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Effects of Phenoxyherbicides and Glyphosate on the Hepatic and Intestinal Biotransformation Activities in the Rat

By

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Abstract: The effects of phenoxyacid herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (4-chloro-2-methylphenoxyacetic acid), clofibrate, and glyphosate on hepatic and intestinal drug metabolizing enzyme activities were studied in rats intragastrically exposed for 2 weeks. The hepatic ethoxycoumarin O-deethylase activity increased about 2-fold with MCPA. Both 2,4-D and MCPA increased the hepatic epoxide hydrolase activity and decreased the hepatic glutathione S-transferase activity. MCPA also increased the intestinal activities of ethoxycoumarin O-deethylase and epoxide hydrolase. Glyphosate decreased the hepatic level of cytochrome P-450 and monooxygenase activities and the intestinal activity of aryl hydrocarbon hydroxylase. Clofibrate decreased the hepatic activities of UDPglucuronosyltransferase with p-nitrophenol or methylumbelliferone as the substrate. Also 2,4-D decreased the hepatic activity of UDPglucuronosyltransferase with p-nitrophenol as the substrate. MCPA decreased the intestinal activities of UDPglucuronosyltransferase with either p-nitrophenol or methylumbelliferone as the substrate. The results indicate that phenoxyacetic acids, especially MCPA, may have potent effects on the metabolism of xenobiotics. Glyphosate, not chemically related to phenoxyacids, seems to inhibit monooxygenases. Whether these changes are related to the toxicity of these xenobiotics remains to be clarified in further experiments.

Key words: Clofibrate – cytochrome P-450 – glutathione-S-transferase – glyphosate – herbicides – intestine – liver – monooxygenases – phenoxyacetic acids – UDPglucuronosyltransferase – rat.

Phenoxyacid herbicides and glyphosate are chemicals widely used in agriculture and forestry to control the growth of weeds and brush. Both types of chemicals are eliminated rather rapidly from the body, usually in urine (Fjeldstad & Wannag 1977; Ramel 1978; Sauerhoff *et al.* 1976). Although phenoxyacids are usually excreted mainly unmetabolized, part of them may be conjugated with glucuronic acid or amino acids (glycine or taurine).

In epidemiological studies some investigators have found evidence suggesting that phenoxyacetic acids are related to an increased incidence of soft tissue sarcomas (Hardell & Sandström 1979; Moses & Selikoff 1981). The results from some studies on experimental animals indicate that 2,4-D (2,4-dichlorophenoxyacetic acid) may be carcinogenic but these studies have had many limitations (Innes *et al.* 1969; Hansen *et al.* 1971). The carcinogenicity of MCPA (4-chloro-2-methylphenoxyacetic acid) has not yet been evaluated. Some studies have suspected that a third phenoxyacetic acid, 2,4,5-T (2,4,5-trichlorophenoxy acetic acid) is weakly carcinogenic, the carcinogenicity possibly due to the presence of tetrachlorodioxin, which is formed in the manufacturing process. However, pure 2,4,5-T has not been shown to be carcinogenic (Kociba *et al.* 1978; Muranyi-Kovács *et al.* 1976). As 2,4,5-T has been taken off the market in several countries,

we did not include it in the present study. No data on the carcinogenicity of glyphosate has been presented thus far. Neither 2,4-D, MCPA, nor glyphosate has been found to be mutagenic to significant degree (see e.g. Seiler 1978 for references).

There are also reports on the possible carcinogenicity of clofibrate (ethyl-2-(p-chlorophenoxy)-2-methyl-propionate), a compound which is structurally related to phenoxyacetic acids and which is used as a hypolipidaemic drug (Reddy *et al.* 1980). There is also evidence that clofibrate acts on blood lipids by increasing the peroxisome concentration in the liver (Anthony *et al.* 1978; Hanefeld *et al.* 1980; Vainio *et al.* 1982) and by inducing peroxisomal β-oxidation (Lazarow *et al.* 1982). Furthermore, clofibrate increases hepatic concentrations of cytochrome P-450 (Anthony *et al.* 1978), and it increases both the NADPH cytochrome c reductase activity and some of the monooxygenase activities (Mellon *et al.* 1976). Also, phenoxyacid herbicides induce hepatic peroxisomal proliferation (Vainio *et al.* 1982).

Even if only a small fraction of these herbicides is metabolized *in vivo,* the herbicides may still modulate the metabolic activation/inactivation capabilities of various organs. By altering the activities of biotransformation enzymes phenoxyacetic acid herbicides may alter the response of the organism to other concomitant compounds. In the present study we examined the effects of two phenoxy acid herbicides (2,4-D and MCPA) and the effects of clofibrate on the drug metabolizing enzyme activities in the liver and the intestine. The effects of glyphosate, a herbicide not structurally related to phenoxy acids, was studied for comparison.

Materials and Methods

Male Wistar rats (358–495 g) were given herbicides, clofibrate or sodium chloride by a gastric gavage 5 days a week for 2 weeks. Of the phenoxyacetic acids, 2,4-dichlorophenoxyacetic acid (2,4-D) was administered as the amine salt at doses of 100 mg/kg and 150–200 mg/kg. Also 4-chloro-2-methylphenoxyacetic acid (MCPA) was given at the same doses as 2,4-D, in the isooctyl ester form. The higher dose of 200 mg/kg was administered for 4 days and was thereafter lowered to 150 mg/kg for the remainder of the administration period. The dose was reduced because rats given 200 mg/kg had become drowsy, and a few died. Glyphosate (N-phosphonomethylglycine) was given as the isopropylamine salt, originally at doses of 500 mg/kg for 4 days and thereafter at 300 mg/kg for the rest of the administration period. The controls received 0.9% saline (4 ml/kg), and the positive controls were given clofibrate (Klofiran®, Remeda Ltd, Kuopio, Finland) at doses of 200 mg/kg throughout the administration period. The glyphosate preparation contained 360 g/l glyphosate (Roundup®, Monsanto), the 2,4-D solution contained 550 g/l 2,4-D, and the MCPA solution contained 500 g/l MCPA (both from Kemira Oy, Finland).

The rats were killed 24 hrs after the last dose; the liver and a segment of the proximal small intestine 20 cm long were removed and placed in ice-cold 0.25 M sucrose. The liver was homogenized in 4-fold wet weight volume of 0.25 M sucrose with a Potter-Elvehjem type glass-Teflon homogenizer and centrifuged in a refrigerated centrifuge at 10,000 g×10 min. to prepare the postmitochondrial supernatant. Both the supernatant and the intestinal segment were stored at −70° for 2 weeks, until further analysis. On the day of analysis the hepatic supernatant was further centrifuged at 105,000 g×60 min. in a refrigerated (4°) ultracentrifuge. This centrifugation isolated the microsomes, which were then resuspended in 0.25 M sucrose in a volume of original sample weight.

This suspension was used for the enzyme and compositional assays, with the exception of glutathione S-transferase, which was assayed in the soluble fraction. The intestinal mucosa was thawed, cleaned and scraped off with an ampoule file. It was weighed, homogenized in 4-fold volume of 0.25 M sucrose, and centrifuged at 10,000 g×10 min. to prepare the postmitochondrial supernatant for analysis.

The cytochrome P-450 concentration of the microsomes was measured by spectrophotometry. A Perkin Elmer double beam spectrophotometer was used to record the difference spectra of Na-dithionate reduced cytochrome P-450 from 400 to 500 nm (Omura & Sato 1964). The activity of NADPH cytochrome c reductase was measured by monitoring the reduction of cytochrome c at 550 nm as described by Phillips & Langdon (1962). The activity of aryl hydrocarbon hydroxylase was determined radiometrically, with 3,4-benzpyrene as the substrates (De Pierre *et al.* 1975). The activity of ethoxycoumarin O-deethylase was measured in an incubation mixture containing 0.2 ml NADPH-generating system and 0.25 ml 0.1 M Tris-HCl buffer, pH 7.6 The 0.1 mM incubation mixture was made with 7-ethoxycoumarin in Tris-HCl buffer, and the reaction was initiated by adding 0.05 ml of 20-fold microsomal dilution or 0.05 ml of intestinal postmitochondrial supernatant. After 10 min. (liver) or 20 min. (intestine) incubation at 37°, 0.05 ml of 5% trichloroacetic acid was added (Aitio 1978; Ullrich & Weber 1972). Fluorescence was determined with an Aminco-Bowman fluorometer set at an excitation wavelength of 390 nm and an emission wavelength of 440 nm, with 7-hydroxycoumarin as the standard. The PPO hydroxylase was measured with diphenyloxazole as the substrate (Ahokas 1976; Cantrell *et al.* 1975). The fluor-

Table 1.

The composition of hepatic microsomes and supernatant fractions and the intestinal postmitochondrial supernatant of rats given various herbicides or clofibrate. The means±S.E.M. are shown. *: $P<0.05$.

	Protein (mg/g w.wt)					
	Liver microsomes				Cholesterol	Phospholipids
	Native	Digitonin	Supernatant	Intestinal PMS	(μmol/g)	(μmol Pi/g)
Controls	26.1±0.4	8.8±0.4	87± 2	80.2±3.3	0.76±0.07	7.6±0.7
Clofibrate	24.4±0.8	9.1±0.6	81± 4	80.4±4.6	0.58±0.08	7.7±0.6
Glyphosate	24.4±1.2	10.3±1.3	93±15	75.5±9.2	0.55±0.10	7.0±0.2
2,4-D						
100 mg/kg	24.3±1.8	8.9±1.3	96± 7	84.5±4.5	0.51±0.10*	6.3±0.8
150–200 mg/kg	31.0±4.5	10.9±1.3	90± 5	78.0±7.3	0.88±0.08	7.2 (2)
MCPA						
100 mg/kg	25.6±3.1	8.6±0.8	86± 5	86.6±7.5	0.77±0.10	7.8±0.4
150–200 mg/kg	25.7±0.7	8.4±0.7	97± 6	60.4±6.4*	0.77±0.50	7.7±1.0

escing metabolites of PPO were measured from an alkaline extraction of the metabolites, using an excitation wavelength of 345 nm and an emission wavelength of 510 nm.

The activity of epoxide hydrolase was measured with styrene oxide as the substrate (Koivusaari *et al.* 1980; Oesch *et al.* 1971). The incubation mixture was composed of ^{3}H-styrene oxide, unlabelled, substrate and 50 μl of hepatic microsomes or 150 μl of intestinal postmitochondrial supernatant. The reaction was stopped by placing the test tubes in an ice-bath after 5 min. (liver) or 15 min. (intestine) incubation, whereafter 5 ml of petroleum ether was added and the mixture was shaken. After the phases separated the lower water phase was frozen. The petroleum ether phase, which contained unmetabolized styrene oxide, was discarded, and the procedure was repeated twice more. The water phase containing formed styrene glycol was rinsed twice with LumaGel® (Lumac AG, Basel, Switzerland) scintillation cocktail; the test tube was then shaken and the solution was poured into a scintillation vial for counting. The activity of UDP-glucuronosyltransferase was determined with 0.35 mM p-nitrophenol as the substrate and a 4,5 mM concentration of UDP glucuronic acid in 0.5 M K-phosphate buffer, pH

Table 2.

The NADPH cytochrome c reductase activity of liver microsomes in rats given various herbicides or clofibrate. For other explanations see Table 1.

	μmol cytochrome c reduced×min.$^{-1}$ ×g (w.wt)$^{-1}$
Controls	1.57±0.39
Clofibrate	1.41±0.09
Glyphosate	0.79±0.11*
2,4-D	
100 mg/kg	1.34±0.18
150–200 mg/kg	1.58±0.38
MCPA	
100 mg/kg	1.90±0.21
150–200 mg/kg	2.03±0.26

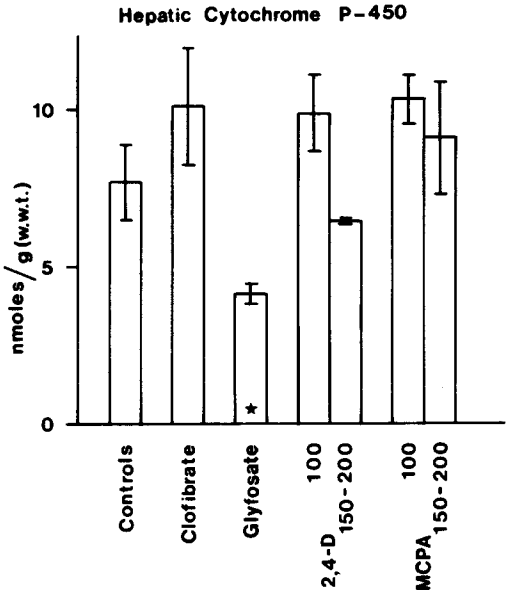


Fig. 1. The concentration of hepatic cytochrome P-450 in rats exposed to clofibrate, glyphosate, 2,4-dichlorophenoxyacetic acid (2,4-D) or 4-chloro-2-methylphenoxyacetic acid (MCPA) for 2 weeks. The doses of 2,4-D and MCPA are also shown. The means±S.E.M. are shown. The statistical significances were analyzed with Student's t-test using controls as a reference group. The following symbol is used: *: $P<0.05$.

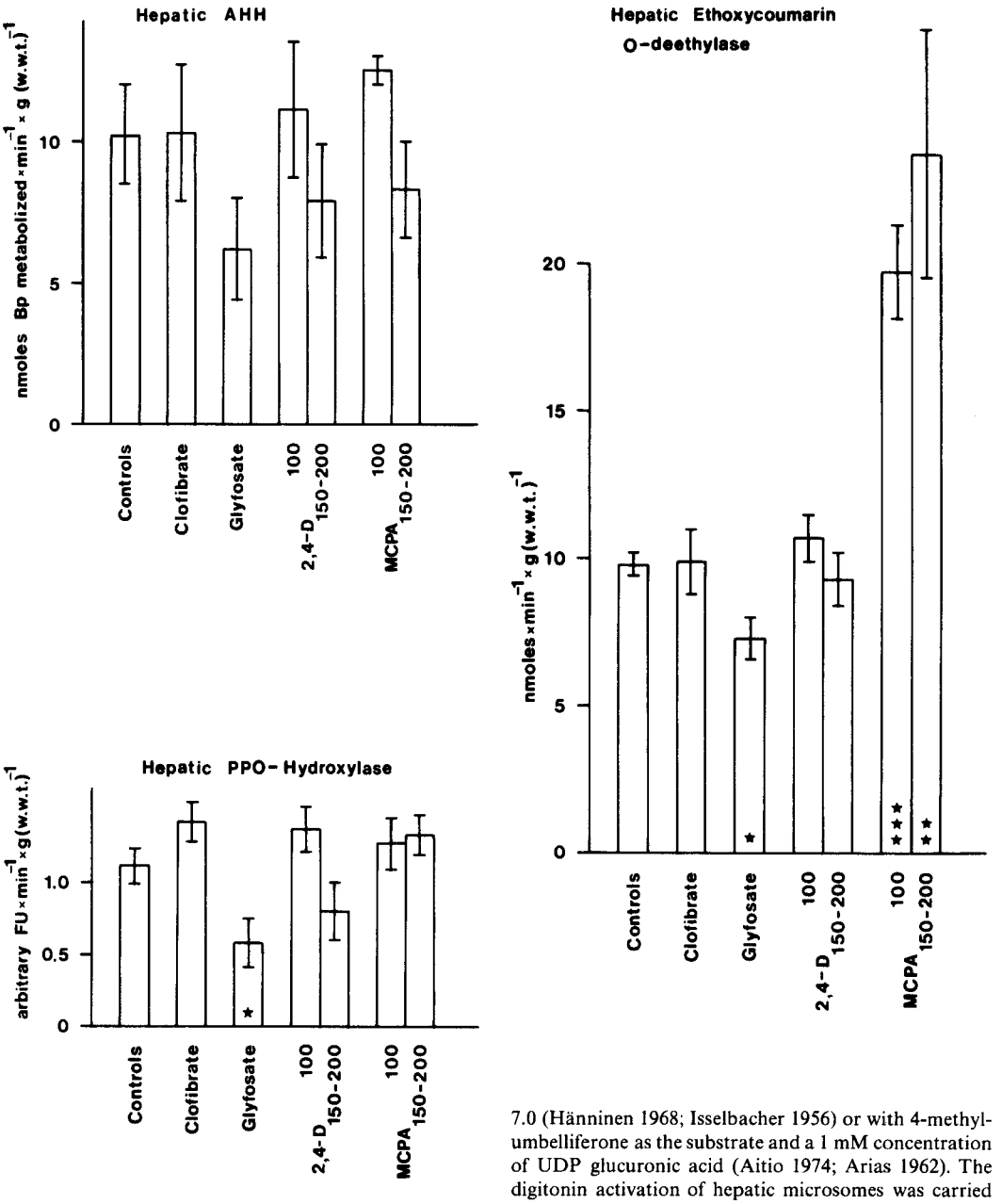


Fig. 2. The hepatic activities of aryl hydrocarbon hydroxylase (A), PPO-hydroxylase (B) and ethoxycoumarin O-deethylase (C) in rats exposed to herbicides or clofibrate for 2 weeks. For further explanations see fig. 1. The following symbols are used: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

7.0 (Hänninen 1968; Isselbacher 1956) or with 4-methylumbelliferone as the substrate and a 1 mM concentration of UDP glucuronic acid (Aitio 1974; Arias 1962). The digitonin activation of hepatic microsomes was carried out as described by Hänninen (1968) in order to measure the latency of the UDPglucuronosyltransferase enzyme. The glutathione S-transferase in the soluble hepatic subcellular fraction or in the postmitochondrial fraction of the intestine was also measured with styrene oxide as the substrate (James *et al.* 1976). Protein was determined by the biuret method, with bovine serum albumin as the reference protein (Layne 1957). Student's t-test was used to evaluate the statistical significance of the results.

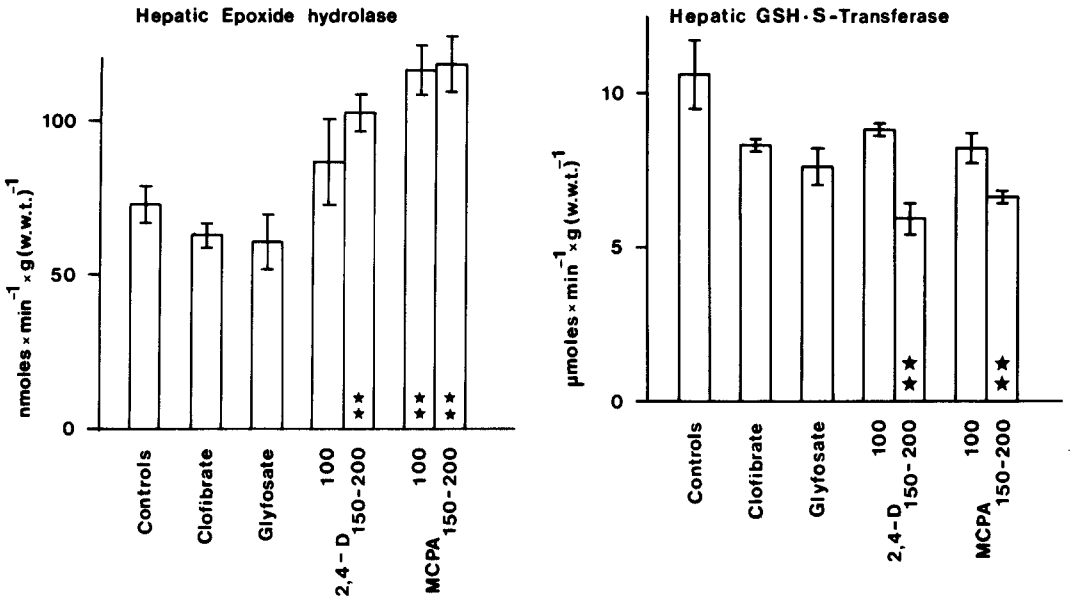


Fig. 3. The hepatic activities of epoxide hydrolase (A) and glutathione S-transferase (B), with styrene oxide as the substrate, in rats exposed to clofibrate or herbicides. For further explanations see figs. 1 and 2.

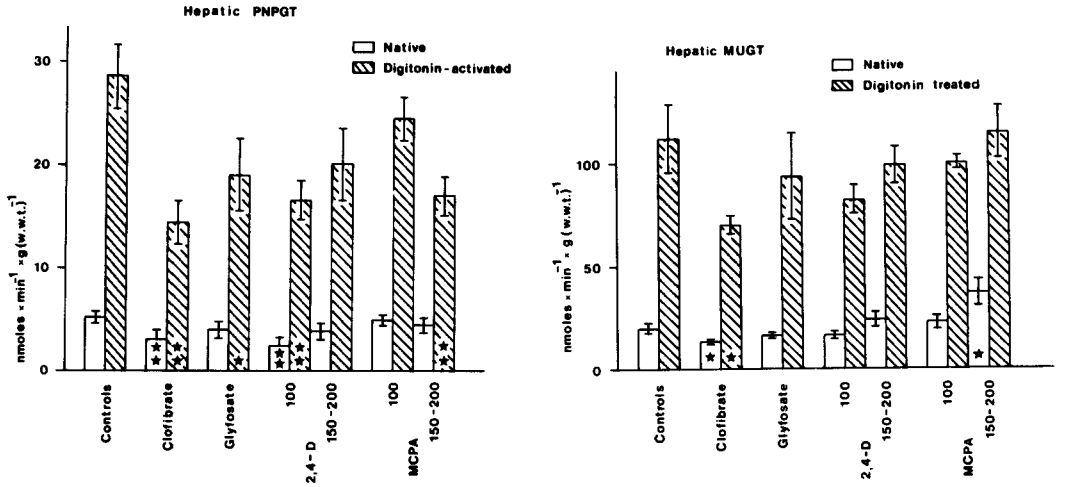


Fig. 4. The hepatic activity of UDPglucuronosyltransferase, with p-nitrophenol (A) or methylumbelliferone (B) as the substrate, in native (open columns) and in digitonin-activated (shaded columns) microsomes in rats exposed to clofibrate or herbicides. For further explanations see figs. 1 and 2.

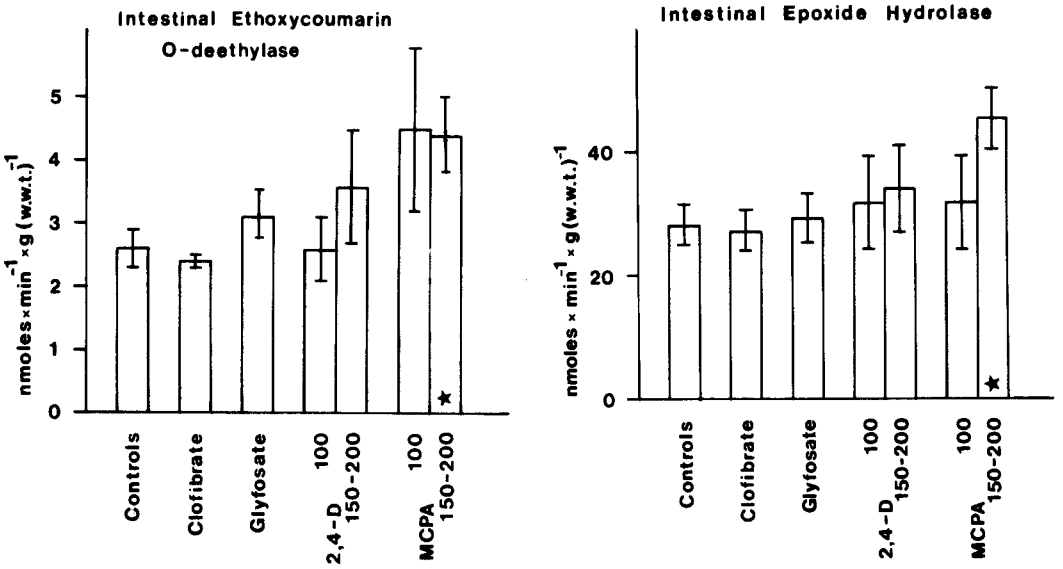
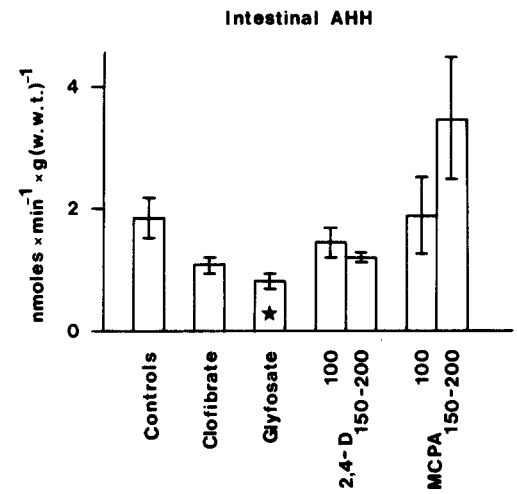


Fig. 5. The intestinal activities of ethoxycoumarin O-deethylase (A), aryl hydrocarbon hydroxylase (B), and epoxide hydrolase (C) in rates exposed to clofibrate or herbicides. For further explanations see figs. 1 and 2.



Results

The levels of microsomal protein, cholesterol and phospholipid in the treated animals are presented in table 1. Both in native microsomes and in the digitonin treated microsomes, the protein content of the liver microsomes was unaltered after the administration of herbicides or clofibrate. Nor were there any changes in the soluble protein contents. The microsomal cholesterol content was significantly decreased in the group treated with 100 mg/kg of 2,4-D. Some decrease was also found in the groups treated with clofibrate or glyphosate, but the decrease was not statistically significant. No changes in the microsomal phospholipid contents could be found. The content of protein in the intestinal mucosa decreased for those rats given high doses of MCPA (table 1).

The concentration of microsomal cytochrome P-450 in the liver did not change significantly with either of the phenoxy acid doses, and clofibrate caused only a minor increase (fig. 1). However, the rats that had been given glyphosate had a lower concentration of cytochrome P-450 in the liver than the controls (fig. 1). No statistically significant changes in the hepatic cytochrome c reductase activity were found in the rats given any of the phenoxy acids, but the activity of those microsomes in the rats treated with glyphosate was lower than in the controls (table 2). The activity of aryl hydrocarbon hydroxylase had not changed in the livers of rats given any of the compounds studied, although the rats treated with glyphosate did have a

trend of lowered activity (fig. 2A). Also the activity of PPO hydroxylase in the liver was lower in rats treated with glyphosate than in the controls (fig. 2B). The activity of ethoxycoumarin O-deethylase was also somewhat lower in the glyphosate treated rats than in the controls (fig. 2C). The most marked changes were found in the rats treated with MCPA; both doses used in the present study increased the activity of ethoxycoumarin O-deethylase more than 2-fold (fig. 2C).

The hepatic activity of epoxide hydrolase was significantly higher after treatment with 2,4-D (higher dose) or MCPA (both doses) than in the controls (fig. 3A). The activity of glutathione S-transferase in the hepatic postmicrosomal fraction of the rats treated with either 2,4-D or MCPA at a dose of 150-200 mg/kg was significantly decreased (fig. 3B). The activities of UDPglucuronosyltransferase are shown in fig. 4A. With p-nitrophenol as the substrate, both rats treated with clofibrate and those treated with the lower dose of 2,4-D had a decrease in the native microsomes. When measured in the digitonin activated microsomes, the activity was also decreased in the rats treated with the higher dose of MCPA (fig. 4A). When 4-methylumbelliferone was used as the substrate, markedly less changes were found; only a slight decrease was found in the rats treated with clofibrate, and a slight increase was found in the native microsomes of the rats treated with MCPA (fig. 4B).

When the monooxygenase activities were measured in the intestinal mucosa, an increase was found in the rats treated with MCPA when ethoxycoumarin was used as the substrate (fig. 5A). When benzopyrene was used as the substrate, a significantly decreased activity was found in the rats treated with glyphosate (fig. 5B). No changes were present in the intestinal activity of glutathione S-transferase (not shown). An increased activity of the epoxide hydrolase was found in the rats treated with the higher dose of MCPA (fig. 5C). The UDPglucuronosyl-transferase activity decreased significantly in the rats treated with the higher dose of MCPA regardless of whether p-nitrophenol (fig. 6A) or 4-methylumbelliferone (fig. 6B) was used as the substrate.

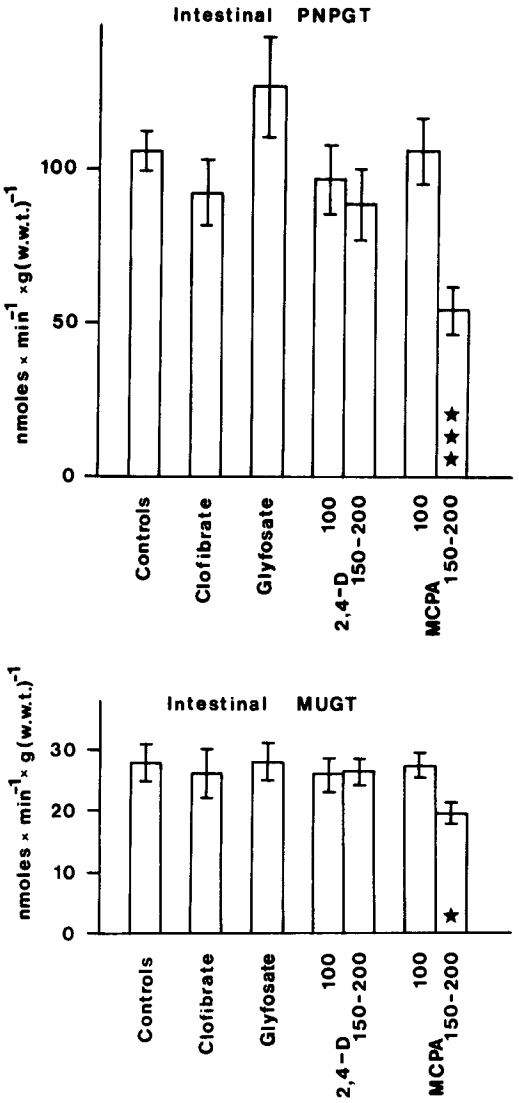


Fig. 6. The intestinal activity of UDPglucuronosyltransferase, with p-nitrophenol (A) or methylumbelliferone (B) as the substrate, in rats exposed to clofibrate or herbicides. For other explanations see figs. 1 and 2.

Discussion

Both phenoxyacetic acids and glyphosate are excreted mainly as such, and only a minor proportion of them is metabolized. The excretion takes place mainly via the kidneys and into the urine. Unlike the phenoxyacetic acids, glyphosate is rather poorly absorbed, and only about 15–40% of the dose is excreted into the urine. Although phenoxyacetic

acids may be conjugated (e.g., with glucuronic acid), in the urine they are found mainly unconjugated. Phenoxyacids and glyphosate act rather differently in plants. Phenoxyacetic acids simulate auxins, the growth hormones of plants, but its action is not controlled. At high doses phenoxyacids may interfere with plant cell membranes (Åberg & Eliasson 1978). Glyphosate acts on plants by inhibiting their synthesis of amino acids (Åberg & Eliasson 1978).

When the toxicity of the herbicides under study is considered, attention must be made to the processes by which they are synthesized so that possible impurities which result from the manufacturing process are detected. Earlier, when 2,4,5-trichlorophenoxyacetic acid was used, the presence of 2,3,7,8-tetrachlorodibenzoparadioxin (TCDD) was considered to be the main problem. TCDD, an impurity found in 2,4,5-trichlorophenoxyacetic acid, is formed when 2,4,5-T is synthesized from tetrachlorobenzene (IARC 1977). However, phenol is the chemical from which 2,4-D is manufactured, and MCPA is synthesized from cresol; the main impurities formed are bis (2,4-dichlorophenoxy)-methane, 2,2',4,6'-tetradiphenoxymethane, 4-chloro-*o*-cresol, and 4,6-dichloro-*o*-cresol. Of these impurities 4-chloro-*o*-cresol, also a degradation product of MCPA, is rather lipid soluble and can be stored in adipose tissue (Hattula *et al.* 1979). In addition, small amounts of the less toxic polychlorodibenzo-*p*-dioxins (PCDD) and polychlorodibenzo-*p*-furans (PCDF) may be formed (Cochrane *et al.* 1982; Rappe 1978). When the reasons for the enzymatic changes in biotransformation enzymes are evaluated, one first suspects that the impurities are partially responsible for the results. However, 2,3,7,8-TCDD is the only known inducer which can be blamed for the induction of biotransformation enzymes (Aitio & Parkki 1978; Madhukar & Matsumura 1981), but this TCDD is not a byproduct of the processes by which the herbicides used in the present study are synthesized. From the practical point of view, whether the changes found in the activities of biotransformation enzymes are due to impurities or the herbicides themselves is not an important issue. What is important is that the preparations sold contain compounds which produce metabolic changes. However, the detailed role of possible impurities in the regulation of biotransformation enzyme activities should be clarified.

Mutagenicity tests have been applied to evaluate the toxicity of herbicides. Only in a few of these tests was 2,4,5-T (not included in the present study) weakly mutagenic (see Grant 1979 for references). No conclusive chromosomal alterations have been found in the lymphocytes of persons exposed to 2,4,5-T and many other pesticides (Högstedt *et al.* 1980; Crossen *et al.* 1978; Yoder *et al.* 1973). When 2,4-D and MCPA were tested in different mutagenicity tests, only weakly positive or negative data were obtained (see Seiler 1978 for references). No induction of sister chromatid exchanges (SCEs) was observed in the peripheral lymphocytes of workers exposed to 2,4-D or MCPA (Linnainmaa 1983). The mutagenicity of glyphosate has been rather poorly studied and no definite data are available.

Despite only weakly positive or negative test results on the mutagenicity of phenoxyacid herbicides, there are some epidemiological data that suggest a link between carcinogenicity and the exposure to phenoxyacetic acid herbicides (Hardell & Sandström 1979; Moses & Selikoff 1981). Although the mechanisms of this suspected carcinogenicity are not known, there are some hypotheses. One hypothesis is that reactive intermediates are produced from other xenobiotics. Our finding that pretreatment with MCPA increased the activity of ethoxycoumarin O-deethylase indirectly supports this idea; however, the unaltered activity of aryl hydrocarbon hydroxylase, which metabolizes polycyclic aromatic hydrocarbons, is contradictory. The diminished inactivation of possible reactive intermediates produced from other xenobiotics was also suggested by our finding that both 2,4-D and MCPA decreased the activity of glutathione S-transferase in the liver. However, the enhanced activity of epoxide hydrolase and minor changes in the activity of UDPglucuronosyltransferase do not directly support this idea. Interestingly, glyphosate decreased both the level of cytochrome P-450 in the hepatic microsomes and the activities of ethoxycoumarin O-deethylase and PPO hydroxylase in the liver. These effects of glyphosate may result from disruption of the cellular membrane in the same way that high doses of glyphosate are known to perturb the cellular

membrane in plants. However, no detailed analysis of membrane composition was done in the present study.

The response of the intestinal enzymes differed from those of the liver. Glyphosate decreased the activity of aryl hydrocarbon hydroxylase in the intestine, whereas the high dose of MCPA decreased the intestinal UDP-glucuronosyltransferase activities whichever substrate was used.

Clofibrate did not significantly change the biotransformation enzyme activities in the rats of our study. However, an approximately 30% increase in the hepatic levels of cytochrome P-450 was observed, but this increase was not statistically significant. As clofibrate induces a specific cytochrome P-450 isozyme (Gibson *et al.* 1982), the purification of that isozyme may have yielded a more clearcut increase. Because the type of cytochrome P-450 induced by clofibrate is one that catalyzes the oxidation of fatty acids (such as lauric acid, which was not measured) it is possible that other catalyzation specific monooxygenases are not induced by clofibrate. Clofibrate is a derivative of phenoxyacetic acid, so 2,4-D and MCPA may have similar properties.

To conclude, phenoxyacid herbicides and glyphosate have partly opposite effects on the activities of drug metabolizing enzymes. However, the changes induced by phenoxyacetic acids in the drug metabolizing enzymes are moderate in comparison to more pronounced effects on other metabolic functions, e.g., the oxidation of fatty acids and lipid metabolism (Reddy *et al.* 1980; Hietanen *et al.* unpublished results).

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