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INDUCTION OF CYTOSOLIC AND MICROSOMAL EPOXIDE HYDROLASES AND PROLIFERATION OF PEROXISOMES AND MITOCHONDRIA IN MOUSE LIVER AFTER DIETARY EXPOSURE TO *p*-CHLOROPHENOXYACETIC ACID, 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4,5-TRICHLOROPHENOXYACETIC ACID*

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Abstract—The effects of dietary exposure to 0.125% (w/w) *p*-chlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid or 2,4,5-trichlorophenoxyacetic acid on the content of peroxisomes and levels of certain xenobiotic-metabolizing enzymes in mouse liver have been investigated.

In agreement with the literature on rat liver 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid were found to cause extensive proliferation of peroxisomes (as judged by the total levels of "mitochondrial" protein, carnitine acetyltransferase, cyanide-insensitive palmitoyl-CoA oxidation and catalase) in mouse liver. On the other hand, exposure to *p*-chlorophenoxyacetic acid did not significantly affect any of these parameters. As with certain other peroxisome proliferators, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid increased total cytochrome oxidase activity as well.

In addition, dietary exposure to 2,4-dichlorophenoxyacetic acid and to 2,4,5-trichlorophenoxyacetic acid resulted in increases in the activities of cytosolic and microsomal epoxide hydrolases in mouse liver and generally less pronounced increases in the total cytosolic gluthathione transferase activity and microsomal content of cytochrome P-450. In the case of cytochrome P-450, this process can be said to be a true induction (i.e. the amount of enzyme protein is increased), because the assay procedure for cytochrome P-450 measures holoenzyme amount. Immunoquantitation demonstrated that this was also the case for the changes in cytosolic epoxide hydrolase.

The dramatic differences in proliferation of peroxisomes and induction of xenobiotic-metabolizing enzymes seen here with compounds differing relatively little in structure may indicate that a receptor mechanism of some kind is involved.

Many of the toxic and genotoxic (mutagenic, carcinogenic and teratogenic) effects of different xenobiotics are now thought to be caused by short-lived reactive intermediates which arise during the metabolism of these foreign compounds by the cytochrome P-450 system [1]. These reactive intermediates, which are usually electrophiles, are thought to cause cellular damage by binding covalently to nucleophilic groups in cellular macromolecules. However, many such intermediates can be rendered comparatively harmless by conversion to dihydrodiols via epoxide hydrolases and/or conjugation with glutathione non-enzymatically and/or via the glutathione transferases. Thus, the cytochrome P-450 system, epoxide hydrolases and glutathione transferases play central roles in determining the toxicity and genotoxicity of xenobiotics and much attention has been directed towards these enzymes in the past two decades.

Of particular interest has been the regulation of these detoxication systems at the genetic level through the process called induction. Studies on enzyme induction can be useful in many different connections, including offering a tool for investigating the genetic regulation of the protein involved, giving hints as to the function(s) of this enzyme, identifying different isozymes and providing increased amounts of enzyme protein for facilitated isolation.

At present, much less is known about the induction of cytosolic epoxide hydrolase than about the induction of the cytochrome P-450 system, microsomal epoxide hydrolase, cytosolic glutathione transferases and UDP-glucuronyltransferases [2–5]. Cytosolic epoxide hydrolase is not induced by the well-known inducers of other xenobiotic-metabolizing systems—including phenobarbital, 3-methylcholanthrene, *trans*-stilbene oxide, 2-acetylaminofluorene, pregnenolone 16,17-oxide and butylated hydroxyanisole [6; J. Meijer and J. W. DePierre, submitted for publication]. The only known inducers of this enzyme, including several widely used hypolipidemic agents, are peroxisome proliferators, i.e. cause an increase in both the size and number of hepatic peroxisomes [6; B. Lundgren, J. Meijer and J. W. DePierre, manuscript in preparation].

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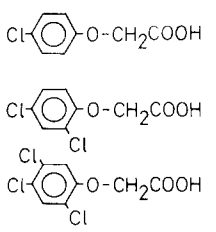

Fig. 1. Structures of the three phenoxyacetic acids which mice received in their diet.

It has recently been demonstrated that both 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid cause proliferation of peroxisomes in rat liver [7–9]. We thus became interested in testing the possibility that these compounds, as well as the structurally related compound *p*-chlorophenoxyacetic acid (see Fig. 1 for structures), induce hepatic cytosolic epoxide hydrolase. At the same time, we have monitored their effects on other xenobiotic-metabolizing enzymes, i.e. microsomal cytochrome P-450 content, microsomal epoxide hydrolase activity and cytosolic glutathione transferase activity. We have also measured the liver-somatic index and the total protein contents in the mitochondrial, microsomal and cytosolic fractions. We followed the effects on carnitine acetyltransferase and the peroxisomal enzymes cyanide-insensitive palmitoyl-CoA oxidation and catalase [10, 11], in order to confirm that peroxisome proliferation occurs in mouse liver and determine its extent. Finally, since certain peroxisome proliferators are also known to cause proliferation of mitochondria [12–14], we assayed cytochrome oxidase after dietary exposure of the mice to these three phenoxyacetic acids.

Both 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid are widely used herbicides and, therefore, their biological effects are of importance to biochemical toxicologists and others in related fields.

MATERIALS AND METHODS

Chemicals. 1-Chloro-2,4-dinitrobenzene and hydrogen peroxide (30%) (E. Merck, Darmstadt, F.R.G.), glutathione, 5,5′-dithiobis(2-nitrobenzoic acid), *S*-acetyl-CoA and L-carnitine, palmitoyl-CoA and antimycin A III (Sigma Chemical Co., St. Louis, MO), *trans*-stilbene oxide (EGA-Chemie, Albuch, F.R.G.), acrylamide and N,N′-methylbisacrylamide (BDH Chemicals Ltd., Poole, U.K.), ^{125}I-labeled protein A (8 μCi/μg = 0.30 MBq/μg) (New England Nuclear, Dreiech, F.R.G.) and *p*-chlorophenoxyacetic acid (Janssen Chimica, Beerse, Belgium) were all obtained from the sources indicated. 2,4-Dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid were gifts from Dan Johnels of the Department of Organic Chemistry, University of Umeå, Umeå, Sweden.

8-(^{3}H)-*trans*- and *cis*-Stilbene oxide (2 Ci/mmol) were synthesized by Dr. Åke Pilotti and Winnie Birberg of the Department of Organic Chemistry, University of Stockholm, according to Gill *et al.* [15] and purified by thin-layer chromatography using hexane:ethylacetate (95:5, v:v) with two developments. The bands containing pure *trans*- or *cis*-stilbene oxide were scraped off the plate and eluted twice with 2 ml ethanol followed by filtration through glass-wool. The final chemical purities were estimated to be 99.9% by NMR. Unlabelled *cis*-stilbene oxide was synthesized by *m*-chloroperoxybenzoic acid oxidation of *cis*-stilbene and purified in the same manner before use.

All other chemicals were of analytical grade and obtained from common commercial sources.

Animals and treatment. Male C57bl/6 mice (ALAB, Sollentuna, Sweden) weighing 20–30 g and about 8 weeks old were used throughout this study. The animals were housed in steel cages in groups of 3–4 with a 12-hr light/dark cycle at 25° and were given free access to commercial food pellets R3, containing 5% fat, 24% protein and 49% carbohydrates (Astra Ewos AB, Södertälje, Sweden) and tap water.

The three phenoxyacetic acids were administered for 4 days (the time period found to give maximal induction of cytosolic expoxide hydrolase [16]) in the diet, which was prepared by thoroughly mixing 0.125% (w/w) powdered substance (half the dose generally used in the literature in order to avoid eventual toxic effects) with powdered chow. Control food was prepared in the same manner, but without addition of any substance. The food was subsequently stored at −20° prior to use. The mice consumed approximately 2 g food per day, which resulted in a dose of about 100 mg/kg body weight. No obvious toxic signs were observed during the treatment period and the increases in body weight in the control and treated groups were not significantly different.

Preparation of subcellular fractions. At the end of the treatment period, the mice were killed by cervical dislocation. This was performed routinely around 7 or 8 o'clock in the morning. The liver was removed, freed from the gallbladder and thereafter placed in ice-cold 0.25 M sucrose and weighed. This tissue was subsequently homogenized in 2 vol. of ice-cold 0.25 M sucrose using 4 up-and-down strokes of a Potter–Elvehjem homogenizer at 440 r.p.m. All further procedures were performed at 4°.

After dilution to 1 g liver/5 ml suspension this material was centrifuged at 600 g_{av} for 10 min and the resulting supernatant then centrifuged at 10,000 g_{av} for 10 min. This 10,000 g_{av} pellet, the "mitochondrial" fraction, was resuspended, washed twice with 0.25 M sucrose by centrifugation, and finally resuspended in 0.25 M sucrose to give a total volume of 2 ml.

The 10,000 g_{av} supernatant was centrifuged at 105,000 g_{av} for 60 min and the resulting pellet, the microsomal fraction, washed once in 0.15 M Tris–

Cl, pH 8.0, by centrifugation to remove adsorbed soluble proteins [17] and finally resuspended in 0.25 M sucrose to give a total volume of 2 ml.

The 105,000 g_{av} supernatant was recentrifuged at 133,000 g_{av} for 60 min and the resulting supernatant (about 3 ml), the cytosol, removed and saved.

Aliquots were taken immediately from each fraction, flushed with nitrogen and stored at −20° for future assays.

The fractions thus obtained have been carefully analyzed using a wide range of marker enzymes and shown to correspond relatively closely to the nuclear, mitochondrial, microsomal and supernatant fractions prepared from rat liver in an analogous manner [J. Meijer and J. W. DePierre, *Biochemical Pharmacology*, in press].

Assays. All enzyme assays were performed with saturating substrate concentrations and conditions of linearity with time and protein as determined with preparations from control animals.

Cytosolic"-mitochondrial" and microsomal epoxide hydrolase activities were measured towards *trans*- and *cis*-stilbene oxide, respectively, as substrates essentially as described by Gill *et al.* [15]. One hundred microliters of buffer (0.1 M potassium phosphate, pH 7.0, in the case of the cytosolic and "mitochondrial" enzymes and 0.1 M glycine, pH 9.0, for the microsomal enzyme) and protein (2.5 μg cytosolic protein, 2.5 μg "mitochondrial" protein or 10 μg microsomal protein) were preincubated for 1 min at 37°. The partitioning of the diols formed between the organic and aqueous phases is 8% and 92%, respectively. Correction for this 8% loss of product has been made in the values reported here. The presence of glutathione conjugates was checked for by extraction with l-hexanol and no such conjugates were observed in this investigation.

Cytochrome P-450 was quantitated according to Omura and Sato [18] and *glutathione transferase* activity was monitored spectrophotometrically at 340 nm with 1-chloro-2,4-dinitrobenzene as the second substrate [19]. *Carnitine acetyltransferase* was measured spectrophotometrically at 412 nm with L-carnitine and *S*-acetyl-CoA as substrates [20]. *Palmitoyl-CoA oxidation* was monitored spectrophotometrically at 340 nm as the reduction of NAD^+ essentially according to Lazarow *et al.* [21] and Gray *et al.* [22], but with 5 ng/ml antimycin A III instead of KCN as inhibitor of mitochondrial β-oxidation [23]. *Catalase* [24], *cytochrome oxidase* [25] and *protein* [26] were assayed using published procedures. *SDS-polyacrylamide gel electrophoresis* was performed according to the method of Laemmli [27].

Cytosolic epoxide hydrolase was purified from mouse liver and antibodies prepared in rabbits as described previously [28].

Immunoblotting was performed according to Towbin *et al.* [29]. Briefly, after the electrophoretic run (14 × 14 cm slab, 1.5 mm thick) the gel was equilibrated for 15 min in blotting buffer (25 mM Tris–192 mM glycine–20% methanol–0.02% sodium dodecyl sulphate) and placed on a prewetted filter paper. A nitrocellulose paper (Millipore GSWP 304FO 0.22 μm) prewetted in the same buffer was placed on the gel, care being taken to remove all trapped air bubbles. A second filter paper also prewetted in the same buffer was placed on the other side of the nitrocellulose paper and this "sandwich" was then placed between two prewetted Scotch-Brite scouring pads held together with plastic holders.

The transfer was performed for 2–3 hr at 250 mA and 4°. The nitrocellulose paper was then blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS). The blot was washed once with PBS and then incubated overnight in the appropriate antibody solution (0.1 mg/ml in PBS and 3% bovine serum albumin). After washing 3 × 15 min, the blot was placed in ^{125}I-labeled protein A (200,000 cpm) in 2% bovine serum albumin–PBS for 1 hr. After three 30-min washes with PBS–0.1% Lubrol PX, the blot was dried. Following autoradiography the bands were cut out and radioactivity determined in a gamma counter. Quantitation was performed by running a standard curve of cytosolic epoxide hydrolase purified from untreated mouse liver in parallel with samples on the same gel.

Statistical analysis. In all groups three animals were used. Data are given as means ± SD and the results of the Student's *t*-test are given where appropriate.

RESULTS

Effects on liver-somatic index and the protein contents of subcellular fractions

As can be seen from Table 1, dietary exposure to both 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid had a significant effect on the liver-somatic index of mice. There were no

Table 1. Effects of dietary treatment with *p*-chlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on liver-somatic index and the protein contents of subcellular fractions

Treatment	Liver weight (g)	Liver-somatic index	"Mitochondrial" protein	Microsomal protein	Cytosolic protein
None (control)	1.24 ± 0.12	0.049 ± 0.006	3.34 ± 1.01	6.89 ± 1.45	30.2 ± 4.7
CPA	1.08 ± 0.14	0.039 ± 0.008	4.24 ± 0.52	8.34 ± 2.48	33.8 ± 2.9
2,4-D	1.25 ± 0.07	0.057 ± 0.001* (116)	8.10 ± 3.72 (243)	9.80 ± 0.50* (142)	37.8 ± 1.0
2,4,5-T	1.33 ± 0.10	0.070 ± 0.006* (143)	15.1 ± 1.5*** (452)	8.59 ± 0.3	32.6 ± 1.0

The values given are means ± SD for 3 animals in each group and the figures in parentheses are percentages of the control value.

Abbreviations used in Table 1: CPA = *p*-chlorophenoxyacetic acid, 2,4-D = 2,4-dichlorophenoxyacetic acid, and 2,4,5-T = 2,4,5-trichlorophenoxyacetic acid. The units for protein are mg/g liver. Significantly different from control group: *$P < 0.05$; ***$P < 0.001$.

effects on the body weights of these animals (and, indeed, no other obvious signs of toxicity under the conditions of exposure employed here), so that these changes reflected hepatic hypertrophy. It is not at present known whether this enlargement involved an increase in the number and/or size of hepatocytes.

2,4-Dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid both also increased the total protein content of the "mitochondrial" fraction—2.4- and 4.5-fold, respectively. This observation will be discussed further below. Furthermore, exposure to these substances caused 40% and 25% increases, respectively, in the total microsomal protein.

None of the three phenoxyacetic acids tested affected cytosolic protein and *p*-chlorophenoxyacetic acid did not significantly alter any of the parameters shown in Table 1.

Effects on carnitine acetyltransferase, cyanide-insensitive palmitoyl-CoA oxidation and catalase

In order to confirm that 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid also cause proliferation of peroxisomes in mouse liver, the activities of carnitine acetyltransferase, palmitoyl-CoA oxidation and catalase were measured in the "mitochondrial" fraction. As can be seen from Table 2, dietary exposure to both of these substances caused significant increases (3–12-fold) in the specific activities of the first two of these enzymes and even more dramatic changes in the total activities (which can be obtained by multiplying the specific activities in Table 2 by the appropriate protein contents in Table 1). The specific activity of catalase was not significantly increased by exposure to 2,4-dichlorophenoxyacetic acid or 2,4,5-trichlorophenoxyacetic acid, but the total activity in the mitochondrial fraction was increased about 2-fold in both cases.

p-Chlorophenoxyacetic acid did not affect any of these parameters significantly.

Of course, there are uncertainties involved in measuring specific enzyme activities in a subcellular fraction containing not only mitochondria and peroxisomes, but also a large portion of the cellular lysosomes and even some fragments of the endoplasmic reticulum. Consequently, the total activities may be more reliable indications of the changes which are occurring.

Effects on cytochrome oxidase

Certain peroxisome proliferators also cause a proliferation of mitochondria, as indicated, among other things, by an increase in cytochrome oxidase activity [12–14]. As shown in Table 2, neither 2,4-dichlorophenoxyacetic acid nor 2,4,5-trichlorophenoxyacetic acid increased the specific activity of cytochrome oxidase in the "mitochondrial" pellet to any great extent. However, the total activity of this enzyme (which may be a better measure; see above) was increased about 3.6- and 3.7-fold, respectively.

p-Chlorophenoxyacetic acid, which did not affect the total protein content of the "mitochondrial" frac-

Table 2. Effects of dietary treatment with *p*-chlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on various hepatic enzymes

Enzyme	Treatment			
	None	CPA	2,4-D	2,4,5-T
Carnitine acetyltransferase[a]	7.59 ± 3.73	4.79 ± 1.61 (63)	70.8 ± 4.4*** (931)	94.7 ± 35.0* (1246)
Palmitoyl-CoA[b] oxidation	10.4 ± 3.3	14.1 ± 4.1 (133)	49.4 ± 9.5** (485)	37.5 ± 14* (368)
Catalase[c]	630 ± 156	805 ± 57 (127)	576 ± 201 (91)	332 ± 99* (53)
Cytochrome oxidase[d]	187 ± 50	396 ± 47** (215)	270 ± 3* (145)	157 ± 23 (83)
Cytosolic epoxide[e] hydrolase	6.3 ± 1.6	6.9 ± 1.2 (110)	10.8 ± 1.2* (172)	11.4 ± 1.3** (181)
"Mitochondrial" epoxide hydrolase[e]	5.6 ± 0.9	5.8 ± 1.8 (103)	6.1 ± 1.4 (108)	6.0 ± 1.5 (100)
Microsomal epoxide[f] hydrolase	2.74 ± 0.93	3.64 ± 0.57 (133)	6.15 ± 0.72*** (225)	10.2 ± 0.5*** (372)
Microsomal cytochrome P-450 content[g]	0.913 ± 0.114	0.911 ± 0.364 (100)	1.21 ± 0.13* (133)	1.47 ± 0.18** (161)
Glutathione transferase[h]	3.92 ± 0.45	3.20 ± 0.71 (82)	4.88 ± 0.70 (124)	7.84 ± 0.97*** (200)

The values given are means ± SD for 3 independent determinations and the figures in parentheses are percentages of the control value. CPA = *p*-chlorophenoxyacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; 2,4,5-T = 2,4,5-trichlorophenoxyacetic acid. The units for protein are mg/g liver. Significantly different from control: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[a]nmol CoA released/min-mg "mitochondrial" protein.
[b]nmol NAD+ reduced/min-mg "mitochondrial" protein.
[c]nmol hydrogen peroxide reduced/min-mg "mitochondrial" protein.
[d]nmol cytochrome *c* oxidized/min-mg "mitochondrial" protein.
[e]nmol *trans*-stilbene oxide metabolized/min-mg cytosolic or "mitochondrial" protein.
[f]nmol *cis*-stilbene oxide metabolized/min-mg microsomalt protein.
[g]nmol/mg microsomal protein.
[h]μmol 1-chloro-2,4-dinitrobenzene conjugated/min-mg cytosolic protein.

tion significantly, did cause a 2-fold increase in the cytochrome oxidase activity of this fraction (see also the Discussion). This finding would seem to indicate that there need not be a direct correlation between mitochondrial proliferation and increases in individual enzyme activities.

Nonetheless, the most probable explanation for the increase in "mitochondrial" protein documented in Table 1 is that dietary exposure to 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid causes proliferation of both peroxisomes and mitochondria in mouse liver.

Effects on cytosolic epoxide hydrolase and other enzymes of xenobiotic metabolism

With regard to enzymes involved in xenobiotic metabolism the largest effects of dietary 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid were on cytosolic (172% and 181% of the control values, respectively) and microsomal (225% and 372%, respectively) expoxide hydrolases (Table 2). On the other hand, "mitochondrial" epoxide hydrolase activity towards *trans*-stilbene oxide was unaffected by these treatments. Microsomal content of cytochrome P-450 was also increased significantly, albeit to a more limited extent. Cytosolic glutathione transferase activity measured with 1-chloro-2,4-dinitrobenzene (which is generally the best second substrate for all isozymes of this enzyme in rat liver [4]) was doubled by treatment with 2,4,5-trichlorophenoxyacetic acid.

Dietary exposure to *p*-chlorophenoxyacetic acid did not significantly alter the levels of any of the xenobiotic-metabolizing enzymes assayed in the present study.

Demonstration that the effects of dietary 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on cytosolic epoxide hydrolase are true inductions

It is possible that the increases in cytosolic epoxide hydrolase activity caused by 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid did not reflect increases in enzyme protein, in which case the processes involved were by definition not true inductions. In order to decide this question we quantitated the effect of these treatments on enzyme protein immunochemically (Fig. 2).

As seen in Table 3, the amount of enzyme protein was found to be increased as least as much as the cytosolic epoxide hydrolase activity. Indeed, the enzyme protein seemed to increase somewhat more than activity, which would mean that the induced form of cytosolic epoxide hydrolase may have a lower specific activity than the enzyme in untreated animals and may thus be another isozyme or post-translationally modified. We are presently refining our methodology in attempts to explore this possibility definitively.

DISCUSSION

In agreement with the literature on rat liver [8, 9] 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid were found to cause extensive proliferation of peroxisomes (as judged by the total levels of "mitochondrial" protein, carnitine acetyltransferase, cyanide-insensitive palmitoyl-CoA oxidation and catalase) in mouse liver. On the other hand, exposure to *p*-chlorophenoxyacetic acid did not result in such changes.

As with certain other peroxisome proliferators [12–14], 2,4-dichlorophenoxyacetic acid and 2,4,5-trichloroacetic acid were also seen to increase total cytochrome oxidase activity. However, *p*-chlorophenoxyacetic acid increases the specific activity of cytochrome oxidase without affecting "mitochondrial" protein. This latter observation suggests that cytochrome oxidase activity can be increased by other factors than increased enzyme protein and that such activity changes must thus be interpreted with care.

Dietary exposure to 2,4-dichlorophenoxyacetic acid and to 2,4,5-trichlorophenoxyacetic acid causes rather dramatic increases in the activities of cytosolic and microsomal epoxide hydrolases in mouse liver and generally less pronounced increases in the cytosolic glutathione transferase activity and microsomal content of cytochrome P-450. In the case of cytochrome P-450, this process can be said to be a true induction (i.e. the amount of enzyme protein is increased), because the assay procedure for cytochrome P-450 measures holoenzyme amount. Immunoquantitation demonstrated that this is also the case for the changes in cytosolic epoxide hydrolases. Unfortunately, antibodies towards mouse liver microsomal epoxide hydrolase are not presently available to us.

Table 3. Immunoquantitation of hepatic cytosolic epoxide hydrolase after dietary exposure of mice to *p*-chlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid or 2,4,5-trichlorophenoxyacetic acid

Treatment	μg cytosolic epoxide hydrolase / mg cytosolic protein	% of control
None (control)	7.0 ± 2.0	100 ± 28
CPA	10.7 ± 3.3	140 ± 42
2,4-D	23.7 ± 4.4***	311 ± 57
2,4,5-T	22.3 ± 0.8***	293 ± 10

The values given are means ± SD for 3 animals in each group. Abbreviations used CPA = *p*-chlorophenoxyacetic acid, 2,4-D = 2,4-dichlorophenoxyacetic acid, and 2,4,5-T = 2,4,5-trichlorophenoxyacetic acid. Significantly different from control ***$P < 0.001$.

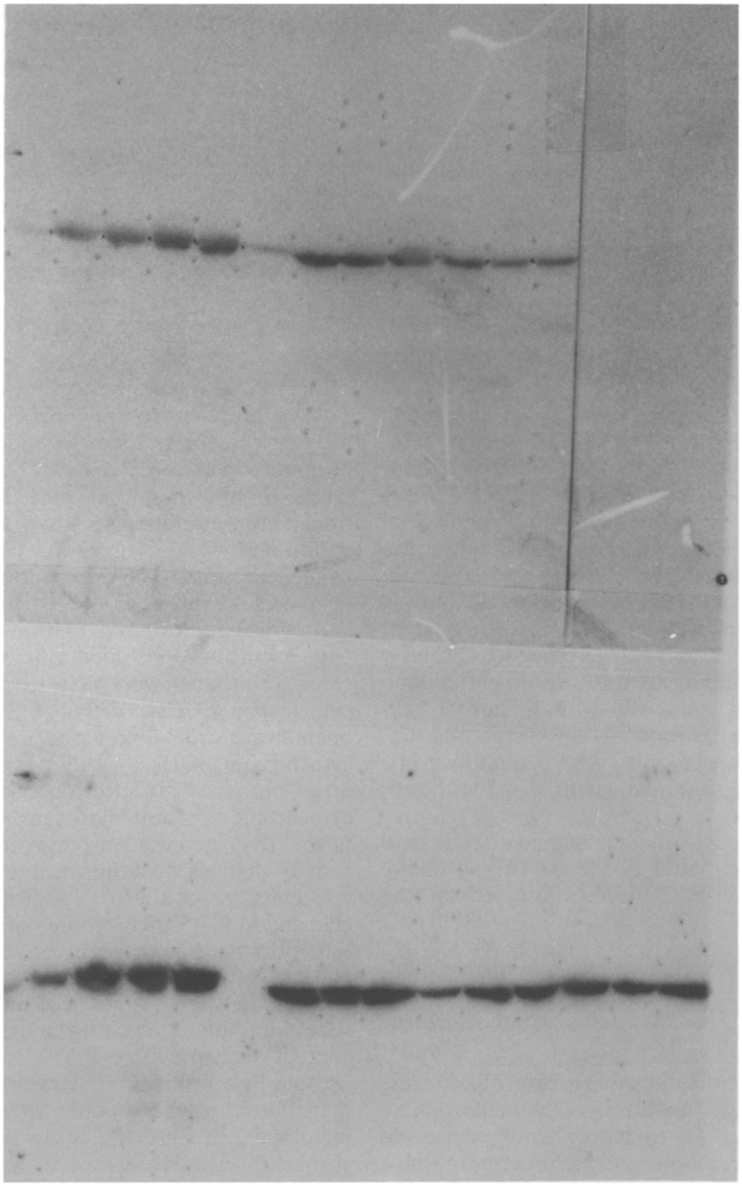

Fig. 2. Immunochemical determination of cytosolic epoxide hydrolase in liver after dietary treatment of mice with phenoxyacetic acids. The immunoblotting was performed as described in Materials and Methods. In both panels the first 5 lanes from the left are the standard curve (0.1, 0.2, 0.3, 0.5 and 1.0 μg) obtained with the purified mouse liver enzyme. The last 6 lanes in the upper panel are, from left to right, 3 aliquots of the cytosolic fraction from untreated mice each containing 50 μg protein, followed by 3 samples of 20 μg cytosolic protein from mice exposed to *p*-chlorophenoxyacetic acid. The last 9 lanes in the lower panel are, from left to right, 3 samples of 50 μg cytosolic protein from control animals, 3 × 20 μg protein after exposure to 2,4-dichlorophenoxyacetic acid, and 3 × 20 μg protein after treatment with 2,4,5-trichlorophenoxyacetic acid.

At present, which isozyme(s) of cytochrome P-450 is induced by 2,4-dichlorophenoxyacetic acid and 2,4,5-trichloroacetic acid is not known. Clofibrate, a hypolipidemic drug and peroxisome proliferator, has been found to induce an isozyme of cytochrome P-450 which is apparently specialized for hydroxylation of fatty acids [30–32], which may be a clue.

The dramatic differences in proliferation of peroxisomes and induction of xenobiotic-metabolizing enzymes seen here with compounds differing only slightly in structure may indicate that a receptor mechanism of some kind is involved. Such small changes in structure would be expected to cause only minor effects on the uptake of these compounds from the gastrointestinal tract, another important factor in these considerations. Of course, it may well be that it is not 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid themselves, but rather a metabolite(s) of these xenobiotics which are directly responsible for the induction of xenobiotic-metabolizing enzymes and/or the proliferation of peroxisomes and/or mitochondria.

To date, all known inducers of cytosolic epoxide hydrolase also cause peroxisome proliferation and it is tempting to speculate that these processes are related. Reddy and coworkers [33, 34] have proposed that proliferation of peroxisomes causes increased lipid peroxidation due to an increased level of active oxygen in the form of hydrogen peroxide in the cell. The source of this hydrogen peroxide would be the increased peroxisomal β-oxidation of fatty acids.

It is likely that a small molecule such as hydrogen peroxide leaks out of the peroxisome, especially since catalase activity is not increased nearly as much as peroxisomal β-oxidation. This free hydrogen peroxide could cause the observed carcinogenic effects of hypolipidemic substances, as well causing lipid peroxidation and the formation, among other things, of fatty acid epoxides. These epoxides can serve as substrates for epoxide hydrolase [35, 36] and may act as the immediate inducer(s).

Finally, the toxic, genotoxic and synergistic implications of the biological effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid described here are not at present known.

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