Effects of ethanol and phenobarbital treatments on the pharmacokinetics of toluene in rats

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Abstract

Rats were exposed to toluene at a wide range of concentrations from 50 to 4000 ppm for six hours, and the effects of ethanol and phenobarbital (PB) treatments on the pharmacokinetics of toluene metabolism were investigated. Ethanol treatment influenced toluene metabolism mainly at low exposure concentrations. Thus ethanol accelerated the clearance of toluene from blood only when the blood concentration of toluene was not high ($<360 \mu\text{M}$), and ethanol increased hippuric acid (HA) excretion in urine more significantly at low ($<250 \text{ ppm}$) than at high atmospheric toluene concentrations. Ethanol also expressed a similar effect on *p*-cresol excretion as on HA, but had little effect on *o*-cresol. Phenobarbital treatment promoted the urinary excretion of all of the metabolites of toluene, especially after exposure to high toluene concentration. As well as HA, benzoylglucuronide (BG) and free benzoic acid were found in urine. These are the products of the side chain metabolism of toluene. Amounts of BG could be detected when the urinary excretion of free benzoic acid exceeded $5 \mu\text{mol/kg/6 h}$, indicating that a great deal of benzoic acid is required for the formation of BG. The Michaelis constant (K_m) and the maximum rate of metabolic excretion in urine during six hours exposure ($V_{\max}$) of isozymes involved in the excretion of toluene metabolites were calculated, and correlated with the subtypes of cytochrome P-450. The significance of the result was suggested in the biological monitoring of exposure to toluene.

Toluene has attracted great attention in recent years because of its wide use as a solvent, synthetic material, and component of gasoline, and its toxic potential on the central nervous system, liver, and kidney.¹⁻³

We reported previously, from in vitro studies,⁴ that toluene is mainly metabolised at its side chain to benzyl alcohol, and at least three isozymes of cytochrome P-450 are involved in the formation of the metabolite; the low ($0.13\text{--}0.17 \text{ mM}$) and high ($0.60\text{--}0.87 \text{ mM}$) K_m isozymes are found in naive rat liver microsomes, with the low K_m being ethanol inducible; another is an isozyme (0.90 mM) inducible by phenobarbital (PB), but absent in naive rats. Metabolites from hydroxylation at ring positions—namely, *o*- and *p*-cresol—share less than 1.5% and 2.8% respectively of total metabolites in rat liver microsomes; the high K_m and low $V_{\max}$ values of isozymes responsible for aromatic hydroxylation may account for the result. The low and high K_m isozymes for benzyl alcohol and *p*-cresol are cytochrome P-450IIE1 and P-450IIC11 respectively; the PB inducible isozyme for the formation of the three metabolites is cytochrome P-450IIB.⁵

In our present study, we investigated the in vivo metabolic pattern of toluene in rats, especially the pattern of urinary excretion of toluene metabolites, at seven exposure concentrations ranging between 50 and 4000 ppm. The effect of ethanol and PB treatments on toluene pharmacokinetics was also studied in that these two chemicals induce different types of cytochrome P-450 that are known to be involved in toluene metabolism in vitro.^{4,5}

Materials and methods

ANIMALS AND TREATMENTS

Male Wistar rats (Nippon SLC Inc, Shizuoka, Japan) were housed individually in stainless steel cages at a constant temperature ($20 \text{ (SD } 2^\circ\text{C)}$) with a 12 hour light dark cycle. Food (Clea CE-2, Nippon Clea, Tokyo) and water were available ad libitum. Rats were acclimatised for two weeks, and at the age of 8 weeks, they were divided into three groups: control, PB, and ethanol groups. The first two groups were fed a nutritionally adequate liquid diet (basal diet) described by DeCarli and Lieber⁶ and slightly modified.⁷ The third group was fed an isocaloric ethanol diet (2 g ethanol/80 ml) in which sucrose content was partially replaced with ethanol and corn oil. The animals were supplied daily with 80 ml (1 kcal/ml) liquid diets per rat at 4.00 pm as their only

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source of nutrition for three weeks. Rats in the PB group were administered PB at 80 mg/kg/day intraperitoneally at 10.00 am for four days before exposure to toluene.

TOLUENE EXPOSURE

Toluene inhalation was conducted in a dynamic flow system as described previously.⁸ Rats were exposed for six hours to toluene vapour at concentrations of 0, 50, 100, 250, 500, 1000, 2000, and 4000 ppm from 10.00 am to 4.00 pm. Toluene concentration in the exposure chamber was monitored every 15 minutes by a gas chromatograph equipped with a hydrogen flame ionisation detector. The variation of toluene concentration remained within 10% of the required value during the six hour exposure. Rats in the 0 ppm group were exposed to solvent free room air in the same chamber. Animals were removed to metabolic cages individually and supplied only with water after each exposure session.

TOLUENE CONCENTRATION IN BLOOD

Blood samples were taken from the tail at 0, one, two, four, and six hours after exposure, and the concentration of toluene in blood was measured by a syringe equilibration method.⁹

MEASUREMENT OF URINARY METABOLITES OF TOLUENE

Urine samples were collected from each rat during the six hour exposure and at four, eight, 12, and 18 hours after termination of each exposure. Hippuric acid concentration was determined by the method of Takeuchi *et al.*¹⁰ with slight modifications in the concentration of acetonitrile, acetic acid, and β -cyclodextrin of the mobile phase, and with the substitution of Unisil C18 for Senshupack ODS as the column packing.

For HA and free benzoic acid (F-BA) measurements, urine samples were centrifuged at 3000 rpm for 15 minutes. After diluting the supernatant with water, 10 μ l of the solution was injected into a high performance liquid chromatograph (HPLC, Hitachi L-6000) with a UV-Vis detector (Hitachi L-4200) and a chromato-integrator (Hitachi E-2500). The analytical conditions were: column, 4.0 ϕ $\times$ 250 mm stainless steel column packed with Unisil C18 (Gasukurokogyo Inc, Tokyo); mobile phase, 25% acetonitrile water solution containing 0.5% β -cyclodextrin and 0.5% acetic acid at a flow speed of 0.8 ml/min; wave length, 228 nm. Under these conditions the detection limit was found to be 11.5 μ g/l for HA and 17.6 μ g/l for F-BA.

Another metabolite was found in urine samples of rats exposed to high concentrations of toluene; this peaked just before the HA peak on the chromatogram, and showed a peak height toluene dose relation. To clarify the unidentified peak, we added 500 IU β -glucuronidase to 1 ml of the urine

samples and found that the peak before HA was completely eliminated from the urine and the peak height of benzoic acid increased correspondingly after incubation for 30 minutes at 37°C. The peak height of HA was not influenced by the treatment. This suggests that the unidentified peak represented benzoylglucuronide (BG), a conjugate of benzoic acid with glucuronic acid. From the difference in the amount of F-BA between intact urine and enzyme treated urine, the amount of BG was calculated.

For *o*- and *p*-cresol measurement, urine samples were hydrolysed by adding 0.5 ml 15% HCl to 1 ml of urine sample in a tube with cap. The mixture was then heated at 100°C for 60 minutes. The samples were centrifuged at 3000 rpm for 15 minutes, and after diluting the supernatant with water, 10 μ l was injected into the HPLC apparatus, with the analytical conditions: column, 4.0 ϕ $\times$ 250 mm stainless steel packed with Unisil C18; mobile phase, 25% acetonitrile solution at a flow speed of 1.2 ml/min; wave length, 200 nm. The detection limits of this method for *o*- and *p*-cresol were 5.2 and 5.0 μ g/l respectively.

STATISTICS

Statistical significance was determined by the Student's *t* test if the variances of data were not uniform, otherwise by Duncan's new multiple range test. The criterion for significance was set at *p* < 0.05.

Results

TOLUENE CONCENTRATION IN BLOOD

Figure 1 shows the blood concentrations of toluene at

Figure 1 Toluene concentration in rat blood at the end of six hour exposures to different concentrations of toluene. $\circ$, control group; $\square$, ethanol group; $\triangle$, PB treatment group. Each symbol represents the mean determined from five rats. **p* < 0.05 (significantly different from control group).

Figure 2 Decay curves of toluene in blood after six hour exposures to different concentrations of toluene. ○, control group; □, ethanol group; △, PB treatment group. Each symbol represents the mean from five rats. *Significantly different from control group ($p < 0.05$).

Figure 3 Excretion of toluene metabolites in urine of rats during six hour exposures to different concentrations of toluene. ○, control group; □, ethanol group; Δ, PB group. Each point represents the mean from five rats. A, Hippuric acid; B, free benzoic acid; C, benzoyl glucuronide; D, o-cresol; E, p-cresol.

the end of six hours of exposure to different concentrations of toluene. At low exposure concentrations (50, 100, and 250 ppm), the blood concentration increased slowly with increasing inhalation concentration in control rats, and then rapidly when the exposure concentration was over 250 ppm. In ethanol treated rats, the blood concentration of toluene was close to that of control rats at 50 and 100 ppm toluene exposures, but the value was significantly lower at 250, 500, and 1000 ppm inhalations than in the control group, indicating that treatment with ethanol accelerates the clearance of toluene from blood. At high exposures (2000 and 4000 ppm) differences in blood concentrations of toluene between ethanol and control groups became insignificant. The blood *v* exposure concentration curve of PB treated rats had a more shallow slope compared with the ethanol group, indicating that PB treatment increased toluene metabolism more than ethanol treatment. This effect was more significant at high exposure concentrations (2000 and 4000 ppm).

On the other hand, blood concentration of toluene decreased exponentially with time after stopping exposure in the three groups (fig 2). At 250–1000 ppm exposures, ethanol treatment decreased toluene concentration in blood significantly at all five time points. At the two highest exposures the difference became significant only when the blood concentration decreased below 360 μM —that is, two hours after 2000 ppm and six hours after exposure to 4000 ppm. This suggests that the effect of ethanol is limited to lower substrate concentrations. Blood concentrations of toluene in PB treated rats, from 250–4000 ppm exposures, were significantly lower than those of control rats at any sampling time.

EXCRETION OF TOLUENE METABOLITES IN URINE

Hippuric acid

Figure 3 shows the excretion patterns of five metabolites (HA, F-BA, BG, *o*-, and *p*-cresol) in urine during six hours of exposure to different toluene concentrations. As shown in fig 3A, the amount of HA in urine increased rapidly at low exposure concentrations (50–250 ppm) and then slowly with increasing toluene concentrations. It reached a maximum after exposure to 2000 ppm toluene. In ethanol treated rats, the excretion pattern was similar to that of controls, but the excreted amount was much larger, especially at low toluene concentrations (50–250 ppm). Excretion of HA increased almost linearly up to 1000 ppm toluene in PB treated rats. At high toluene concentrations, especially at 4000 ppm exposure, PB treated rats looked weak and walked unsteadily even during exposure. The volume of urine excreted in the first four hours after stopping exposure in the PB group was more than twice that in the control or in the ethanol group, and was thereafter decreasing. Of five animals, three rats died

within 12 hours of stopping 4000 ppm exposure. The decrease in the excretion of HA and other urinary metabolites may be caused by heavy intoxication.

HA excretion decreased with time (data not shown), and almost to the level of non-exposed rats 18 hours after stopping exposure to 50 and 100 ppm. At 2000 and 4000 ppm exposures, urinary HA concentration did not decrease even after stopping exposure, indicating the vast accumulation of toluene in adipose tissue and other organs.

Free benzoic acid

Free benzoic acid was detected even in non-exposed rats. Ethanol treatment significantly increased the urinary excretion of F-BA when toluene exposure exceeded 100 ppm. Phenobarbital had a more moderate effect (fig 3B).

Benzoylglucuronide

Benzoylglucuronide was firstly detected at 2000, 500, and 500 ppm exposure in control, ethanol, and PB groups respectively, when F-BA excretion in urine was above 5 $\mu\text{mol/kg}$, and HA excretion was above 500 $\mu\text{mol/kg}$. Both ethanol and PB increased BG excretion significantly (fig 3C).

o-Cresol

o-Cresol was detected only in urine of toluene exposed rats. It increased with toluene exposure up to 1000 ppm exposure (fig 3D). Ethanol increased *o*-cresol excretion only at high toluene exposures (1000, 2000, and 4000 ppm), whereas PB showed this effect from 50 ppm.

p-Cresol

p-Cresol was detected even in non-exposed rats. In the control group, urinary *p*-cresol increased with increasing toluene concentration up to 1000 ppm exposure (fig 3E). Ethanol treatment significantly increased *p*-cresol excretion from 100 ppm exposure and PB promoted excretion from 250 to 2000 ppm toluene exposure.

Total metabolism

Table 1 lists the cumulative amounts of five metabolites of toluene in 24 hours after the start of six hours of exposure to different concentrations of toluene. Hippuric acid made up 90–93% of all metabolites at exposure concentrations of 100–2000 ppm. At exposure to 4000 ppm, it decreased to 84% because of the large formation of BG, but was still the main metabolite. The percentage of *o*-cresol varied between 0.5 and 1.1% without any distinct tendency, whereas the percentage of *p*-cresol decreased gradually with increase in exposure. The real amount of *p*-cresol excretion did not obviously increase until exposure to 250 ppm toluene; above this level it increased with toluene concentration.

Table 1 Cumulative amount of urinary metabolites in 24 hours during and after a six hour exposure to different concentrations of toluene

Toluene (ppm)	Total	HA	F-BA	BG	<i>o</i> -Cresol	<i>p</i> -Cresol
<i>Control group</i>						
0	40.9 (100)	12.7 (2.9) (31.1)	2.4 (1.2) (5.9)	ND	ND	25.8 (10.8) (63.0)
50	175.9 (100)	146.3 (11.7) (83.2)	3.7 (1.4) (2.1)	ND	0.9 (0.1) (0.5)	25.0 (2.0) (14.2)
100	348.5 (100)	315.8 (62.5) (90.6)	5.1 (4.0) (1.5)	ND	1.7 (0.3) (0.5)	26.0 (3.5) (7.5)
250	594.1 (100)	545.6 (38.4) (91.8)	5.6 (2.4) (0.9)	ND	4.5 (0.9) (0.8)	38.4 (6.7) (6.5)
500	858.7 (100)	802.3 (126.8) (93.4)	7.5 (2.7) (0.9)	ND	5.8 (2.1) (0.7)	43.1 (5.7) (5.0)
1000	1221.7 (100)	1136.0 (94.6) (93.0)	19.7 (4.0) (1.6)	ND	12.5 (0.9) (1.0)	53.5 (12.7) (4.4)
2000	3121.1 (100)	2869.7 (252.2) (91.9)	61.7 (14.3) (2.0)	86.0 (6.0) (2.8)	25.8 (9.4) (0.8)	77.9 (5.8) (2.5)
4000	4050.9 (100)	3407.1 (504.1) (84.1)	122.3 (59.5) (3.0)	351.5 (32.9) (8.7)	45.6 (6.7) (1.1)	124.4 (13.8) (3.1)
<i>Ethanol group</i>						
0	61.0 (100)	18.3 (4.2)* (30.1)	0.9 (0.1) (1.4)	ND	ND	41.8 (7.0)* (68.5)
50	249.3 (100)	202.3 (46.9)* (81.1)	3.5 (1.4) (1.4)	ND	1.4 (0.1)* (0.6)	42.1 (11.2)* (16.9)
100	436.9 (100)	379.5 (14.5) (86.9)	3.9 (1.7) (0.9)	ND	2.0 (0.5) (0.5)	51.5 (3.9)* (11.8)
250	804.0 (100)	738.7 (111.1)* (91.9)	6.9 (0.7) (0.9)	ND	3.8 (0.5) (0.5)	54.6 (10.3)* (6.8)
500	1128.7 (100)	1040.1 (121.1)* (92.1)	17.2 (6.8)* (1.5)	11.1 (5.1) (1.0)	4.3 (0.5) (0.4)	56.0 (7.3)* (5.0)
1000	1599.1 (100)	1443.2 (156.7)* (90.3)	36.6 (19.2) (2.3)	44.6 (8.7) (2.8)	9.3 (1.4) (0.6)	65.4 (13.8) (4.1)
2000	3441.4 (100)	2914.5 (305.1)* (84.7)	118.3 (49.9)* (3.4)	275.1 (31.4)* (8.0)	35.2 (16.7) (1.0)	98.3 (8.6)* (2.9)
4000	5641.6 (100)	4355.0 (735.3)* (77.2)	167.9 (30.2) (3.0)	907.3 (58.7)* (16.1)	67.4 (14.6)* (1.2)	144.0 (22.6) (2.5)
<i>PB group</i>						
0	37.4 (100)	16.5 (1.2) (44.2)	1.9 (1.0) (5.0)	ND	ND	19.0 (10.7) (50.8)
50	200.3 (100)	175.5 (39.9) (87.6)	2.9 (1.2) (1.4)	ND	1.4 (0.2)* (0.7)	20.5 (9.9) (10.2)
100	436.3 (100)	405.8 (55.3)* (93.0)	5.1 (2.0) (1.2)	ND	2.6 (0.3)* (0.6)	22.8 (14.0) (5.2)
250	849.0 (100)	795.9 (117.5)* (93.7)	5.8 (3.1) (0.7)	ND	5.2 (0.6) (0.6)	42.1 (3.6) (5.0)
500	1477.8 (100)	1322.7 (42.0)* (89.5)	20.1 (9.0)* (1.4)	66.2 (2.0) (4.5)	10.3 (1.2)* (0.7)	58.5 (9.1) (4.0)
1000	2750.5 (100)	2353.8 (192.6)* (85.6)	81.0 (48.4)* (2.9)	213.3 (34.7) (7.8)	24.5 (7.4)* (0.9)	77.9 (6.3)* (2.8)
2000	5224.7 (100)	3430.4 (93.2)* (65.7)	294.0 (147.8)* (5.6)	1286.5 (57.5)* (24.6)	77.2 (4.7)* (1.5)	136.6 (5.7)* (2.6)
4000	3481.3 (100)	2232.8 (64.1)	48.5 (1.4)	957.6 (27.5)	89.9 (2.6)	152.5 (4.4)

The numbers ($\mu\text{mol/kg}$) represent the mean (SD) in five rats except for that of 4000 ppm exposure in PB group, where the mean for two rats is presented. Other values in parentheses are percentages of total metabolites.

HA = Hippuric acid; F-BA = free benzoic acid; BG = benzoyl glucuronide.

*Significantly different from that of respective control group ($p < 0.05$); ND = not detected.

Ethanol treatment increased the amount of HA, but did not express so much influence on the percentage of HA in total metabolites, with the exception of the two highest exposures (2000 and 4000 ppm) where the percentage decreased to 84.7 and 77.2% with a simultaneous increase in the percentage of BG to 8.0 and 16.1% respectively. The percentage of *p*-cresol was not apparently influenced by ethanol treatment, although the amount of this metabolite increased significantly at all exposures except to 1000 and 4000 ppm. The cumulative *o*-cresol excretion

was not affected by ethanol treatment except at exposures to 50 and 4000 ppm toluene.

Phenobarbital had a similar effect on the cumulative excretion of HA, F-BA, and BG. It increased the percentage of BG to as high as 24.6 and 27.5% at 2000 and 4000 ppm exposure respectively. By comparison with ethanol treatment, PB increased the cumulative amount of *o*-cresol significantly at almost all exposures and *p*-cresol excretion only at high toluene exposures. As in untreated rats, the excreted amount of *p*-cresol in 24 hours showed no

Table 2 The kinetic constants *in vivo* for urinary metabolites of toluene during six hour exposure*

Treatment	HA + F-BA + BG		<i>o</i> -Cresol		<i>p</i> -Cresol	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Control	182 600	394 664	407	6	86	15
Ethanol	522 882	1291 1342	414	8	122 431	29 43
PB	1634	3453	1622	30	729	62

*Values represent the mean determined from five rats.

HA = Hippuric acid; F-BA = free benzoic acid; BG = benzoyl glucuronide; K_m = Michaelis constant (ppm); V_{max} = maximal velocity ($\mu\text{mol/kg}$).

significant increase at low exposure concentrations (until 250 ppm).

Kinetics of toluene metabolism

Table 2 lists the Michaelis constants (K_m) (the concentration of toluene exposure at which excretion proceeds at half maximal velocity) and maximal velocities (V_{max}) (the maximum rate of metabolite excretion in urine during a six hour exposure), calculated using the least squares method. Three urinary metabolites, HA, F-BA and BG, were summed as one route of metabolism because they originated from the same intermediate of toluene, benzoic acid. For this side chain hydroxylation, two isozymes were calculated for control and ethanol treated groups. In the control group one had a low K_m (182 ppm), and the other a high K_m (600 ppm). In ethanol treated rats the K_m values for both isozymes were larger (522 and 882 ppm respectively) with higher V_{max} values. Only one enzyme was detected for the side chain hydroxylation in PB treated rats, and this had much greater K_m and V_{max} values.

Only one enzyme was detected for *o*-cresol (K_m , 407 ppm) in control rats, with a small V_{max} value. Ethanol treatment seemed to have little effect on this enzyme (K_m , 414 ppm). In PB treated rats, the enzyme involved in *o*-cresol metabolism showed a much larger K_m , and the V_{max} was also increased.

One enzyme was detected for *p*-cresol in control rats, with smaller K_m (85 ppm) and V_{max} (15 $\mu\text{mol/kg}$) in comparison with that of side chain metabolism. Ethanol treatment increased the V_{max} value by about 2.5 times, and furthermore, one more isozyme with a larger K_m value (515 ppm) was detected in this group at high toluene concentrations. In PB treated rats, there was one enzyme for *p*-cresol with a larger K_m (728 ppm) than that of the high K_m type in the ethanol group, indicating that PB may express an effect on *p*-cresol metabolism only at high toluene concentrations.

Discussion

Benzyl alcohol is the most essential metabolite in *in vitro* toluene metabolism.^{4,5} Most of this intermediate

is transformed to benzoic acid and excreted in urine as HA after conjugation with glycine *in vivo*.^{11,12} This was confirmed in the present experiment with a wide range of exposure concentrations of toluene.

The enzyme that metabolises toluene at its side chain to benzyl alcohol and eventually to benzoic acid has been confirmed in previous experiments to be cytochrome P-450.^{4,5} The activity of this enzyme is intimately correlated with the excreted amounts of metabolites from side chain transformation. Ethanol and PB treatment in the present study, for example, greatly influenced the amount of HA, F-BA, and BG in urine through the enhancement of isozyme activity. Therefore, it could be said that the transformation of toluene to benzyl alcohol catalysed by cytochrome P-450 is the rate limiting step in the side chain metabolism of toluene.

In the present study, with wide toluene concentrations, an increase in benzoic acid after β -glucuronidase treatment of a urine sample was found when the exposure concentration of toluene was raised to 2000 ppm in control rats. This result gives indirect supporting evidence that benzoic acid could conjugate with glucuronic acid, as well as glycine, to form a separate metabolite from HA at higher exposures to toluene. Quistad *et al*¹³ recently reported that a small amount of benzoylcarnitine was detected in the urine of rats dosed with ¹⁴C benzoic acid. Whether the conjugate of benzoic acid with carnitine is included in the urinary metabolites, has to be confirmed.

We noted that BG could be detected when the cumulative amount of F-BA during six hours of exposure was more than 5 $\mu\text{mol/kg}$ in all the three groups. Fishbein¹⁴ and Riihimäki¹⁵ suggested that conjugation of benzoic acid with glucuronic acid occurs when glycine conjugation is saturated. We think that this conjugating reaction with glucuronic acid depends on the amounts of benzoic acid accumulated and the glycine conjugation reaction is not saturated. In agreement with this view excretion of HA still increased with toluene exposure concentration when BG was detected in urine. This may indicate the existence of an enzyme with a much larger K_m value mediated in the glucuronic acid

conjugating reaction, compared with the K_m value of glycine conjugating enzyme. The conjugating reaction of benzoic acid with glucuronic acid may be another step to limit the side chain metabolism of toluene, especially at high toluene concentrations.

The amount of F-BA in urine increased with the increase in toluene concentration, so F-BA could be thought of as another metabolite of toluene, although its amount was minute compared with HA.

Treatment with ethanol enhanced the excretion of HA, as reported elsewhere.¹⁶ At low toluene concentration—for example, 250 ppm—the amount of HA in urine during six hours of exposure was increased more than twofold by ethanol, whereas at high exposure—for example, 2000 ppm—it increased only 1.5-fold. Ethanol treatment had little effect on V_{max} and K_m of isozyme for *o*-cresol. At toluene exposures up to 1000 ppm, urinary *o*-cresol was not increased by ethanol treatment. At high toluene concentrations, the amount of *o*-cresol excreted in six hour urine was significantly increased in the ethanol group, but the amount in 24 hour urine was not increased except at 4000 ppm. Ethanol increased *p*-cresol excretion. From the result of blood determinations, it was found that ethanol treatment increased toluene clearance from blood only when the concentration in blood was not high. These results indicate that treatment with ethanol enhances the side chain hydroxylation of toluene, especially at low toluene concentrations; increases ring oxidation at the *p* position; and has little effect on *o*-cresol metabolism.

Phenobarbital treatment induced an isozyme for side chain metabolism with large K_m and V_{max} values. It significantly increased HA excretion. For *o*-cresol metabolism, PB treatment also expressed a significant promoting effect at all exposure concentrations. For ring metabolism at the *p* position, it showed an effect only at high toluene concentration. These results were in agreement with in vitro experiments.⁴

A variety of chemicals including organic solvents have been reported to be subject to perfusion limited metabolism at low exposure concentrations¹⁷ (the metabolism rate is controlled by blood flow to the liver, and not influenced by enzyme induction). In this study, even after exposure to low toluene concentrations, the excretion of HA and F-BA, and *o*-cresol in six hour urine was significantly increased by ethanol and PB treatments (fig 3A, B, D), although the two treatments did not promote toluene clearance from blood below 250 ppm exposure (fig 1). This suggests that the metabolism of toluene is perfusion limited only at very low exposure concentrations.

Sato and Nakajima¹⁸ reported that PB treatment increases the activity of UDP-glucuronyltransferase in rat liver, and ethanol treatment also increased the activity of the enzyme although the difference was not significant. Yost and Finley¹⁹ found a significant induction of UDP-glucuronyltransferase activity by

ethanol in rabbits. In this study, the excretion of BG was promoted in both ethanol and PB treated rats. This may be explained with increase in the production of benzoic acid, which is one of the substances of the enzyme and the increased activity of UDP-glucuronyltransferase involved in the conjugation of benzoic acid with glucuronic acid.

The excretion of *p*-cresol in urine did not show an increase with increasing exposure concentration when toluene concentration was low (50 and 100 ppm) in the three groups (table 1). We investigated the cause in vitro with liver microsomes of rats. It has been found that *p*-cresol was cleared from the reaction system by the microsome mediated metabolism, and a new peak was detected in the HPLC chromatogram (data not shown). This phenomenon was more significant with microsomes from ethanol and PB treated rats. A similar finding was observed in urine samples of rats. This means that *p*-cresol is not the end metabolite. The available five isomers of dihydroxytoluene (DHT: 2,3-DHT, 2,5-DHT, 2,6-DHT, 3,4-DHT, and 3,5-DHT) were used in an attempt to identify the substance but it was not any of these isomers. The identification of the new metabolite is therefore to be further undertaken.

With monoclonal antibodies to different types of cytochrome P-450, we found that cytochromes P-450IIE1 and P-450IIC11²⁰ are mainly responsible for the formation of BA and *p*-cresol at high- and low affinity sites of toluene respectively.⁵ Cytochrome P-450IIE1 is also an ethanol inducible enzyme.²¹ PB inducible P-450IIB contributes to the formation of the three metabolites. The low K_m isozyme for side chain metabolism in this experiment in control and ethanol treated rats may be P-450IIE1, although the K_m value in the ethanol group is larger. The high K_m isozymes in the two groups may reflect P-450IIC11, which is also the constructive cytochrome P-450 in microsomes as P-450IIE1 and which acts mainly at high toluene concentrations. Similarly, the low (86–122) and high (431) K_m isozymes in *p*-cresol formation may be cytochrome P-450IIE1 and P-450IIC11 respectively. The isozyme responsible for *o*-cresol formation in control and ethanol treated rats is not identified yet. The PB induced enzyme that acts in the metabolism of the side chain and in the formation of *o*- and *p*-cresol is cytochrome P-450IIB.

Three of the five rats died at exposure to 4000 ppm toluene in the PB group. With PB treatment, the clearance of the solvent was significantly enhanced (fig 1) suggesting that it is a metabolite of toluene that mediates the toxicity. At this exposure, the most remarkable occurrence was a significant decrease in the excretion of side chain metabolites compared with that at exposure to 2000 ppm and this excretion was even less than that of ethanol treated rats. These results may indicate the accumulation of acidic side chain metabolites. Exposure to a high concentration

of toluene has been reported to lead to metabolic acidosis.²² It seems that the death of rats in our present study may be related to life threatening metabolic acidosis as a result of high concentration of acids such as HA and F-BA in PB treated rats. The mechanism whereby acidosis may be caused and how renal function is involved, requires further study.

Hippuric acid has been used in the biological monitoring of people exposed to toluene.²³ The physiological presence of HA in the urine of non-exposed subjects originating from food, drugs etc reduces its importance as an index of biological monitoring, especially at low exposure.²⁴ Some investigators have attempted to use *o*-cresol as the monitoring index.¹² In this experiment, *o*-cresol showed a relatively specific, constant small amount of excretion in urine (0.5–1.1% of total metabolites), and was hardly influenced by ethanol. On the other hand, HA corresponded to 92% of total metabolites even at low exposure to toluene. The correlation coefficient between HA in six hour urine and toluene concentration (0.90) was not inferior to that between *o*-cresol and toluene concentration (0.89). Studies in human subjects showed that the coefficient between HA and toluene was larger than that between *o*-cresol and toluene concentration.^{11, 25} Taking these advantages of HA into consideration, it could be suggested that HA is still a valuable index of biological monitoring of exposure to toluene, and it may be more reasonable to determine HA and free benzoic acid in urine than HA only, at low exposure.

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