Jones & Sweeney, 1980; Smith *et al.*, 1981). In our study with chick liver cultures we have concluded that it is the induction by the biphenyls of the *P*-448 isoenzyme (as measured by ethoxyresorufin de-ethylase activity) that is obligatorily required before uroporphyrinogen decarboxylase activity is inhibited.

Debets *et al.* (1980) also reported that pretreatment of the chick liver cultures with the *P*-448 inducers, MC and β-naphthoflavone increased the rate of porphyrin accumulation (presumably due to induction of ALA synthase) after exposure to hexachlorobenzene or a polybrominated biphenyl mixture. By using the ALA-loading assay, shift to URO accumulation in cells exposed to hexachlorobenzene or 2,4,5,2',4',5'-hexachlorobiphenyl was unaffected by pre-exposure to phenobarbital (Debets *et al.*, 1981). They also repeated our previous finding (Sinclair & Granick, 1974) that piperonyl butoxide, an inhibitor of various isoenzymes of cytochromes *P*-450, would block inhibition of the enzyme. However, no conclusions were drawn concerning which isoenzyme of *P*-450 was involved. Carpenter *et al.* (1984) found that the porphyria caused by hexachlorobenzene in Japanese quail developed more rapidly in birds pretreated with β-naphthoflavone and did not occur in birds pretreated with phenobarbital.

In contrast, de Verneuil *et al.* (1983*a*) found that pretreatment of the chick liver culture with phenobarbital (but not with β-naphthoflavone), followed by treatment with TCDD, caused increased inactivation of uroporphyrinogen decarboxylase and synergistic induction of ALA synthase. They concluded that the phenobarbital-induced *P*-450 isoenzyme rather than the β-naphthoflavone-induced *P*-448 isoenzyme is involved in the inactivation of the decarboxylase. Furthermore, they reported a small decrease in the decarboxylase activity caused by phenobarbital itself. We also found a synergistic induction of ALA synthase caused by the combination of the phenobarbital-like *P*-450 inducer PIA and an inhibitor of uroporphyrinogen decarboxylase (TCB or PBB) (Fig. 5). Our experiments in which synergistic induction of ALA synthase and ALA loading were followed, showed clear additional effects of pretreatment with a *P*-448 inducer (Table 3, Fig. 5) and an effect of the *P*-450 inducer alone (Table 3). We attribute the effect of PIA in the synergistic induction of ALA synthase to be due to some other action of the *P*-450 inducer [such as to increase binding of free haem to newly induced

apocytochrome (see review by Bonkovsky, 1982)] rather than to increased inhibition of uroporphyrinogen decarboxylase. The further decreases in uroporphyrinogen decarboxylase caused by combining phenobarbital with TCDD reported by de Verneuil *et al.* (1983*a*) were small and may have been caused by the massive amount of uroporphyrin present in these cells due to the highly induced ALA synthase. In contrast with our results showing effects of preinducing the *P*-448 isoenzyme (Tables 2 and 3; Figs. 4 and 5), de Verneuil *et al.* (1983*a*) found that pretreatment with β-naphthoflavone had no effect on the actions of TCDD. Our experiments showed that the effects of the pretreatment are only seen soon after addition of the halogenated compound, since TCDD itself, like TCB, is a good inducer of the *P*-448 isoenzyme. They also found extracts of cells treated with phenobarbital contained URO and heptacarboxyl porphyrins. We agree with Marks *et al.* (1983) that such cultures also accumulate in the culture medium an amount of coproporphyrin equal to the URO accumulated within the cells. This is consistent with the finding of de Verneuil *et al.* (1983*a*) that there is only a partial inhibition of uroporphyrinogen decarboxylase in phenobarbital-treated cells.

Kawanishi *et al.* (1981, 1983) found that all the halogenated biphenyls they tested directly inhibited purified chicken erythrocyte uroporphyrinogen decarboxylase, although some of the biphenyls did not cause URO accumulation in the chick liver culture. They argued that some of these compounds without effect in the culture, such as 2,4,2′,4′-tetrachlorobiphenyl, do not enter the cells and, hence, do not inhibit the enzyme. This is unlikely, since this biphenyl highly induces cytochrome *P*-450 and Phenol Red glucuronidation in the culture (P. Sinclair & J. Bement, unpublished work). Other workers have found no direct inhibition of the enzyme by TCDD (de Verneuil *et al.*, 1983*b*) or by TCB (Swain *et al.*, 1983).

The role of the cytochrome *P*-448 isoenzyme in the mechanism of the decrease in uroporphyrinogen decarboxylase activity caused by halogenated biphenyls is not known. Our results show that mere induction of the *P*-448 isoenzyme does not, by itself, affect the enzyme, since polycyclic aromatic hydrocarbons, such as benz[*a*]pyrene and MC, that induced *P*-448-catalysed ethoxyresorufin de-ethylase to high activities, had no effect on porphyrin patterns. Furthermore, only 1/2500th the concentration of TCDD is required to induce the *P*-448 isoenzyme in the chick liver culture (Niwa *et al.*, 1975) as is needed for inhibition of uroporphyrinogen decarboxylase (de Verneuil *et al.*, 1983*a*). Thus some action, in addition to induction of the *P*-448 isoenzyme, seems essential for the biphenyl-mediated inhibition of the decarboxylase that leads to URO accumulation. Many workers have suggested that this other action is due to a metabolite or reactive intermediate of the halogenated compound that inhibits the enzyme (Sinclair & Granick, 1974; Debets *et al.*, 1980; Swain *et al.*, 1983; de Verneuil *et al.*, 1983*a*), although there is no direct evidence for covalent binding of any metabolite to the enzyme. Evidence in favour of the metabolite hypothesis includes the requirement for action of cytochrome *P*-448, and the prevention of the effect by known inhibitors of cytochrome *P*-448 (Table 3). However, up to this time, there is no evidence that biphenyls are metabolized in the chick liver culture. Debets *et al.* (1981) showed metabolism of hexachlorobenzene in the culture. We have found that TCB is converted into water-soluble products by MC- but not PIA-pretreated cells, a conversion sensitive to piperonyl butoxide and ellipticine (P. Sinclair, W. J. Bement, R. Lambrecht & J. Frezza, unpublished work). Thus far we have failed to detect PBB metabolism in the culture, a finding that agrees with animal studies of highly halogenated biphenyls (see, e.g., Mühlebach & Bickel, 1981). Our method could detect disappearance of 20% or more of the added PBB. In recent experiments (Sinclair *et al.*, 1984*b*), we have found the inhibitors of cytochrome *P*-448 (piperonyl butoxide and ellipticine, Table 3) not only prevented inhibition of uroporphyrinogen decarboxylase by the biphenyls, but also rapidly reversed the inhibition. This finding makes it unlikely that the inhibition involves covalent binding of metabolites to the enzyme.

Amount of cytochrome P-448 required

This study has shown that some inhibition of uroporphyrinogen decarboxylase, as detected by the ALA loading assay, can occur with much less than maximally induced levels of cytochrome *P*-448 (e.g. Figs. 1–5). Thus caution should be observed, particularly in animal studies seeking to dissociate inducibility of cytochrome *P*-448 from the decrease in the enzyme (Smith & Francis, 1983), since rather a small amount of induction of cytochrome *P*-448 may be all that is required for inhibition of uroporphyrinogen decarboxylase by the halogenated compounds.

Effect on haem synthesis

Figs. 2 and 3 show that there was a decrease in ethoxyresorufin de-ethylase activity at the high concentrations of TCB and PBB. We speculate that this decrease corresponds to the attainment of maximum inhibition of uroporphyrinogen decarboxylase, perhaps making haem limiting for synthesis or assembly of the holocytochrome of

cytochrome *P*-448. In support of the maximum decrease in haem synthesis being achieved is the result in Fig. 5, which shows that synergistic induction of ALA synthase occurred when PIA was combined with the biphenyls at high concentrations. As discussed above, this synergism depends on loss of feedback repression by haem of ALA synthase. We have found that the biphenyls decrease incorporation of radioactive ALA into haem (Sinclair *et al.*, 1984*b*).

Actions of inhibitory compounds: chelators and antioxidants

We have reinvestigated the actions of a number of compounds that we and others have used previously in attempts to determine the mechanism of the enzyme decrease. We found a serious pitfall in their use, namely that when used at times and concentrations which prevented induction of cytochrome *P*-448, they prevented biphenyl-mediated inhibition of the enzyme decrease by indirect or non-specific mechanisms. For example, it was previously concluded that actions of chelators to prevent URO accumulation indicated a role of iron in the mechanism (Sinclair & Granick, 1974; de Verneuil *et al.*, 1983*a*). Our results (Tables 4 and 5) showed the chelators acted by inhibiting synthesis of cytochrome *P*-448, since the chelators were not effective if added after MC-pretreatment. Similar effects on cytochrome *P*-448 and uroporphyrinogen decarboxylase were caused by butylated hydroxyanisole, a powerful antioxidant in this system (Shedlofsky *et al.*, 1983), and chromate, a DNA-damaging agent in this culture (Tsapakos *et al.*, 1983).

Conclusion

Inhibition of uroporphyrinogen decarboxylase by the halogenated biphenyls PBB and TCB in chick embryo liver cultures is obligatorily associated with induction of a *P*-448 isoenzyme of cytochrome *P*-450, which perhaps produces an inhibitory metabolite. The way in which induction of this cytochrome leads to the inhibition is not yet clear. Interpretation of the effects of some chemical probes of the mechanism requires caution.

We thank Dr. G. Elder, Dr. R. Lambrecht, Dr. S. Shedlofsky and Dr. E. L. Smith for their helpful comments. Mr. S. Howell and Dr. N. Kagan performed the h.p.l.c. and t.l.c. analyses. Mrs D. Burke, Mr. R. Chapman and Miss P. Walden typed the manuscript. The work was supported by the U.S. Veterans Administration and by grants from the National Institutes of Health (CA 25012 and HL 28177).

References

Althaus, F., Sinclair, J., Sinclair, P. & Meyer, U. (1979) *J. Biol. Chem.* **254**, 2148–2153

Bonkovsky, H. (1982) in *Hepatology* (Zakim, D. & Boyer, T., eds.), p. 351, Saunders, Philadelphia

Carpenter, H., Williams, D., Henderson, M., Bender, R. & Buhler, D. (1984) *Biochem. Pharmacol.* in the press

Debets, F., Hamers, W. & Strik, J. (1980) *Int. J. Biochem.* **12**, 1019–1025

Debets, F., Reinders, J., Debets, A., Lossbroek, T., Strik, J. & Koss, G. (1981) *Toxicology* **19**, 185–196

de Verneuil, H., Sassa, S. & Kappas, A. (1983*a*) *Biochem. J.* **214**, 145–151

de Verneuil, H., Sassa, S. & Kappas, A. (1983*b*) *J. Biol. Chem.* **258**, 2454–2460

Elder, G. & Shepperd, D. (1982) *Biochem. Biophys. Res. Commun.* **109**, 113–120

Elder, G. & Wyvill, P. (1982) *Enzyme* **28**, 186–195

Elder, G., Evans, J. & Matlin, S. (1976) *Clin. Sci. Mol. Med.* **51**, 71–80

Grandchamp, B., Deybach, J., Grelier, M., de Verneuil, H. & Nordmann, A. (1980) *Biochim. Biophys. Acta* **629**, 577–586

Granick, S., Sinclair, P., Sassa, S. & Grieninger, G. (1975) *J. Biol. Chem.* **250**, 9215–9225

Jones, K. & Sweeney, G. (1980) *Toxicol. Appl. Pharmacol.* **53**, 42–49

Jones, K., Cole, F. & Sweeney, G. (1981) *Toxicol. Appl. Pharmacol.* **61**, 74–88

Kawanishi, S., Seki, Y. & Sano, S. (1981) *FEBS Lett.* **129**, 93–96

Kawanishi, S., Seki, Y. & Sano, S. (1983) *J. Biol. Chem.* **258**, 4285–4292

Lesca, P., Rafidinarivo, E., Lecointe, P. & Mansuy, D. (1979) *Chem.-Biol. Interact.* **24**, 189–198

Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951) *J. Biol. Chem.* **193**, 265–275

Lu, A. & West, S. (1980) *Pharmacol. Rev.* **31**, 277–295

Marks, G., Follows, S., Zelt, D. & Cole, S. (1983) *Can. J. Physiol. Pharmacol.* **61**, 546–553

Mauzerall, D. & Granick, S. (1958) *J. Biol. Chem.* **232**, 1141–1162

Mühlebach, S. & Bickel, M. (1981) *Xenobiotica* **11**, 249–257

Niwa, A., Kumaki, K. & Nebert, D. (1975) *Mol. Pharmacol.* **11**, 399–408

Pimstone, N. (1982) *Semin. Liver Dis.* **2**, 132–142

Poland, A. & Kappas, A. (1971) *Mol. Pharmacol.* **7**, 697–705

Schoenfield, N., Greenblat, Y., Epstein, O. & Atsmon, A. (1982) *Biochim. Biophys. Acta* **721**, 408–417

Shedlofsky, S., Bonkovsky, H., Sinclair, P., Sinclair, J., Bement, W. & Pomeroy, J. (1983) *Biochem. J.* **212**, 321–330

Sinclair, P. & Granick, S. (1974) *Biochem. Biophys. Res. Commun.* **61**, 124–133

Sinclair, J., Sinclair, P., Smith, E., Bement, W., Pomeroy, J. & Bonkovsky, H. (1981) *Biochem. Pharmacol.* **30**, 2805–2809

Sinclair, J., Sinclair, P., Healey, J., Smith, E. & Bonkovsky, H. (1982) *Biochem. J.* **204**, 103–109

Sinclair, P., Elder, G., Bement, W., Smith, S., Bonkovsky, H. & Sinclair, J. (1983) *FEBS Lett.* **152**, 217–221

Sinclair, P., Bement, W., Bonkovsky, H. & Sinclair, J. (1984*a*) *Clin. Res.* **32**, 491*a*

Sinclair, P., Bonkovsky, H., Bement, W., Sinclair, J., Urquhart, A. & Elder, G. (1984*b*) *Toxicologist* **4**, 132
Smith, A. & Francis, J. (1983) *Biochem. J.* **214**, 909–913
Smith, A., Francis, J., Kay, S. & Greig, J. (1981) *Biochem. Pharmacol.* **30**, 2825–2830
Swain, M., Follows, S. & Marks, G. (1983) *Can. J. Physiol. Pharmacol.* **61**, 105–108
Taljaard, J., Shanley, B. & Joubert, S. (1971) *Life Sci.* **10**, 887–893
Testa, B. & Jenner, P. (1981) *Drug Metab. Rev.* **12**, 1–117
Tsapakos, M., Hampton, T., Sinclair, P., Sinclair, J., Bement, W. & Wetterhahn, K. (1983) *Carcinogenesis* **4**, 959–966
Voorman, R., Jensen, R., Sleight, S. & Aust, S. (1983) *Toxicologist* **3**, 98