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METABOLISM OF VINYLTOLUENE IN THE RAT: EFFECT OF INDUCTION AND INHIBITION OF THE CYTOCHROME P-450

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Abstract—Metabolism of vinyltoluene was studied in rats after injecting different doses of vinyltoluene. The main metabolites excreted in urine of rats after vinyltoluene treatment were: thioethers, p-methylmandelic acid, p-methylphenylglyoxylic acid, p-methylbenzoyl glycine, p-methylphenylacetyl glycine and p-vinylbenzoyl glycine. The highest excretion rate was obtained with doses of 50, 250 and 500 mg/kg already within the first six hours. However, the dose of 500 mg/kg did not increase the excretion rates of these metabolites compared to the dose of 250 mg/kg suggesting that the metabolic pathways begin to be saturated with the amount of 250 mg/kg. At the dose of 50 mg/kg 55% of the dose was detected as urinary metabolites within 23 hr, mainly within the first 6 hr. The amounts of the excreted metabolites expressed as per cent of the injected dose (250 mg/kg or 500 mg/kg) were lower than that caused by 50 mg/kg, and a noticeable amount of the total sums were excreted within 11–23 hr suggesting that the excretion was still continued with the doses of 250 and 500 mg/kg 23 hr after the injection. The excretion of all analyzed metabolites of vinyltoluene was prevented by the pretreatment of the rats with 1-phenylimidazole, an inhibitor of cytochrome P-450 monooxygenases. This indicates that these metabolites were formed as catalyzed by cytochrome P-450. The structures of the analyzed metabolites suggest that the main reactive intermediate of vinyltoluene is vinyltoluene-7,8-oxide. Furthermore, the amounts of the excreted metabolites showed that the main detoxification pathways of vinyltoluene-7,8-oxide were the conjugation with reduced glutathione and hydration to diols. Pretreatment of the rats with PCBs increased the excretion rates of the metabolites. However, the PCB-pretreated rats excreted less thioethers (62%) compared to the rats treated only with the same amount of vinyltoluene whereas the total sum of the other metabolites was about the same in these both groups. This result suggests that PCBs change the metabolism of vinyltoluene to some other pathway which could be glucuronide conjugation because PCBs increased the activity of UDPglucuronosyltransferase in a dose-dependent manner.

Vinyltoluene (methylstyrene) has been shown to be metabolized in rodents to electrophilic intermediates which have been thought to be the respective vinyl side-chain epoxides formed by cytochrome P-450 monooxygenases [1]. Due to their electrophilic character, epoxides tend to bind to cellular nucleophiles, e.g. nucleic acids and proteins. This binding may lead to toxic, mutagenic and carcinogenic effects [2–4]). Vinyltoluene-7-8-oxide alkylates guanosine and a synthetic nucleophile, 4-(p-nitrobenzyl)-pyridine, *in vitro* [5], and it is mutagenic in bacteria and in Chinese hamster V79 cells [6]. Both vinyltoluene and vinyltoluene-7,8-oxide may induce sister chromatid exchanges in human lymphocytes [7].

Glutathione (GSH) is available in many organisms [8], and it is obviously the most common free sulfhydryl compound in the tissues of mammalian species [9, 10]. Glutathione functions as an important cellular defense mechanism against electrophilic epoxides by conjugating with them. The conjugation may occur both non-enzymatically and enzymatically, catalyzed by GSH-transferases [11–13]. Glutathione conjugates are excreted as thioethers, i.e. acetylated or nonacetylated cysteine conjugates [14]. Another detoxification pathway for epoxides is hydration to diols catalyzed by epoxide hydrolase [2, 15, 16]. Diols are usually further metabolized to more water-soluble acids or amino acid conjugates. A recent study with isolated perfused rat liver [17] showed that GSH-transferase and epoxide hydrolase are equally important in the metabolism of styrene-7,8-oxide, the active metabolite of styrene. When styrene glycol was added to the perfusion system, its major metabolite was mandelic acid. *In vivo* the metabolic pattern of styrene may be different. The main metabolites of styrene in rat urine were mandelic acid, phenylglyoxylic acid, hippuric acid, glucuronide conjugates and thioethers [18, cf. 19].

The aim of the present study was to investigate the metabolism of p-vinyltoluene by measuring the contents of the main urinary metabolites. The urinary metabolites analyzed were: thioethers, p-methylmandelic acid; p-methylphenylglyoxylic acid; the glycine conjugates of p-methylbenzoic acid, p-methylphenylacetic acid; and p-vinylbenzoic acid. The role of cytochrome P-450 in the formation of these metabolites was studied by inhibiting its catalytic activity. The effect of vinyltoluene on the metabolic balance between the detoxification pathways of vinyl side-chain epoxides (i.e. GSH-conjugation and hydration to diols) was studied by measuring the

content of urinary metabolites after the administration of different doses of p-vinyltoluene to rats. Furthermore, the metabolic balance was also studied by the use of an inducer of drug metabolism: a mixture of PCBs (polychlorinated biphenyls), which increases, e.g. the activity of cytochrome P-450 monooxygenases and epoxide hydrolase but does not change the content of glutathione in the liver or in the kidneys [20].

MATERIALS AND METHODS

Animals. Wistar male rats (350–400 g) were used. They were fed commercial pelleted diet obtained from Astra-Ewos (Södertälje, Sweden). Food and tap water were given freely.

Chemicals given to the animals. p-Vinyltoluene (purity > 99%) was obtained from Fluka AG (Buchs SG, Switzerland), PCBs (Clophen C, a mixture of polychlorinated biphenyls with an average chlorination degree of 42%) from Bayer AG (Leverkusen, FRG), olive oil (pure) from Yliopiston Apteekki (Helsinki, Finland) and dimethylsulfoxide (DMSO, pure) from Merck (Darmstadt, FRG). 1-Phenylimidazole was a gift from Dr. A. J. Baars (The Netherlands).

Exposure of the animals. All the chemicals were given as a single intraperitoneal injection. Three kinds of experiments were performed: In the first experiment vinyltoluene dissolved in olive oil was given at doses of 50, 250, 500 or 1000 mg/kg body weight. Four rats were killed after 12 hr at each dose level and three rats at the dose levels of 50, 250 or 500 mg/kg after 23 hr. In the second experiment the rats were given either vinyltoluene (500 mg/kg in olive oil) or 1-phenylimidazole (50 mg/kg in DMSO) or both. The control rats received either olive oil or DMSO alone. 1-Phenylimidazole and DMSO were given 1.5 hr before the administration of vinyltoluene. Four rats in each group were then killed 12 hr after the injection of vinyltoluene, and three rats in each group were killed after 23 hr. In the third experiment the rats were given either PCBs (500 mg/kg in olive oil) or vinyltoluene (500 mg/kg in olive oil) or both. The control rats received olive oil only. The dose of PCBs was given five days before the administration of vinyltoluene. The animals (three in each group) were killed 23 hr after the injection.

In each experiment the amounts of the vehicle (olive oil and DMSO) per kg body weight were constant. The urine was collected during the exposure from all the rats in each group. After the rats were decapitated the livers were removed and chilled. They were packed in air-free plastic bags and stored at −70°. The urine was centrifuged (5000 *g*) after collection and stored in closed polyethylene tubes at −20°.

Parameters measured. Ca^{2+}-aggregated microsomes were made as described by Aitio and Vainio [21]. The contents of hepatic and renal glutathione (measured as free nonprotein sulfhydryl groups [22], hepatic cytochrome P-450 [23], and renal cytochrome P-450 [24] and the activities of NADPH-cytochrome *c* reductase [25], 7-ethoxycoumarin *O*-deethylase [26], epoxide hydrolase [27] and UDPglucuronosyltransferase [28] were measured as described earlier [20]. The protein content was measured by the method of Lowry *et al.* [29].

The content of urinary thioethers was measured by the method of van Doorn *et al.* [30] as modified by Heinonen *et al.* [31]. The concentrations of p-methylbenzoyl glycine (p-methylhippuric acid), p-methylphenylacetyl glycine, and p-vinylbenzoylglycine were measured according to the method of Engström *et al.* [32], with 2,3-dimethylbenzoic acid as the internal standard. The concentration of p-methylmandelic acid was measured by the method of Engström and Rantanen [33] with cinnamic acid as the internal standard. The concentration of p-methylphenylglyoxylic acid was measured by the method of Engström and Rantanen [33], which was modified. Before the extraction of p-methylphenylglyoxylic acid from the urine, p-methylphenylglyoxylic acid was allowed to react with o-phenyldiamine to form o-phenyldiamine conjugates in order to get the p-methylphenylglyoxylic acid better silylated. The concentration was measured with phenylglyoxylic acid as the standard. These methods were originally used for measuring the corresponding non-methylated metabolites derived from styrene. p-Methylbenzoyl glycine (p-methylhippuric acid) was the only compound for which an authentic standard was available. p-Methylmandelic acid and p-methylphenylglyoxylic acid were quantitated with the corresponding mandelic and phenylglyoxylic acids as the standards. The effect of the additional methyl group on the detector response was estimated from the difference between p-methylbenzoyl glycine and benzoyl glycine. The concentrations of p-vinylbenzoyl glycine and p-methylphenylacetyl glycine were calculated by comparing the peak areas to those of the internal standard, 2,3-dimethylbenzoic acid, only. The identity of the specified metabolites was verified by combined gas chromatograph/mass spectrometer (LKB-9000, SE-30 capillar column), with electron energy of 70 eV. The temperature of the ionization source was 290°.

RESULTS

Effects of vinyltoluene on drug metabolizing enzymes, the concentration of glutathione in rat liver, and the excretion of urinary thioethers in 12 hr. A dose-dependent decrease in the content of hepatic glutathione was seen 12 hr after the rats had been given an intraperitoneal dose of 50, 250, or 500 mg/kg body weight of vinyltoluene (Fig. 1a). The decrease was, however, about the same for the doses of 500 and 1000 mg/kg (Fig. 1a). The excretion of urinary thioethers increased remarkably after the administration of 250 mg/kg when compared to that of 50 mg/kg (Fig. 1b). The doses of 500 and 1000 mg/kg, however, did not increase the excretion more than the dose of 250 mg/kg. When the rats were treated with 1-phenylimidazole—a known inhibitor of the activity of cytochrome P-450 [34]—before the administration of vinyltoluene (500 mg/kg), there were no changes in the contents of glutathione and urinary thioethers compared to those of the controls (Figs. 2a and b). Thus, a decrease in the content of glutathione was reflected as an increase in the content of urinary thioethers.

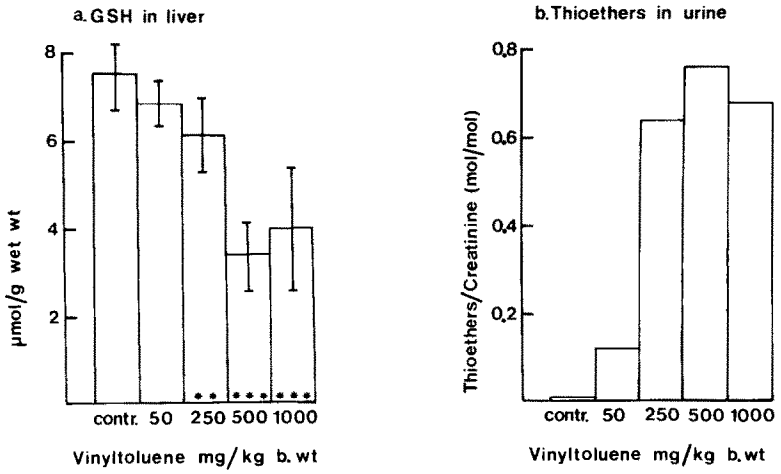

Fig. 1. The contents of hepatic glutathione (GSH) (μmol/g wet wt) (a) and the urinary thioethers (moles/mole creatinine) (b) after the administration of vinyltoluene to rats as an intraperitoneal dose of 50, 250, 500 or 1000 mg/kg. Each bar is the mean ± S.D. of four animals (a) and the total value of thioethers/creatinine from the pooled urine of four animals (b). The rats were decapitated 12 hr after the administration of vinyltoluene. The urine was collected during the whole exposure period. **P < 0.01, ***P < 0.001.

The content of cytochrome P-450 and the activity of 7-ethoxycoumarin *O*-deethylase increased significantly at some doses of vinyltoluene but showed no dose-dependence, whereas the activities of NADPH-cytochrome *c* reductase and epoxide hydrolase did not change (data not shown). The activity of UDPglucuronosyltransferase was increased in a dose-dependent manner: 14, 38, 53, and 74% 12 hr after exposure to 50, 250, 500, or 1000 mg/kg of vinyltoluene, respectively. These doses of vinyltoluene did not change the content of glutathione, the content of cytochrome P-450, nor the activity of epoxide hydrolase in the kidneys (data not shown).

* These compounds were analyzed after the hydrolysis of the glycine moiety; thus the corresponding free acids are included in the figures.

Urinary excretion of the vinyltoluene metabolites in 23 hr. The urine samples were collected during 0–6, 6–11, and 11–23 hr after the treatment of the rats with a single dose of 50, 250, or 500 mg/kg of vinyltoluene. Thioethers comprised 6–25% of the dose of vinyltoluene given. The other main metabolites which were characterized in the urine by gas-chromatography–mass spectrophotometry were p-methylmandelic acids, p-methylbenzoyl glycine*, p-methylphenylglyoxylic acids, p-methylphenylacetyl glycine*, and p-vinylbenzoyl glycine*. The mass spectra of these metabolites were similar to those obtained by Bergemalm-Rynell and Steen [35] from vinyltoluene metabolites. The excretion of these metabolites are shown in Fig. 3 (a–f), respectively.

In general, at each dose, all the metabolites (except p-methylmandelic acid at the dose of 500 mg/kg

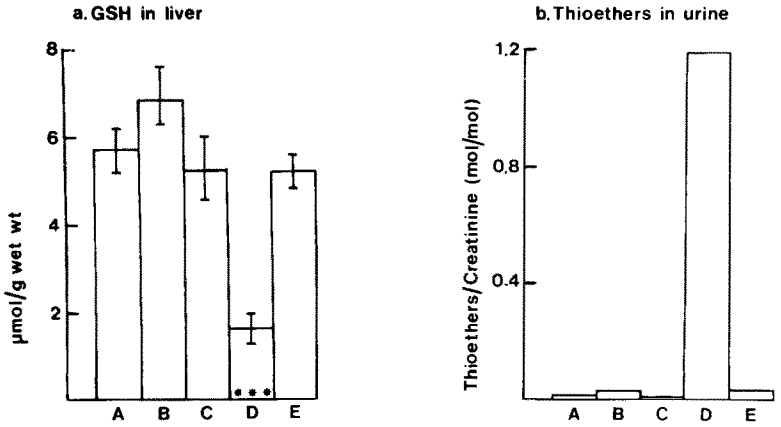

Fig. 2. The contents of hepatic glutathione (GSH) (μmoles/g wet wt) (a) and urinary thioethers (moles/mole creatinine) (b). Each bar is the mean ± S.D. of four animals (a) and the total value of thioethers/creatinine from the pooled urine of four animals (b). A, olive oil; B, DMSO; C, 1-phenylimidazole (50 mg/kg); D, vinyltoluene (500 mg/kg); E, vinyltoluene (500 mg/kg) plus 1-phenylimidazole (50 mg/kg). The rats were decapitated 12 hr after the administration of vinyltoluene. The urine was collected during the whole exposure period. ***P < 0.001.

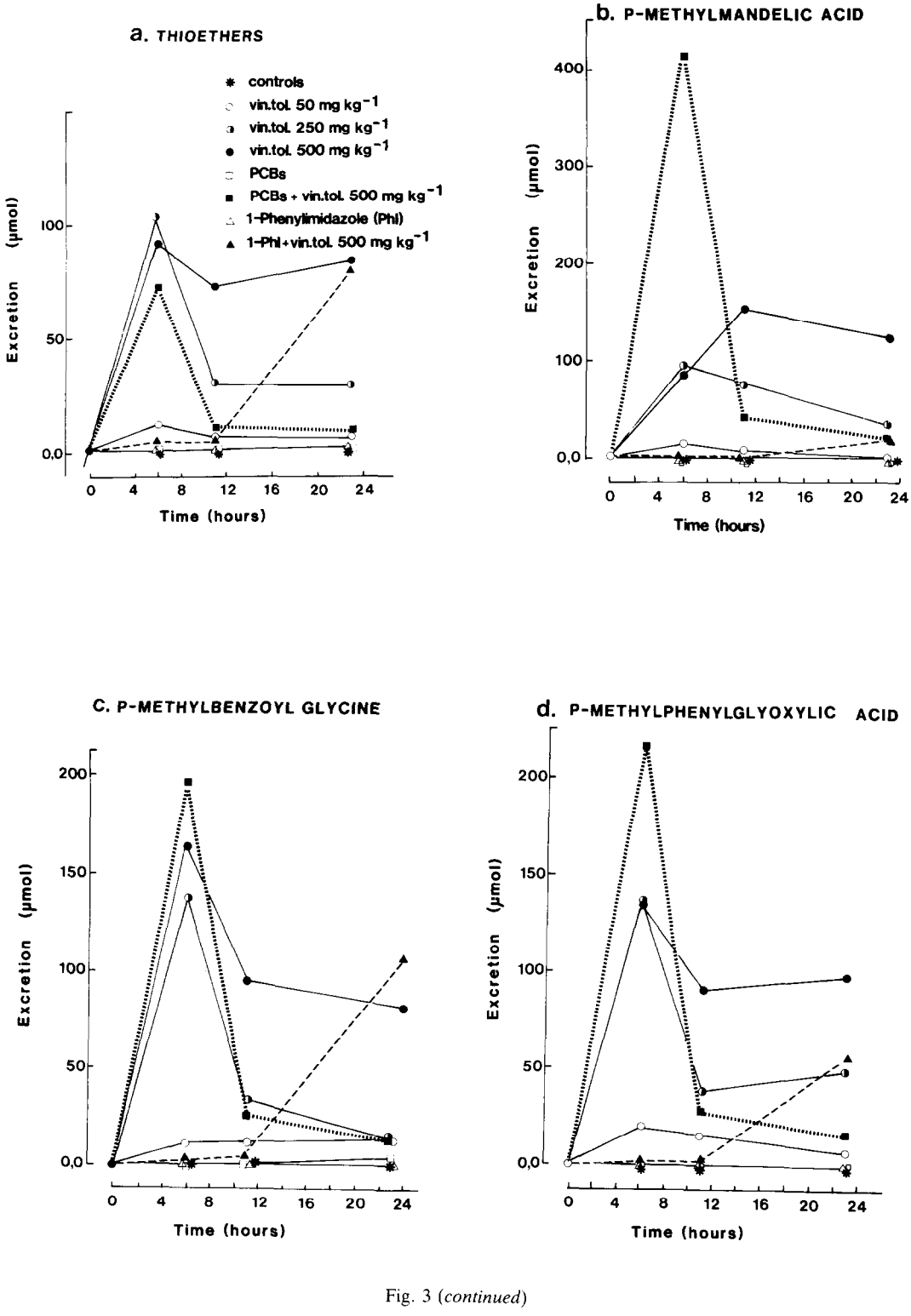

Fig. 3 (*continued*)

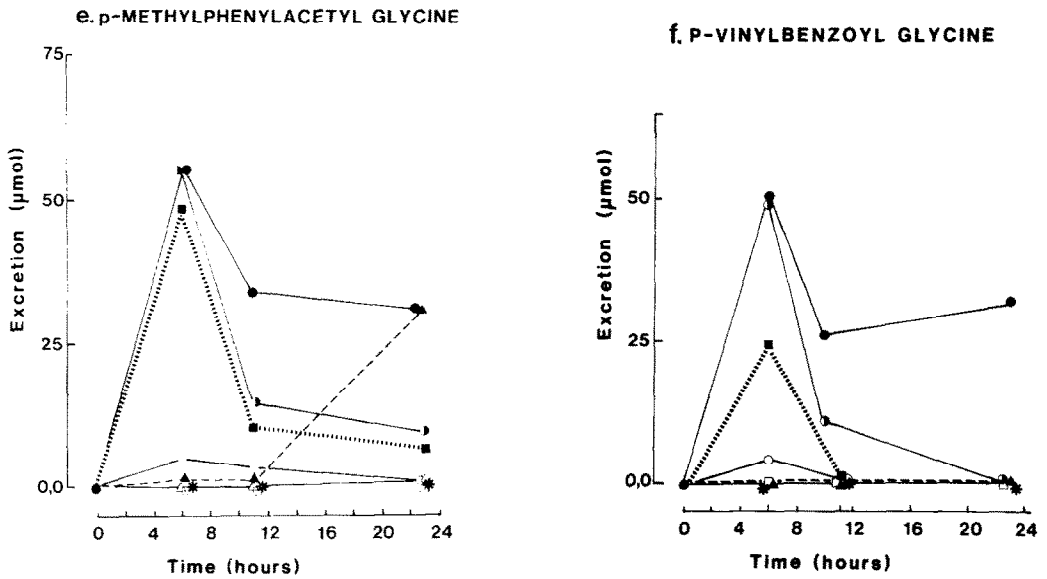

Fig. 3. The excretion of thioethers (a), p-methylmandelic acid (b), p-methylbenzoyl glycine (c), p-methylphenylglyoxylic acid (d), p-methylphenylacetyl glycine (e) and p-vinylbenzoyl glycine (f) in the urine after exposure to different doses of vinyltoluene (50, 250 or 500 mg/kg). The urine samples were collected during 0–6, 6–11 and 11–23 hr after the injection from three animals in each group. Treatment with PCBs was done 5 days and 1-phenylimidazole treatment 1.5 hr before the injection of vinyltoluene (500 mg/kg). The rats were decapitated 23 hr after the administration of vinyltoluene.

of vinyltoluene) reached the highest excretion rates already over 6 hr. The dose of 50 mg/kg was excreted almost completely already within the first six hours. The total excretion of this dose was 55% of the injected amount during the 23 hr. Vinyltoluene at the dose of 500 mg/kg caused about the same amounts of urinary metabolites as the dose of 250 mg/kg over six hours. After six hours the concentrations of these metabolites decreased to almost the control level in 23 hr in rats which received 250 mg/kg vinyltoluene. At 500 mg/kg of vinyltoluene, the excretion decreased to some extent but remained on a quite high level. The rats pretreated with PCBs excreted the vinyltoluene metabolites studied faster than the rats treated only with vinyltoluene. In particular, PCB-pretreatment increased the first six-hour excretion of p-methylmandelic acids and p-methylphenylglyoxylic acids (Figs. 3 b and d). The excretion of thioethers during 6–11 and 11–23 hr and the excretion of vinylbenzoyl glycine were lower in PCB-pretreated rats than in the rats treated only with vinyltoluene (Figs. 3 a and f). The rats treated with 1-phenylimidazole before the administration of vinyltoluene (500 mg/kg) did not excrete any metabolite of vinyltoluene during 0–11 hr (Fig. 3). However, after 11 hr the contents of all the metabolites except that of p-vinylbenzoyl glycine were significantly higher in the urine than those in the urine of controls. Especially the contents of thioethers, p-methylbenzoyl glycine and p-methylphenylacetyl glycine were increased.

Table 1 shows the total amounts of metabolites excreted in the rats over 23 hr in relation to their treatment. In PCB-pretreated rats only 2.8% of the given vinyltoluene dose (500 mg/kg) was excreted as thioethers, whereas the rats not pretreated excreted 7% of the injected dose as thioethers (Table 1). Furthermore, the excretion of p-methylmandelic acid was slightly increased in PCB-pretreated rats when compared to those without pretreatment whereas other metabolites analyzed were excreted slightly less in rats treated with vinyltoluene and PCBs than in rats treated only with the same amount of vinyltoluene (Table 1).

When the excretion of thioethers (expressed as % of the injected dose) during 23 hr is compared to the respective sum of the other metabolites it was shown that over 40% of all the metabolites analyzed were excreted as thioethers in rats which received 50 mg/kg vinyltoluene, whereas in rats which received 500 mg/kg, the respective value was only 17% (Table 2). The total amount of the metabolites excreted over 23 hr was 23% lower in rats which were treated with PCBs before the administration of vinyltoluene than in rats without PCB-treatment. Pretreatment of the rats with PCBs drastically (about 60%) diminished the excretion of thioethers compared to the amount in non-pretreated rats, whereas the sum of the amounts of the other metabolites remained the same.

DISCUSSION

The urine is obviously the main excretory route for the vinyl compound such as styrene, because after the injection of (β-C^{14})styrene to rats over 70% of the radioactivity appeared in the urine within 24 hr, whereas less than 3% was found in feces [18]. Accordingly, this finding suggests that the urine is also the main excretory route for vinyltoluene,

Table 1. The contents of the main urinary metabolites of vinyltoluene (VT) after rats were administered different doses of vinyltoluene (50, 250 or 500 mg/kg body weight) and the effect of PCBs (500 mg/kg, 5 days before the administration of vinyltoluene) or 1-phenylimidazole (PhI 50 mg/kg, 1.5 hr before the administration of vinyltoluene) treatments on the excretion caused by a vinyltoluene dose of 500 mg/kg

Treatment	Metabolites excreted by 3 rats in 23 hr (μmole)					
	Thioethers	p-Methyl mandelic acid	p-Methyl benzoyl glycine	p-Methylphenyl glyoxylic acid	p-Methylphenyl acetyl glycine	p-Vinylbenzoyl glycine
50 mg/kg (%*)	95 (25)	22 (5.7)	36 (9.3)	46 (11.9)	~10 (~2.5)	~4 (~1)
250 mg/kg (%*)	163 (9)	205 (11.3)	192 (10.6)	226 (12.5)	~80 (~4)	~60 (~3)
500 mg/kg (%*)	250 (7)	365 (10.0)	339 (9.3)	325 (8.9)	~120 (~3)	~109 (~2.5)
PCBs + VT (500 mg/kg) (%*)	95 (2.8)	479 (14.4)	240 (7.2)	262 (7.9)	~65 (~2)	~24 (~0.7)
PhI + VT (500 mg/kg) (%*)	93 (2.1)	44 (1.0)	109 (2.6)	59 (1.4)	~33 (~0.8)	~139 (~3)
PCBs	6.2	0	10	0	1.9	0
PhI	12.9	0	~2	0	0	0
Control	7	0	~1	0	0.8	0

* The amount of the metabolite as (%) of the injected dose of vinyltoluene

Table 2. The content of thioethers compared to the sum of the other metabolites (see Table 1)

Treatment	Excreted by three rats in 23 hr (% of dose)			
	Thioethers	Others	Total sum	Thioethers/others
50 mg/kg	25	30	55	0.8
250 mg/kg	9	41	50	0.2
500 mg/kg	7	33	40	0.2
PCBs + VT (500 mg/kg)	2.8	32	35	0.09
PhI + VT (500 mg/kg)	2.1	9	11	0.2

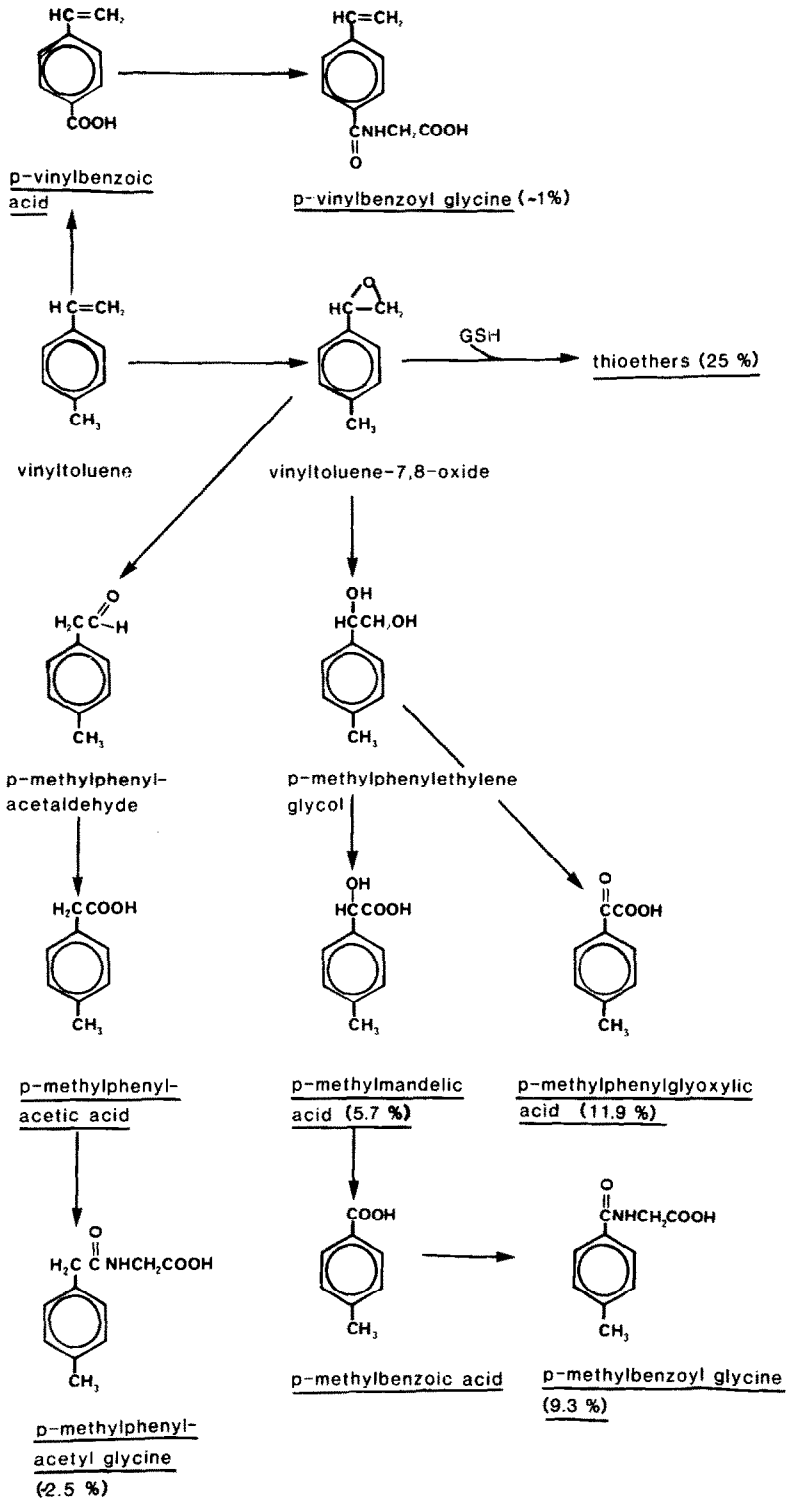

Fig. 4. The main metabolic pathways of vinyltoluene. The compounds underlined were measured in this study. In order to emphasize the importance of each pathway, the amounts of the metabolites are expressed as per cent (%) of the given dose (50 mg/kg body weight) in parentheses.

which is the compound most closely related to styrene. A good picture of the metabolism of these compounds could be achieved by the characterization of the urinary metabolites and by the analysis of their contents. Figure 4 presents a suggestion for the main metabolic pathways of vinyltoluene. The schema is based on the amounts and structures of vinyltoluene metabolites characterized in the present study and on the previous reports on the metabolism of styrene and vinyltoluene [18, 19, 35, 36, 37].

Vinyltoluene-7,8-oxide is suggested to be formed as catalyzed by cytochrome P-450 monooxygenases. As vinyltoluene-7,8-oxide is electrophilic it binds easily to cellular nucleophiles. Nucleophilic glutathione is regarded as the key molecule in the detoxification of electrophilic compounds [38]. Glutathione conjugation has also been shown to be an important pathway when the vinyltoluene intermediates are detoxified: Inhalation studies with rats have shown that vinyltoluene decreases the content of liver glutathione with a concomitant excretion of urinary thioethers [20, 39]. This study confirmed the previous results and showed that, depending on the dose of vinyltoluene given, up to 25% of the dose (50 mg/kg) was excreted as urinary thioethers within 23 hr.

The present study indicated that the other main detoxification pathway of vinyltoluene epoxides is hydration to diols. The major urinary metabolites of vinyltoluene detected in this study which are metabolized via diols were p-methylmandelic acid, p-methylphenylglyoxylic acid and p-methylbenzoyl glycine*. The total amount of these metabolites excreted during 23 hr after the administration of vinyltoluene was 27% of the dose (50 mg/kg). p-Methylphenylacetyl glycine* corresponds to the metabolites formed from vinyltoluene-7,8-oxide via its corresponding vinylaldehyde (Fig. 4). The significance of this route seems to be of minor importance compared to that of epoxide hydrolase and glutathione conjugation, as only 2.5% of the injected dose (50 mg/kg) of vinyltoluene was detected as this metabolite. These results are supported by those of Delbressine *et al.* [37], who found 1.4% of the injected dose of styrene (250 mg/kg) in rat urine as phenylacetyl glycine. The third metabolic route detected in this study is the oxidation of the methyl group of vinyltoluene. This route, which does not go via vinyltoluene-7,8-oxide, also seems to be of minor importance.

All the metabolites detected were excreted in a dose-dependent manner. The excretion of the metabolites was inhibited by 1-phenylimidazole, a finding which confirmed the role of cytochrome P-450 as the key enzyme system in the metabolism of vinyltoluene. The structures of the metabolites (those metabolized via epoxide hydrolase) indicated that vinyltoluene-7,8-oxide is the most important intermediate (Fig.4). The thioethers excreted after the administration of vinyltoluene have been characterized by Bergemalm–Rynell and Steen [35] and they were identified as N-acetylcysteine conjugates of methylphenylethanol. This result also confirms the significance of vinyltoluene-7,8-oxide as the first main intermediate of vinyltoluene. Vinyltoluene at the dose of 500 or 1000 mg/kg virtually did not increase the excretion of the metabolites over the first 6–12 hr compared to the excretion caused by the dose of 250 mg/kg. This phenomenon suggests that the dose of 250 mg/kg is the amount the metabolic pathways began to be saturated.

A recent *in vitro* study has shown that about half of styrene-7,8-oxide is metabolized in perfused rat liver to glutathione conjugates and the other half to diols [17]. When rat liver was perfused with styrenediols, the major metabolite was mandelic acid [17]. Similar results were also obtained in this study *in vivo* with a dose of vinyltoluene of 50 mg/kg. About 50% of the vinyltoluene metabolites were detected as thioethers, and the rest of the metabolites could be expected mainly to be metabolized via epoxide hydrolase. However, when the dose was increased, more reactive vinyltoluene intermediates (epoxides) appeared to go via the epoxide hydrolase pathway than via that of glutathione conjugation. The content of liver glutathione in each rat of this study was about 80 μmoles; thus about 40% of the total hepatic glutathione reservoir was consumed by 50 mg/kg of vinyltoluene. The turnover of hepatic glutathione in the rat is 2–4 hr [8]. When glutathione was depleted in isolated hepatocytes by vinyltoluene-7,8-oxide the biosynthesis of glutathione started within 30 min to produce glutathione [40]. Typically, when hepatic glutathione is depleted *in vivo* the biosynthesis starts to produce new glutathione so efficiently that the content of glutathione increases to an even higher level than that in the controls [1, 14]. Thus, the increased biosynthesis obviously maintained the excretion of thioethers on a high level 6 hr after the injection in the rats which received 500 mg/kg of vinyltoluene.

The activities of the enzymes of the detoxification pathways were changed by the pretreatment of the rats with PCBs, which induces, e.g. cytochrome P-450 monooxygenase activity and epoxide hydrolase activity but which does not affect the content of glutathione [20]. This study supported the suggestion made on the basis of the *in vivo* studies [20] that PCBs change the metabolism of reactive vinyltoluene intermediates more to diol formation than to conjugation with glutathione. However, PCB-pretreatment diminished the total amount of metabolites excreted. This result indicates that PCBs increased the metabolism of vinyltoluene by some other detoxification pathway. One possibility is glucuronidation, as the activity of UDPglucuronosyltransferase increased in a dose-dependent manner.

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* The fractions contained the corresponding free acids, too.

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