Uroporphyrin accumulation produced by halogenated biphenyls in chick-embryo hepatocytes

Reversal of the accumulation by piperonyl butoxide

Peter R. SINCLAIR,*† William J. BEMENT,* Herbert L. BONKOVSKY,*‡|| Richard W. LAMBRECHT,* Joan E. FREZZA,*† Jacqueline F. SINCLAIR,*† Andrew J. URQUHART§ and George H. ELDER§

* Veterans Administration, White River Junction, VT 05001, Departments of †Biochemistry and ‡Medicine, Dartmouth Medical School, Hanover, NH 03756, U.S.A., and §Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, Wales, U.K.

Cultures of chick-embryo hepatocytes were used to study the mechanism by which 3,4,3',4'-tetrachlorobiphenyl and 2,4,5,3',4'-pentabromobiphenyl cause accumulation of uroporphyrin. In a previous paper, an isoenzyme of cytochrome *P*-450 induced by 3-methylcholanthrene had been implicated in this process [Sinclair, Bement, Bonkovsky & Sinclair (1984) *Biochem. J.* **222**, 737–748]. Cells treated with 3,4,3',4'-tetrachlorobiphenyl and 5-aminolaevulinate accumulated uroporphyrin and heptacarboxyporphyrin, whereas similarly treated cells accumulated protoporphyrin immediately after piperonyl butoxide was added. Piperonyl butoxide also restored haem synthesis as detected by (i) incorporation of radioactive 5-aminolaevulinate into haem, and (ii) decrease in drug-induced 5-aminolaevulinate synthase activity. The restoration of synthesis of protoporphyrin and haem by piperonyl butoxide was not affected by addition of cycloheximide, indicating recovery was probably not due to protein synthesis *de novo*. Piperonyl butoxide also reversed uroporphyrin accumulation caused by 3,4,5,3',4',5'-hexachlorobiphenyl, mixtures of other halogenated biphenyls, lindane, parathion, nifedipine and verapamil. The effect of piperonyl butoxide was probably not due to inhibition of metabolism of these compounds, since the hexachlorobiphenyl was scarcely metabolized. Other methylenedioxyphenyl compounds, as well as ellipticine and acetylaminofluorene, also reversed the uroporphyrin accumulation caused by 3,4,3',4'-tetrachlorobiphenyl. SKF-525A (2-dimethylaminoethyl-2,2-diphenyl valerate) did not reverse the uroporphyrin accumulation caused by the halogenated biphenyls, but did reverse that caused by phenobarbital and propylisopropylacetamide. We conclude that the mechanism of the uroporphyrin accumulation cannot be due to covalent binding of activated metabolites of halogenated compounds to uroporphyrinogen decarboxylase.

INTRODUCTION

A human disease, porphyria cutanea tarda, is associated with hepatic accumulation of the oxidized porphyrinogens, URO and heptacarboxyporphyrin and with decreased activity of uroporphyrinogen decarboxylase in the liver [for reviews, see Bonkovsky (1982) and Kappas *et al.* (1983)]. Activity of this enzyme is also decreased in liver homogenates from animals treated with hexachlorobenzene (Elder *et al.*, 1976), TCDD (Jones & Sweeney, 1980) or with some polybrominated biphenyls (Voorman *et al.*, 1983), and this decrease is associated with hepatic accumulation of URO and heptacarboxyporphyrin. Immunological studies indicate that, in treated animals accumulating these porphyrins, the concentration of immunoreactive enzyme is equivalent to that in controls (Elder & Sheppard, 1982), suggesting that decreased enzyme activity is due to inactivation rather than a decrease in the amount of the enzyme.

A number of halogenated cyclic compounds cause URO accumulation when added to cultures of chick-embryo hepatocytes [see review by Marks (1985)]. Recently, we showed that URO accumulation by intact

cells caused by the halogenated biphenyls TCB and PBB is dependent on the induction of an isoenzyme of cytochrome *P*-450 that is induced by these compounds or by MC (Sinclair *et al.*, 1984a). We also reported that prior or simultaneous addition of the inhibitors of this *P*-450 isoenzyme, piperonyl butoxide or ellipticine, prevented URO accumulation caused by the halogenated compounds (Sinclair *et al.*, 1984a). From these and similar experiments by others using chick liver culture (de Verneuil *et al.*, 1983a; Swain *et al.*, 1983; Debets *et al.*, 1980) or intact animals (Debets *et al.*, 1981a; Wainstock de Calmanovici *et al.*, 1984) it has been suggested that URO accumulation involves metabolism of the halogenated compounds by the *P*-450 isoenzyme to reactive intermediates which bind covalently to and inhibit uroporphyrinogen decarboxylase. As yet, there is no direct evidence to support this hypothesis.

In the present paper we show that URO accumulation caused by a number of compounds (including TCB and PBB) in cultures of intact chick hepatocytes was rapidly reversed by piperonyl butoxide and other inhibitors of cytochrome *P*-450. This reversal of URO accumulation was detected primarily by change in the pattern of

Abbreviations used: ALA, 5-aminolaevulinic acid; DMSO, dimethyl sulphoxide; HCB, 3,4,5,3',4',5'-hexachlorobiphenyl; MC, 3-methylcholanthrene; PBB, 2,4,5,3',4'-pentabromobiphenyl; PROTO, protoporphyrin; SKF-525A, 2-dimethylaminoethyl-2,2-diphenyl valerate; TCB, 3,4,3',4'-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; URO, uroporphyrin; PIA, 2-propyl-2-isopropylacetamide.

|| Present address: Clinical Research Facility, Emory University Hospital, Atlanta, GA 30322, U.S.A.

accumulation of porphyrins, from URO to PROTO, after addition of ALA. In addition, the synthesis of haem from ALA was restored to control levels, and feedback repression by intracellular haem of induced ALA synthase was restored.

Our results are incompatible with current mechanistic proposals for URO accumulation that invoke irreversible inhibition of uroporphyrinogen decarboxylase by halogenated hydrocarbons due to covalent binding of metabolites to the enzyme. The implications of these studies for development of the URO accumulation in animals and humans caused by halogenated hydrocarbons are discussed.

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

MATERIALS AND METHODS

Materials

Sources of most of the chemicals were given previously (Sinclair *et al.*, 1984a). Aroclor-1254 and parathion were from Ultra-Science, Hope, RI, U.S.A. Firemaster and 3,4,5,3',4',5'-hexabromobiphenyl were generously given by Dr. S. Aust, Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A. Lindane (99.9%) was from Chevron Chemical Co., San Francisco, CA, U.S.A. [4-¹⁴C]ALA (53.2 mCi/mmol and [¹⁴C]TCB (27.3 mCi/mmol) were from New England Nuclear, Boston, MA, U.S.A. Ellipticine was synthesized by Dr. G. Gribble, Department of Chemistry, Dartmouth College, Hanover, NH, U.S.A. Isosafrole and biphenyl were from Pfaltz and Bauer, Stamford, CT, U.S.A. Safrole, methylenedioxybenzene and 2-acetylaminofluorene were from Aldrich, Milwaukee, WI, U.S.A. n-Butyldioxalane was generously given by Dr. E. Hodgson, North Carolina State University, Raleigh, NC, U.S.A. SKF-525A was a gift from Smith, Kline and French Co., Philadelphia, PA, U.S.A. Desferrioxamine mesylate was purchased from Ciba-Geigy, Camden, NJ, U.S.A. CaMg-EDTA was prepared as described by Sinclair & Granick (1977). [¹⁴C]HCB (12.1 mCi/mmol) was generously given by Dr. M. J. Vodick, Department of Pharmacology, Medical College of Wisconsin, Milwaukee, WI, U.S.A. Nifedipine and verapamil were from Sigma, St. Louis, MO, 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 3.5 cm-diameter plastic dishes unless otherwise indicated. Dishes contained 0.5–0.6 mg of protein per 3.5 cm dish or 1.5–1.7 mg of protein per 6 cm dish. Chemicals were added during the second day of culture after cells had been rinsed twice with Williams E medium containing dexamethasone and 3,3',5-tri-iodothyronine, but not insulin. Compounds of poor water solubility were added to cultures in DMSO (not exceeding 4 µl/ml). In later experiments, we added piperonyl butoxide in 50% (v/v) ethanol (6 µl/ml of medium). Controls always included appropriate amounts of solvents.

Assay of porphyrins formed by intact cells

The spectrofluorometric method was described in detail previously (Sinclair *et al.*, 1984a).

H.p.l.c. of porphyrins

Cells and medium together were extracted with an equal volume of conc. HCl/acetone (1:39, v/v). After centrifugation, the pH of the supernatant was adjusted to about 4.0 with 1M-NaOH. A 50 µl portion was injected on to a 5 µ C-18 reversed-phase Radial Pak column (Waters Associates, Milford, MA, U.S.A.). Porphyrins were eluted by using the following conditions: solvent A, methanol/0.1 M-ammonium phosphate (11:14, v/v), pH 3.5; solvent B, 100% methanol; linear gradient, 30–100% B over 12 min, at a flow rate of 1.5 ml/min (Ford *et al.*, 1981) and detected by fluorescence (excitation 398 nm, emission 620 nm; Shimadzu fluorescence detector, model RF-530). Recovery for each porphyrin using this method was (%): URO, 67; heptacarboxyl, 74; hexacarboxyl, 77; pentacarboxyl, 76; copro, 79; and meso, 73, based on results with a standard mixture (Porphyrin Products, Logan, UT, U.S.A.) added to cells and medium before extraction.

Toxicity of chemicals in hepatocyte cultures

Concentrations of chemicals were regarded as toxic if they decreased the accumulation of porphyrins induced by the combination of 0.14 mM-propylisopropylacetamide and 30 µM-desferrioxamine (Granick *et al.*, 1975). Chemicals to be tested for toxic effects were added 1 h before the inducing chemicals, and accumulated porphyrins due to the induced ALA synthase were measured after 7 h further incubation. We have found this method to be more sensitive for detection of toxicity than microscopic examination, Trypan Blue uptake or release of lactate dehydrogenase.

H.p.l.c. of TCB and HCB

Purity of the radioactive TCB and HCB was determined by h.p.l.c. using a 10 µ C-18 Radial Pak column and an isocratic liquid phase of methanol/water (9:1, v/v) and a flow rate of 1 ml/min. Both compounds were eluted as single peaks. Fractions (1 ml) were collected, added to 10 ml of scintillation fluid (ACS; Amersham/Searle, Des Plaines, IL, U.S.A.), and radioactivity was determined in a Searle-Analytic Mark III scintillation spectrometer. Metabolism of these compounds by the cell cultures to water-soluble products was determined by a single extraction of the cells plus medium with 2 vol. of dichloromethane. The radioactivity in both layers was determined as described above. The remaining organic layer was evaporated under N₂ with heating (40 °C) and a portion was injected on to the C-18 column. The eluate from this column was collected (1 ml fractions) and radioactivity determined.

ALA synthase

Activity in intact cells was measured as described previously (Sinclair & Granick, 1977).

Incorporation of [¹⁴C]ALA into cellular haem

Cells were treated with [4-¹⁴C]ALA (1 µCi, 82 µM). After a 2 h labelling period, cells were rinsed rapidly with cold 0.15 M-NaCl and haem was extracted with 2.5 ml of acetone/HCl (acetone/conc. HCl/water, 20:1:4, by vol.), containing a small amount of freshly added red-cell haemolysate to supply carrier haem. After 5 min the acetone/HCl extract was mixed with 5 ml of diethyl ether in a glass scintillation vial and washed twice with 2 ml of

2 M-HCl to remove porphyrins and ALA. The diethyl ether was evaporated at 37 °C and the haem dissolved in 10 ml of ACS for determination of radioactivity.

RESULTS

Reversal of URO accumulation by piperonyl butoxide as detected by porphyrin composition

Previously (Sinclair *et al.*, 1984a) we demonstrated that URO accumulation in intact cultured hepatocytes caused by halogenated biphenyls such as TCB could be detected by changes in the composition of porphyrins generated from added ALA. Cells treated with ALA alone accumulated predominantly PROTO, whereas cells treated with both ALA and TCB accumulated mainly URO and heptacarboxyporphyrins. We also showed that if inhibitors of cytochrome *P*-450 isoenzymes such as piperonyl butoxide were added before TCB, URO accumulation was prevented. Fig. 1 shows that piperonyl butoxide can also reverse the URO accumulation. Cells treated first with TCB or PBB and then with ALA accumulated URO. After piperonyl butoxide was added to these cultures, URO accumulation stopped and PROTO accumulation was restored almost to the control rate. In the absence of piperonyl butoxide, no PROTO was detected and URO accumulation continued. In the absence of any other treatment, piperonyl butoxide did not induce accumulation of porphyrin and hence had no effect alone. Values for coproporphyrin are not shown, since concentrations of this porphyrin varied minimally with the different treatments. In a similar experiment, SKF-525A, an inhibitor of the major phenobarbital-induced *P*-450 isoenzyme (Testa & Jenner, 1981), had no effect on the porphyrins accumulating from ALA in TCB-treated cells (results not shown). This finding confirms the previous implication of an essential role for MC-inducible isoenzymes in the URO accumulation

caused by halogenated biphenyls (Sinclair *et al.*, 1984a). These experiments suggest that URO accumulated in the cells as the porphyrin and not the porphyrinogen, since uroporphyrinogen would have been further metabolized by uroporphyrinogen decarboxylase, whereas Fig. 1 indicates URO levels in the cells treated with either polyhalogenated biphenyl plus piperonyl butoxide remained constant over the indicated time period. Previous work has shown URO remains in the cells (Granick *et al.*, 1975; Kawanishi *et al.*, 1983; Sinclair *et al.*, 1983) possibly in lysosomes (Sandberg & Romslo, 1982).

Fig. 2 shows the amount of piperonyl butoxide required to reverse the URO accumulation caused by TCB. These doses of piperonyl butoxide were non-toxic by the criterion of induced porphyrin accumulation used in the present paper (see the Materials and methods section). Concentrations of piperonyl butoxide greater than 45 μ M were not soluble in the culture medium.

Restoration of TCB-inhibited haem synthesis by piperonyl butoxide

Table 1 shows that the TCB-mediated decrease in haem synthesized by intact cells was almost completely reversed by addition of piperonyl butoxide. It is not clear why the treatment with piperonyl butoxide increased haem radioactivity over control; however, other inducers of cytochrome *P*-450 also increase haem radioactivity (S. Shedlofsky, P. Sinclair, H. Bonkovsky, J. Sinclair & J. Healey, unpublished work).

Restoration of haem synthesis by piperonyl butoxide detected by changes in ALA synthase activities

It is now widely accepted that induction by drugs of ALA synthase, the first and rate-limiting enzyme of hepatic haem biosynthesis, can be augmented by inhibiting one of the subsequent steps of haem biosynthesis and thus decreasing feedback repression of

Fig. 1. Porphyrin accumulation from exogenous ALA after addition of piperonyl butoxide to hepatocytes treated with TCB or PBB

Cells on 3.5 cm dishes were treated for 17 h with DMSO alone (a), 3.4 μ M-TCB (b) or 1.8 μ M-PBB (c). ALA (150 μ M) was added, and after 2 h, piperonyl butoxide was added to a final concentration of 30 μ M. Porphyrin composition was measured as described in the Materials and methods section. Values are means for three dishes. If not shown, the s.d. fell within symbol size. Symbols: $\circ$, PROTO in the absence of piperonyl butoxide; $\bullet$, PROTO in the presence of piperonyl butoxide; $\triangle$, URO in the absence of piperonyl butoxide; $\blacktriangle$, URO in the presence of piperonyl butoxide.

Fig. 2. Dose response of piperonyl butoxide to reverse the URO accumulation caused by TCB

Cells on 3.5 cm dishes were treated with $3.4 \mu\text{M}$ -TCB for 18 h and then with $150 \mu\text{M}$ -ALA for 1 h before adding piperonyl butoxide for a further 5 h incubation. Porphyrin composition was determined as described in the Materials and methods section. Cells not treated with TCB accumulated 0.09 nmol of URO and 0.98 nmol of PROTO/dish. Data are presented as means and range of values from two dishes. Δ , URO; $\circ$, PROTO.

Table 1. Restoration of TCB-inhibited haem synthesis by piperonyl butoxide

Cells in 6 cm dishes were pretreated where indicated with $0.85 \mu\text{M}$ -TCB or DMSO for 4 h. Then, where indicated, $30 \mu\text{M}$ -piperonyl butoxide (PipB) was added and, after 1.7 h, [^{14}C]ALA (1 μCi , 82 μM) was added for 2 h further incubation. Haem radioactivity was determined as described in the Materials and methods section. Excess ALA (82 μM) was used to swamp any endogenous pools. Values represent the means for duplicate dishes (the numbers in parentheses are the individual values).

Treatment	$10^{-3} \times$ Haem radioactivity (d.p.m.)
DMSO	7.9 (7.4, 8.3)
TCB	4.0 (3.8, 4.2)
DMSO + PipB	9.1 (9.1, 9.1)
TCB + PipB	11.8 (11.6, 11.9)

ALA synthase by haem [for reviews, see Bonkovsky (1982) and De Matteis (1978)]. When chick hepatocytes in culture are treated with chlorinated aromatic hydrocarbons in combination with phenobarbital, high levels

of ALA synthase are induced (de Verneuil *et al.*, 1983a). Also, rats treated with hexachlorobenzene and phenobarbital have highly induced levels of hepatic ALA synthase (Blekkenhorst *et al.*, 1980; Wainstock de Calmanovici *et al.*, 1984). If piperonyl butoxide restored haem synthesis in the chick liver cultures as well as preventing URO accumulation, then addition of piperonyl butoxide should lead to a decrease in ALA synthase activity by restoring the regulatory haem pool. Table 2 shows the results of an experiment in which ALA synthase activity was synergistically induced by combining PIA, a phenobarbital-like inducer in this system (Sinclair *et al.*, 1981) either with TCB (which causes URO accumulation), or with the iron chelator CaMg-EDTA (which inhibits the last step of the haem-synthetic pathway, ferrochelatase). The data show subsequent addition of piperonyl butoxide to cells pretreated with PIA and TCB caused a decrease in the activity of ALA synthase. This was most probably due to reversal of URO accumulation and restored haem synthesis (cf. Table 1) that thereby restored the normal feedback repression of ALA synthase. Desferrioxamine, when added with piperonyl butoxide, allowed maintenance of induced ALA synthase activity (Table 2, line 5). This was most probably because haem synthesis remained inhibited at the later ferrochelatase step of the pathway. When PIA was combined with CaMg-EDTA, piperonyl butoxide had little or no effect (Table 2, line 6), ruling out

Table 2. Restoration of haem synthesis by piperonyl butoxide as indicated by decrease in 5-aminolaevulinate synthase activity

Cells on 3.5 cm dishes were pre-treated with inducers as indicated for 11 h. Then piperonyl butoxide (PipB) ($30 \mu\text{M}$), or cycloheximide (CX) ($1.8 \mu\text{M}$), or desferrioxamine (Des) ($30 \mu\text{M}$) were added to some dishes and the incubations continued for 4 h. ALA synthase was measured in the intact cells as described in the Materials and methods section. For ease of comparison, data have been normalized. An activity (nmol of ALA/h per mg of protein) of 100% for treatment with 0.14 mM -PIA + $3.4 \mu\text{M}$ -TCB was 14.2 ± 0.7 in Expt. 1 and 12.1 ± 1.12 in Expt. 2. Activities in cells treated with PIA or TCB alone were 1.0 and 1.33 respectively for Expt. 2. These activities were not determined in Expt. 1. An activity of 100% for treatment with 0.14 M -PIA + 7.5 mM -CaMg-EDTA was 8.2 ± 0.1 in Expt. 1 and 6.8 ± 0.4 in Expt. 2. Data are presented as enzyme activity remaining after 4 h treatment (100% being the 'untreated' value at 4 h) and represent means $\pm$ s.d. —, 'not done'.

Pretreatment	Later treatment	Relative ALA synthase activity (%)	
		Expt. 1	Expt. 2
PIA + TCB	None	100 ± 5	100 ± 10
	PipB	32 ± 1	47 ± 6
	CX	20 ± 1	18 ± 5
	Des	—	86 ± 7
	PipB + Des	—	83 ± 2
PIA + CaMg-EDTA	None	100 ± 9	100 ± 6
	PipB	89 ± 1	82 ± 13
	CX	10 ± 0	11 ± 6
	Des	—	124 ± 13
	PipB + Des	—	134 ± 19

non-specific inhibition of ALA synthase by piperonyl butoxide. The cycloheximide treatments (Table 2, lines 3 and 8) showed that, without continual induced protein synthesis, ALA synthase activity rapidly fell, consistent with the known short half-life of the enzyme (Ades *et al.*, 1983).

Piperonyl butoxide also reversed URO accumulation caused by other compounds

Table 3 shows that piperonyl butoxide was also effective in reversing the URO accumulation caused by TCB and other halogenated compounds, including mixtures of polyhalogenated biphenyls (Aroclor, Firemaster), 3,4,5,3',4',5'-hexabromobiphenyl or lindane (γ -hexachlorocyclohexane). This was indicated by resumption of PROTO accumulation and prevention of further accumulation of URO. Table 4 shows that piperonyl butoxide also reversed URO accumulation caused by

treatment of cells with either PIA or phenobarbital (de Verneuil *et al.*, 1983a; Marks *et al.*, 1983). The recently reported URO accumulations caused by the organophosphorus compound parathion (Nichol & Angel, 1984) and by the Ca^{2+} -channel-blocking drugs nifedipine and verapamil (Schoenfeld *et al.*, 1985), were also reversible by piperonyl butoxide (results not shown). SKF-525A, the selective inhibitor of the major phenobarbital-induced isoenzyme of cytochrome *P*-450, reversed the URO accumulation caused by PIA and phenobarbital, but not that caused by TCB.

Further characteristics of the reversal of URO accumulation by piperonyl butoxide

Even after 44 h of TCB pretreatment followed by ALA, piperonyl butoxide almost completely restored PROTO synthesis. When cells were incubated for 19 h with $68 \mu\text{M}$ -TCB (the limit of solubility) as used by Swain

Table 3. Reversal by piperonyl butoxide of URO accumulation caused by other compounds

Cells in 3.5 cm dishes were treated with the chemicals indicated for 18 h then were either harvested or treated with $30 \mu\text{M}$ -piperonyl butoxide (PipB). After 1 h, ALA (0.15 mM) was added for 6 h further incubation. Porphyrin composition was determined as described in the Materials and methods section. Results are means of two determinations whose values varied less than 5%. —, 'Not done'. Abbreviation: HBB, 3,4,5,3',4',5'-hexabromobiphenyl.

Treatment	Porphyrin composition (nmol/dish)					
	at 18 h		at 25 h			
			No PipB		+ PipB	
			URO	PROTO	URO	PROTO
None	—	—	0.13	1.07	0.05	1.12
TCB (1 $\mu\text{g}/\text{ml}$)	0.69	0	1.22	0.06	0.69	0.90
HBB (0.5 $\mu\text{g}/\text{ml}$)	0.19	0	0.89	0.05	0.33	0.79
Aroclor (6 $\mu\text{g}/\text{ml}$)	1.16	0	1.70	0.09	1.08	0.62
Firemaster (6 $\mu\text{g}/\text{ml}$)	1.10	0.07	1.81	0.01	1.20	0.58
Lindane (5 $\mu\text{g}/\text{ml}$)	0.87	0	1.40	0.10	0.87	0.41
β -Naphthoflavone (4 $\mu\text{g}/\text{ml}$)	—	—	0.60	0.07	0.08	0.85

Table 4. Effects of SKF-525A and piperonyl butoxide on the URO accumulation caused by PIA, phenobarbital and TCB

Cells on 3.5 cm dishes were treated with $3.5 \mu\text{M}$ -TCB, 0.14 mM-PIA or 2 mM-sodium phenobarbital (PB) for 17 h. Some cells were harvested as indicated (zero time). To others, 0.15 mM-ALA, $30 \mu\text{M}$ -piperonyl butoxide (PipB) or $22 \mu\text{M}$ -SKF-525A were added as indicated. After 5 h, porphyrin composition was determined as described in the Materials and methods section. Values are means $\pm$ S.D. for three dishes. —, 'not determined'.

Pretreatment	Later treatment	Porphyrin composition (nmol/dish)			
		At zero time		5 h after addition of ALA + PipB or SKF-525A	
		URO	PROTO	URO	PROTO
None	ALA	—	—	0.07 ± 0.02	0.98 ± 0.05
TCB	ALA	0.32 ± 0.05	0	0.77 ± 0.16	0
	ALA + PipB	—	—	0.35 ± 0.04	0.78 ± 0.02
PIA	ALA + SKF-525A	—	—	0.76 ± 0.06	0
	ALA	0.02 ± 0.01	0.02 ± 0.01	0.21 ± 0.03	0.16 ± 0.02
	ALA + PipB	—	—	0.06 ± 0.04	0.70 ± 0.04
	ALA + SKF-525A	—	—	0.03 ± 0.04	0.84 ± 0.11
PB	ALA	0.24 ± 0.04	0.02 ± 0.04	0.53 ± 0.05	0.01 ± 0.01
	ALA + PipB	—	—	0.24 ± 0.04	0.52 ± 0.05
	ALA + SKF-525A	—	—	0.33 ± 0.08	0.52 ± 0.01

et al. (1983) and then treated with ALA and piperonyl butoxide, URO accumulation was stopped and PROTO synthesis restored. In other experiments, the presence or absence of insulin did not affect the results. The ability of piperonyl butoxide to prevent or reverse URO accumulation caused by $3.4 \mu\text{M}$ -TCB was lost after 17.5 h. However, a second addition of piperonyl butoxide to such cells at 17.5 h again reversed URO accumulation, possibly indicating that the loss of effect of piperonyl butoxide after 17.5 h was due to metabolism of piperonyl butoxide itself. This was not investigated further.

Reversal of URO accumulation of piperonyl butoxide is independent of protein synthesis

Table 5 shows that addition of cycloheximide with piperonyl butoxide did not prevent the reversal of URO accumulation. The effectiveness of cycloheximide as an inhibitor of protein synthesis was shown by its inhibition of induced porphyrin accumulation (see legend to Table 5). Cycloheximide at this concentration decreases incorporation of labelled leucine into protein by 85% (J. Sinclair *et al.*, 1984). In other experiments, addition of cycloheximide 1 h before adding piperonyl butoxide also did not affect the reversal by piperonyl butoxide of URO accumulation.

Reversal of URO accumulation by analogues of piperonyl butoxide

Two structural analogues of piperonyl butoxide, safrole and isosafrole, were also effective at reversing URO accumulation, although their potency and efficacy were lower. For example, 0.2 mM-isosafrole was about as effective as 0.03 mM-piperonyl butoxide. Both safrole and isosafrole were tested (up to concentrations of 0.6 mM); neither was found to be toxic to porphyrin induction (measured as described in the Materials and methods section). The parent ring compound, methylenedioxybenzene, up to 1 mM, was ineffective at reversing the URO accumulation, as was 0.8 mM-*n*-butyldioxalane, an analogue to the oxygen-containing ring of piperonyl butoxide (results not shown).

Table 5. Effect of cycloheximide on the reversal by piperonyl butoxide of URO accumulation

Cells on 3.5 cm dishes were treated with DMSO or with $3.4 \mu\text{M}$ -TCB for 26.5 h. During the last 4.5 h, 0.15 mM-ALA, 1.8 μM -cycloheximide (CX) and 30 μM -piperonyl butoxide (PipB) were present as indicated. Values are means $\pm$ S.D. for three dishes. In the same experiment, 1.8 μM -CX inhibited by 93% a 9 h induction of porphyrin accumulation caused by PIA (140 μM) plus $3.4 \mu\text{M}$ -TCB (total induced porphyrin was 0.53 nmol/dish). Porphyrins were determined as described in the Materials and methods section.

Treatment	Porphyrin composition (nmol/dish)	
	URO	PROTO
ALA	0.09 ± 0.02	0.81 ± 0.12
TCB + ALA	0.59 ± 0.03	0.00 ± 0.00
TCB + ALA + PipB	0.30 ± 0.02	0.64 ± 0.04
TCB + ALA + PipB + CX	0.28 ± 0.02	0.64 ± 0.05

Fig. 3. Effect of ellipticine on the porphyrin pattern after treatment of cells with TCB

Cells on 6 cm dishes were treated with DMSO or $3.4 \mu\text{M}$ -TCB for 18 h. Then 150 μM -ALA and, where indicated, 20 μM -ellipticine, were added, and the incubation was continued for 7 h. Porphyrins in cells and media were analysed by h.p.l.c. as described in the Materials and methods section. (a) ALA; (b) TCB and ALA; (c) TCB, ALA and ellipticine. Peak identification: 8, URO; 7, heptacarboxyporphyrin; 6, hexacarboxyporphyrin; 5, pentacarboxyporphyrin; 4, coproporphyrin; 2, PROTO.

Reversal of URO accumulation by ellipticine

Previously we found that only inhibitors of MC-inducible *P*-450 isoenzymes would prevent URO accumulation by TCB (Sinclair *et al.*, 1984a). Ellipticine, the specific inhibitor of MC-inducible cytochrome *P*-450 (Lesca *et al.*, 1979), reversed URO accumulation caused by TCB as seen by accumulation of coproporphyrin and PROTO (Fig. 3). In this case, h.p.l.c. analysis of porphyrins was required to demonstrate the recovery of PROTO synthesis, because ellipticine has a fluorescence emission spectrum similar to PROTO, a fact that made the spectrofluorometric assay for porphyrins unusable. Ellipticine, like piperonyl butoxide, caused a decrease in

Table 6. Reversal of URO accumulation by acetylaminofluorene

Cells on 3.5 cm dishes were treated with $3.4 \mu\text{M}$ -TCB or DMSO alone for 20 h, then 0.15 mM-ALA was added with 0.1 mM-2-acetylaminofluorene (AAF) as indicated and cells harvested after 6 h further incubation. Porphyrin composition was determined as described in the Materials and methods section. Values represent means $\pm$ S.D. for triplicate dishes.

Pretreatment	Later treatment	Porphyrin composition (nmol/dish)	
		URO	PROTO
DMSO	ALA	0.05 ± 0.01	0.87 ± 0.05
	ALA + AAF	0.05 ± 0.01	0.86 ± 0.03
TCB	No addition	0.35 ± 0.01	0.00 ± 0.00
	ALA	0.90 ± 0.07	0.03 ± 0.02
	ALA + AAF	0.44 ± 0.04	0.57 ± 0.10

Table 7. Effect of piperonyl butoxide on metabolism of TCB and HCB and on URO accumulation

Cells on 6 cm dishes were treated with 0.96 μM -MC for 18.5 h then with 30 μM -piperonyl butoxide (PipB) as indicated. After 1 h, 0.6 μM - ^{14}C TCB or 0.7 μM - ^{14}C HCB were added. Cells and medium were harvested together after 8 h further incubation, 0.15 mM-ALA being present for the last 2 h. Portions were assayed for porphyrin or extracted with dichloromethane as described in the Materials and methods section. Radioactivity of the aqueous fraction was counted. H.p.l.c. analysis of the dichloromethane fraction showed that most of the radioactivity was parent compound ($> 80\%$ for TCB, $\sim 100\%$ for HCB). In piperonyl butoxide-treated cells, all the radioactivity in the dichloromethane fraction was in the parent compound. Total porphyrin ranged from 0.81 to 1.05 nmol/dish. Values are means for three dishes.

Treatment	Radioactivity in aqueous phase (% of total)	Porphyrin composition (nmol/dish)	
		URO	PROTO
TCB	22 $\pm$ 0.4	0.60 $\pm$ 0.03	0.07 $\pm$ 0.02
TCB + PipB	6 $\pm$ 0.1	0.08 $\pm$ 0.03	0.85 $\pm$ 0.07
HCB	4 $\pm$ 2.0	0.59 $\pm$ 0.07	0.09 $\pm$ 0.03
HCB + PipB	4 $\pm$ 1.0	0.24 $\pm$ 0.06	0.59 $\pm$ 0.02

ALA synthase activity after treatment with PIA and TCB in experiments analogous to those shown in Table 2 (results not shown).

Reversal of URO accumulation by acetaminofluorene

Previously we reported that 2-acetaminofluorene is rapidly metabolized by chick liver culture and that piperonyl butoxide, ellipticine and SKF-525A inhibit this metabolism, depending on the *P*-450 isoenzymes present in the cells (Sinclair *et al.*, 1984a). Table 6 shows that 2-acetaminofluorene added to cells pretreated with TCB and ALA restored PROTO synthesis in a way analogous to the action of piperonyl butoxide. Acetaminofluorene was not toxic at 100 μM in the induced-porphyrin test described in the Materials and methods section. Other substrates of cytochrome *P*-450, including biphenyl (up to 64 μM), aminopyrine (0.87 mM), and benzo[*a*]pyrene (20 μM) did not reverse the URO accumulation caused by TCB (results not shown).

Piperonyl butoxide and metabolism of TCB and HCB

The major hypothesis for the mechanism of URO accumulation is that activated metabolites of halogenated compounds react with uroporphyrinogen decarboxylase [see review by Marks (1985)]. Table 7 shows that 22% of the TCB was metabolized to water-soluble products, whereas HCB was scarcely metabolized. Nevertheless, URO accumulation by the cells caused by either compound was reversible by piperonyl butoxide, which measurably affected the metabolism of TCB only. Thus there was no demonstrable relationship between the action of piperonyl butoxide to reverse the URO accumulation and to inhibit metabolism of the halogenated biphenyls.

DISCUSSION

In the present paper we have shown by several different criteria that URO accumulation by cultures of chick-embryo liver cells treated with polyhalogenated aromatic hydrocarbons was reversed almost immediately after piperonyl butoxide was added to the cultures (Fig. 1). Compounds such as ellipticine, which also bind to and inhibit the activity of the isoenzyme of cytochrome *P*-450 induced by methylcholanthrene, were also effective

(Fig. 3). These results conflict with the generally held hypotheses that the mechanism of the URO accumulation caused by polyhalogenated aromatic compounds is mediated via metabolites that covalently bind to uroporphyrinogen decarboxylase (de Verneuil *et al.*, 1983a; Swain *et al.*, 1983; Wainstock de Calmanovici *et al.*, 1984; Cantoni *et al.*, 1984a; Sinclair *et al.*, 1984a; Marks, 1985) or that oxidize some essential thiol group of this enzyme (Debets *et al.*, 1981b).

The action of piperonyl butoxide in reversing URO accumulation in the intact cells was confirmed by two independent assays of haem synthesis: (i) the conversion of ^{14}C ALA into haem (Table 1), and (ii) the decrease of ALA synthase activity induced by combination of PIA and TCB (Table 2). These results taken together strongly indicate that, in the intact cells, conversion of uroporphyrinogen into coproporphyrinogen and hence haem was restored by the action of piperonyl butoxide.

The reversal of URO accumulation by piperonyl butoxide was achievable under all the conditions by which we and others have reported URO accumulation in the intact chick-embryo liver cells. These include treatment (i) with halogenated compounds for 2 days, (ii) with high TCB concentrations (Swain *et al.*, 1983), (iii) with a variety of halogenated compounds (Table 3), (iv) with PIA and phenobarbital (Table 4), (v) with parathion, an organophosphorous compound that causes URO accumulation (Nichol & Angel, 1984) and (vi) with the Ca^{2+} -channel-blocking agents nifedipin and verpamil (Schoenfeld *et al.*, 1985). Recently we found URO accumulation caused by TCDD (Sinclair & Granick, 1974; de Verneuil *et al.*, 1983a) was also reversible by piperonyl butoxide (Lambrecht *et al.*, 1986). We have shown that accumulation of URO from exogenous ALA is detectable by treatment of chick hepatocyte cultures with PBB at concentrations lower than those required to cause detectable decreases in uroporphyrinogen decarboxylase activity as assayed by a conventional assay *in vitro* (Sinclair *et al.*, 1983). The present results show that piperonyl butoxide restored normal haem synthesis at all concentrations of halogenated biphenyl studied.

Some comments on the validity of assays in the intact cells are appropriate. In these and previous studies (Sinclair *et al.*, 1983, 1984a), porphyrin composition was determined by the fluorimetric assay of Grandchamp

et al. (1980). By using reverse-phase h.p.l.c. we have now further validated that fluorimetric assay (Fig. 3). Haem synthesis in intact cells was measured by exposing cells to excess [^{14}C]ALA (82 μM) for 2 h (Table 1). With this concentration of ALA, a maximal rate of accumulation of radioactivity as haem was produced. In the intact cells, haem is continually being degraded to biliverdin by intracellular haem oxygenase. In recent experiments (P. Sinclair, S. Howell, J. Healey & H. Bonkovsky, unpublished work) we have measured radioactivity in biliverdin as well as in haem and have found that not only haem, but also biliverdin, synthesis, was restored to normal by addition of piperonyl butoxide to cells previously treated with TCB. We conclude that normal rates of haem synthesis and catabolism were restored by treatment of the cells with piperonyl butoxide.

Previously (Sinclair *et al.*, 1984a) we showed that the induction of the *P*-450 isoenzyme induced by methylcholanthrene is a prerequisite for URO accumulation caused by the halogenated biphenyls. Here we have demonstrated that either inhibitors of cytochrome *P*-450 (piperonyl butoxide, some methylenedioxybenzene analogues and ellipticine) or a highly metabolized substrate of cytochrome *P*-450 (acetylaminofluorene) can reverse URO accumulation (Figs. 1 and 3; Table 6). One explanation for the action of these compounds is that they prevent formation of an inhibitor of uroporphyrinogen decarboxylase derived from the halogenated biphenyl. However, a problem with this hypothesis is that, although one compound that caused URO accumulation, TCB, was measurably metabolized in the culture, another more halogenated biphenyl, HCB, was scarcely metabolized at all (Table 7). Indeed, h.p.l.c. of the organic phase indicated no other HCB-derived compound except HCB itself. Therefore, if the inhibitor were a metabolite of HCB, it would have to be extremely potent and specific, since we calculate that, on a molar basis, the amount of water-soluble metabolite of HCB could be no greater than the amount of uroporphyrinogen decarboxylase in the cells. The calculation is based on published values for the M_r and the specific activity of human uroporphyrinogen decarboxylase (de Verneuil *et al.*, 1983b; Elder *et al.*, 1983) with pentacarboxyl-porphyrinogen III as substrate. Assuming that the specific activity and M_r of human and chick enzymes are similar, we calculate that 1 mg of chick hepatocyte-cell protein contains 1–4 pmol of uroporphyrinogen decarboxylase.

Consistent with data of other workers (de Verneuil *et al.*, 1983a; Marks *et al.*, 1983), we showed that URO accumulation followed treatment of the cells with inducers of other isoenzymes of cytochrome *P*-450, PIA or phenobarbital (Table 4). de Verneuil *et al.* (1983a) suggested a role for the phenobarbital-induced *P*-450 isoenzyme in the mechanism of URO accumulation caused by phenobarbital. This isoenzyme may also be involved in the URO accumulation caused by aliphatic alcohols in this cell-culture system (Sinclair *et al.*, 1986). Participation of this *P*-450 isoenzyme is supported by our finding that SKF-525A reversed the URO accumulation caused by phenobarbital (Table 4). However, SKF-525A was not effective against the URO accumulation caused by TCB (Table 4) (Debets *et al.*, 1980), indicating that, in the URO accumulation caused by the biphenyls, another isoenzyme of cytochrome *P*-450 must be involved (Sinclair *et al.*, 1984a).

Since cycloheximide failed to affect the ability of

piperonyl butoxide to reverse URO accumulation (Table 5) and since the reversal was so rapid (Fig. 1), it seems that synthesis *de novo* of uroporphyrinogen decarboxylase was not involved in the mechanism of the reversal.

It is not yet known whether piperonyl butoxide administered to animals will reverse the hepatic accumulation and excretion of URO caused by halogenated aromatic compounds. In rodents, it may be difficult to maintain effective concentrations of piperonyl butoxide, since it is known that the effects of piperonyl butoxide to inhibit drug metabolism are lost in mice within 12 h (Skrinjaric-Spoljar *et al.*, 1971). Even in culture, the effect of piperonyl butoxide did not persist beyond 18 h. In intact rodents the accumulation of URO is over a longer period (weeks to months) (Elder *et al.*, 1976; Jones & Sweeney, 1980) than in our culture experiments (maximum 4 days) in which reversibility by piperonyl butoxide was maintained.

Conclusions on mechanism of URO accumulation

The present results show that, in the intact chick hepatocyte, accumulation of URO caused by some halogenated biphenyls is rapidly reversed by compounds that are known to bind to, and inhibit, the *P*-448 isoenzyme of cytochrome *P*-450. These results conflict with virtually all the current proposals to explain the mechanism of URO accumulation. In particular, these data seem to exclude irreversible covalent binding to uroporphyrinogen decarboxylase by an inhibitory metabolite of the biphenyl as the mechanism for URO accumulation. Direct inhibition of the enzyme by the biphenyls has been proposed (Kawinishi *et al.*, 1983), and this is not excluded by our data. The presence of stable inhibitors of uroporphyrinogen decarboxylase in livers from hexachlorobenzene-treated rats (Rios de Molina *et al.*, 1980) and TCDD-treated mice (Cantoni *et al.*, 1984b) have also been reported. However, as previously discussed (Sinclair *et al.*, 1984a), we and others have produced evidence that a cytochrome *P*-450 isoenzyme plays an essential role in the uroporphyrin caused by halogenated biphenyls. Since a direct role of the biphenyl metabolites is unlikely, we suggest that the role of the *P*-450 isoenzymes is to bind the biphenyls and to generate a form of activated oxygen. Piperonyl butoxide and acetylaminofluorene would prevent binding of the biphenyl and the oxygen species would then decay or be degraded by cellular protective enzymes. The activated oxygen species may reversibly attack uroporphyrinogen decarboxylase itself. We consider it more likely that the oxygen species may oxidize the substrates (uroporphyrinogen and heptacarboxyporphyrinogen) to porphyrins that are themselves not substrates of uroporphyrinogen decarboxylase. A central role of the oxidation of porphyrinogen substrates in the development of porphyria cutanea tarda was suggested previously (Heikel *et al.*, 1958) and has been recently revived (Pimstone, 1982; Mukerji *et al.*, 1984). There is evidence that microsomal cytochrome *P*-450s produce H_2O_2 owing to uncoupling of the mono-oxygenase reaction (Kuthan *et al.*, 1978; Kuthan & Ullrich, 1982), particularly when non-metabolizable substrates are bound. Ferioli *et al.* (1984) have also recently proposed this possibility. Isolated rat hepatocytes generate H_2O_2 during metabolism of hexobarbital (Eklöw *et al.*, 1984). Evidence in favour of this oxidant hypothesis to explain the action of TCB and PBB in the chick culture is that URO, rather than the

reduced porphyrinogen, accumulated in cells (Fig. 1). Recent experiments in our laboratory (P. Sinclair, unpublished work) show that uroporphyrinogen I is oxidized in the presence of NADPH and TCB by microsomal cytochrome *P*-450 from MC-treated chick embryos. Further work is needed to examine the oxidation hypothesis and to determine whether measurements of activities of uroporphyrinogen decarboxylase in cell homogenates correlate with the observations with intact living cells described here.

We are grateful to Dr. L. Smith and Dr. S. Shedlofsky for critical comments and to Mrs. D. Burke and Mr. R. Chapman who typed the manuscript. We thank Ms. S. Wood and Mr. S. Howell for h.p.l.c. analyses of porphyrins. This work was supported by research funds from the Veterans Administration and grants from the National Institutes of Health (CA 25012 and HL 28177).

REFERENCES

- Ades, I., Harpe, K. & Stevens, T. (1983) *Biochem. J.* **214**, 967–974
- Blekkenhorst, G., Day, R. & Eales, L. (1980) *Int. J. Biochem.* **12**, 1013–1017
- Bonkovsky, H. (1982) in *Hepatology* (Zakim, D. & Boyer, T., eds.), pp. 351–393, Saunders, Philadelphia
- Cantoni, L., Dal Fiume, D. & Ruggieri, R. (1984a) *Int. J. Biochem.* **16**, 561–565
- Cantoni, L., Dal Fiume, D., Rizzardini, M. & Ruggieri, R. (1984b) *Toxicol. Lett.* **20**, 211–217
- De Matteis, F. (1978) in *Heme and Hemoproteins* (De Matteis, F. & Aldridge, W., eds.), pp. 129–150, Springer-Verlag, Berlin
- de Verneuil, H., Sassa, S. & Kappas, A. (1983a) *Biochem. J.* **214**, 145–151
- de Verneuil, H., Sassa, S. & Kappas, A. (1983b) *J. Biol. Chem.* **258**, 2454–2460
- Debets, F., Hamers, W. & Strik, J. (1980) *Int. J. Biochem.* **12**, 1019–1025
- Debets, F., Reinders, J., Koss, G., Seidel, J. & Strik, J. (1981a) *Chem-Biol. Interact.* **37**, 77–94
- Debets, F., Reinders, J., Debets, A., Lossbroek, T., Strik, J. & Koss, G. (1981b) *Toxicology* **19**, 185–196
- Eklöw, L., Moldéus, P. & Orrenius, S. (1984) *Eur. J. Biochem.* **138**, 459–463
- Elder, G. & Sheppard, D. (1982) *Biochem. Biophys. Res. Commun.* **109**, 113–120
- Elder, G., Evans, J. & Matlin, S. (1976) *Clin. Sci. Mol. Med.* **51**, 71–80
- Elder, G., Tovey, J. & Sheppard, D. (1983) *Biochem. J.* **215**, 45–55
- Feroli, A., Harvey, C. & De Matteis, F. (1984) *Biochem. J.* **224**, 769–777
- Ford, R., Ou, C. & Ellefson, R. (1981) *Clin. Chem.* **27**, 397–401
- Grandchamp, B., Deybach, J., Grellet, M., de Verneuil, H. & Nordmann, A. (1980) *Biochim. Biophys. Acta* **629**, 577–586
- Granick, S., Sinclair, P., Sassa, S. & Griener, G. (1975) *J. Biol. Chem.* **250**, 9215–9225
- Heikel, T., Lockwood, W. & Rimington, C. (1958) *Nature (London)* **182**, 313
- Jones, K. & Sweeney, G. (1980) *Toxicol. Appl. Pharmacol.* **53**, 42–49
- Kappas, A., Sassa, S. & Anderson, K. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J., Wyngaarden, J., Frederickson, D., Goldstein, J. & Brown, M., eds.), pp. 1301–1384, McGraw-Hill, New York
- Kawanishi, S., Seki, Y. & Sano, S. (1983) *J. Biol. Chem.* **258**, 4285–4292
- Kuthan, H. & Ullrich, V. (1982) *Eur. J. Biochem.* **126**, 583–588
- Kuthan, H., Tsuji, H., Graf, H., Ullrich, V., Werrigloer, J. & Estabrook, R. (1978) *FEBS Lett.* **91**, 343–345
- Lambrecht, R., Bement, W., Sinclair, P., Sinclair, J. & Bonkovsky, H. (1986) *Toxicologist* **6**, 41
- Lesca, P., Rafidinarivo, E., Lecoite, P. & Mansuy, D. (1979) *Chem-Biol. Interact.* **24**, 189–198
- Marks, G. (1985) *Crit. Rev. Toxicol.* **15**, 151–179
- Marks, G., Follows, S., Zelt, D. & Cole, S. (1983) *Can. J. Physiol. Pharmacol.* **61**, 546–553
- Mukerji, S., Pimstone, N. & Burns, M. (1984) *Gastroenterology* **87**, 1248–1254
- Nichol, A. & Angel, L. (1984) *Biochem. Pharmacol.* **33**, 2511–2515
- Pimstone, N. (1982) *Semin. Liver Dis.* **2**, 132–142
- Rios de Molina, M., Wainstock de Calmanovici, R. & San Martin de Viale, L. (1980) *Int. J. Biochem.* **12**, 1027–1032
- Sandberg, S. & Romslo, I. (1982) *Photobiochem. Photobiophys.* **4**, 95–106
- Schoenfeld, N., Aelion, J., Beigel, Y., Epstein, O. & Atsmon, A. (1985) *Clin. Sci.* **69**, 581–586
- 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, J., Wiebkin, P., Zaitlin, L., Smith, E. & Sinclair, P. (1984) *Biochem. Pharmacol.* **33**, 187–190
- Sinclair, J., Zaitlin, L., Smith, E., Howell, S., Bonkovsky, H. & Sinclair, P. (1986) *Biochem. J.* **234**, 405–411
- Sinclair, P. & Granick, S. (1974) *Biochem. Biophys. Res. Commun.* **61**, 124–133
- Sinclair, P. & Granick, S. (1977) *Anal. Biochem.* **79**, 380–393
- Sinclair, P., Elder, G., Bement, W., Smith, E., Bonkovsky, H. & Sinclair, J. (1983) *FEBS Lett.* **152**, 217–221
- Sinclair, P., Bement, W., Bonkovsky, H. & Sinclair, J. (1984a) *Biochem. J.* **222**, 737–748
- Sinclair, P., Bonkovsky, H., Bement, W., Sinclair, J., Urquhart, A. & Elder, G. (1984b) *Toxicologist* **4**, 132
- Skrinjaric-Spoljar, M., Matthews, H., Engel, J. & Casida, J. (1971) *Biochem. Pharmacol.* **20**, 1607–1618
- Swain, M., Follows, S. & Marks, G. (1983) *Can. J. Physiol. Pharmacol.* **61**, 105–108
- Testa, B. & Jenner, P. (1981) *Drug Metab. Rev.* **12**, 1–117
- Voorman, R., Jensen, R., Sleight, S. & Aust, S. (1983) *Toxicologist* **3**, 98
- Wainstock de Calmanovici, R., Rios de Molina, M., Taira de Yamasato, M., Tomio, M. & San Martin de Viale, L. (1984) *Biochem. J.* **218**, 753–763

Received 27 November 1985/17 February 1986; accepted 27 February 1986