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KINETIC STUDIES ON BENZENE METABOLISM IN RAT LIVER—POSSIBLE PRESENCE OF THREE FORMS OF BENZENE METABOLIZING ENZYMES IN THE LIVER

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Abstract—The effects of food deprivation, ethanol consumption and phenobarbital (PB) administration on *in vitro* benzene metabolism in rat liver were studied by using benzene concentration ranging from 0.0055 to 6.25 mM. The kinetic analysis suggested that the liver of normally-fed rats contained two forms of benzene hydroxylases each with a K_m value of 0.01 mM or 0.07 mM. The isozyme with a K_m of 0.01 mM disappeared following one-day food deprivation, but the deprivation enhanced the activity of the other isozyme. Ethanol treatment markedly increased the activity of both normally-existing enzymes. On the other hand, PB treatment induced the synthesis of another benzene-metabolizing enzyme with a high K_m value of 4.5 mM, the presence of which was indistinct in normal rats. The treatment had no influence on the activity of either of the normally-occurring low-K_m isozymes. The combined effects of PB with food deprivation were additive, suggesting that the induction of low- and high-K_m isozymes is each independent of the other.

We have recently demonstrated that phenobarbital (PB) has no influence on the metabolism of benzene, although it causes a marked increase both in microsomal protein and cytochrome P-450 contents in the liver of rats [1, 2], whereas food deprivation [3, 4] or ethanol consumption [5–7] markedly enhances the metabolism with little or no increase in microsomal protein and cytochrome P-450 concentrations. This finding is in sharp contrast to those of others that indicate that benzene hydroxylase activity is increased by treating animals with PB [8–11]. The benzene concentrations we used in the metabolism assay system greatly differ (0.035–0.113 mM) from those of others (2–10 mM). This suggests that (1) there exist at least two forms of benzene-metabolizing enzymes in liver microsomes; one preferentially acts at low benzene concentrations (low-K_m form) and the other at high benzene concentrations (high-K_m form), and (2) ethanol consumption or food deprivation induces isozymes with low-K_m values, while the major type of PB-induced isozymes has high-K_m values toward benzene metabolism. The present report deals with metabolism studies *in vitro* which suggest that at least three forms of enzymes, each with distinct kinetic property for benzene, are involved in the metabolism.

MATERIALS AND METHODS

Rats. Male Wistar strain rats were kept individually in stainless steel wire-bottom cages in an air-conditioned room (20 ± 2°) with artificial lighting from 6 a.m. to 6 p.m. They were maintained on pellet food (Clea CE-2, Nippon Clea, Tokyo) and water *ad libitum*. At 8 weeks of age, they were switched to two different liquid diets; one group of rats to a well-balanced liquid diet (basal diet) and the other group to an ethanol-containing diet (ethanol diet). Basal diet contained casein 3.47 g, oil (a mixture of corn and olive oils) 2.79 g, sucrose 9.72 g, L-cystine 41 mg, DL-methionine 25 mg, ethyl linoleate 212 mg, vitamin mixture 407 mg, DL-α-tocopherol 2 mg, mineral mixture 813 mg and carragheenin 212 mg per 80 ml (1 kcal/ml). Ethanol diet had the same composition as the basal diet except that the sucrose content was reduced to 1.74 g with addition of 2.0 g ethanol. Rats were given one of these liquid diets (80 ml each) daily at 4 p.m. as the only source of food and water. The food was consumed by both groups of rats by 10 a.m. on the next day. Three weeks later, rats were killed by decapitation at 10 a.m. and the liver was removed. A 25% (w/v) liver homogenate in 1.15% KCl was centrifuged at 10,000 *g* for 10 min. The supernatant fraction was aerated with N_2 and stored frozen at −85° until use. Before killing, one half of the rats kept on a basal diet were administered PB (80 mg/kg/day) for 4 days each at 10 a.m. (PB-treated rats). The last dose of PB preceded killing by 24 hr. On the day before they were killed, one half of both the PB-treated and untreated rats were given 80 ml of water in place of their daily food (fasted rats). Thus, we used 5 groups of rats in all, each consisting of 10 (5 for measurement of metabolic rate and 5 for a kinetic study): fed group (rats maintained on the basal diet until death), fast group (rats deprived of food on the day before killing), PB-fed group (rats maintained on the basal diet until death and treated with PB for 4 days), PB-fast group (rats treated with PB for 4 days and deprived of food on the day before killing) and ethanol group (rats maintained on ethanol diet until death).

Benzene metabolism assay. Benzene metabolism

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was assessed by measuring both the rate of substrate (benzene) disappearance and the rate of product (phenol and hydroquinone) formation. The rate of benzene disappearance was determined according to the method of Sato and Nakajima [12] with a slight modification that the final volume of the reaction mixture was reduced to 1 ml consisting of 0.5 ml of enzyme (10,000 *g* liver supernatant fraction), 0.3 ml of cofactor solution and 0.2 ml of substrate solution. The reaction mixture contained, at the final concentration, 0.5 mM NADP, 10 mM glucose 6-phosphate, 25 mM $MgCl_2$, 50 mM K-phosphate buffer (pH 7.4), and 0.0055–0.2 mM benzene.

For measuring the rate of product formation, the same incubation system as that in the substrate disappearance measurement was used. After a 10-min incubation, 3 ml of 0.3 N H_2SO_4 was added to the incubation mixture to stop the reaction. Five milliliters of ethyl ether was then measured into the incubation vessel, and shaken vigorously for 10 min with a vertical shaking machine. The mixture was centrifuged at 1800 *g* for 5 min to facilitate separation of the organic from the aqueous layers. Three-and-a-half milliliters of the ether phase were transferred to a test tube and the ether was evaporated by placing the tube in a thermoregulated water bath (37°) for 15 min. After evaporation, the residue was dissolved in 0.5 ml of acetonitrile/water (3/7) solution, and 50 μl of the solution was injected into a high-performance liquid chromatograph (HPLC) with a UV detector (Hitachi 633A). The analytical condition was the same as described elsewhere [13] except for the use of a solution of acetonitrile/water (1/9) as the mobile phase. Standard solutions were made by adding a known quantity of phenol or hydroquinone into incubation mixtures which were devoid of NADPH-generating system. The peak area thus obtained was essentially linear to the concentration. The mean recovery ratio of phenol and hydroquinone was 97% and 85%, respectively. The amount of these metabolites formed were confirmed before the enzyme assay to increase linearly with time for at least 10 min of incubation and the rate of benzene metabolism was proportional to enzyme concentration.

Microsomal protein and cytochrome P-450 contents. A portion of liver 10,000 *g* supernatant fraction was further centrifuged at 105,000 *g* for 60 min to obtain microsomal pellets. The protein content was measured according to the method of Lowry *et al.* [14] as modified by Miller [15]. The concentration of cytochrome P-450 was determined by the spectrophotometric method of Omura and Sato [16].

Statistics. Means were tested by Student *t*-test. The 0.05 level of probability was the criterion of significance.

RESULTS

Microsomal protein and cytochrome P-450

PB markedly increased the protein content as shown in Table 1. Ethanol also caused a significant increase, but food deprivation had no influence on the content, findings which are in good agreement with the previous report [1]. Combination of PB-treatment with one-day food deprivation did not augment the increases caused by PB-treatment alone. Microsomal cytochrome P-450 content was increased by PB and ethanol, and slightly by the one-day fast. The fast seemed to enhance the effect of PB.

Table 1. Microsomal protein and cytochrome P-450 contents

Treatment*	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmol/mg protein)
Control	26.9 ± 1.4†	0.64 ± 0.04
Fasted	25.1 ± 1.5	0.74 ± 0.03‡
PB-fed	33.7 ± 3.0‡	1.31 ± 0.15‡
PB-fasted	37.1 ± 1.9‡	1.55 ± 0.03‡
Ethanol	31.0 ± 3.6‡	1.17 ± 0.02‡

* Control, rats were maintained on the basal diet until death; Fasted, rats on the basal diet were deprived of food on the day before killing; PB-fed, rats maintained on the basal diet were treated with PB for 4 days; PB-fasted, rats treated with PB for 4 days were deprived of food on the day before killing; Ethanol, rats were maintained on the ethanol diet until death.

† The values represent the mean ± SD for 5 rats.

‡ Significantly different ($P < 0.05$) from control.

Benzene metabolism

Benzene metabolism was assessed at three benzene concentrations, 0.02, 0.23 and 6.26 mM. The metabolism at 0.02 mM was assayed by measuring the rate of substrate disappearance, that at 6.26 mM by measuring the rate of product formation, and that at 0.23 mM by measuring both the substrate and products. Overnight fasting and ethanol consumption elevated the metabolism at all concentrations studied. On the other hand, PB had no influence on the metabolism assessed either at 0.02 or 0.23 mM. The rate of metabolism per unit concentration of microsomal protein or cytochrome P-450 was even lower than the rate determined with enzymes from normally-fed control rats, which suggests that PB-treatment rather suppresses benzene metabolism at these lower substrate concentrations. By contrast, at a higher concentration of 6.26 mM, PB markedly increased the metabolism per unit weight of liver, to almost 4-fold that of control rats. This tendency was also noted even when the rate of metabolism was expressed on the basis of unit microsomal protein or cytochrome P-450 concentration.

When PB-treated rats were fasted overnight, the treatment achieved a high metabolic rate at all benzene concentrations compared with the rate determined with enzymes from control rats. However, no significant difference was noted in the rates measured at 0.02 and 0.23 mM between PB-treated, fasted and PB-non-treated, fasted rats. The former group of rats showed a higher rate than the latter only at the highest benzene concentration of 6.26 mM. Comparison between PB-treated fed and fasted rats indicates that the enhancement at higher benzene concentration is primarily due to PB-treatment whereas the increase at lower concentrations is exclusively produced by fasting itself. This also suggests that the effects of PB and fasting may be independent of each

Table 2. Metabolic rates of benzene at varying substrate concentrations

Treatment*	0.02 mM†	0.23 mM†	0.23 mM‡	6.26 mM‡
		nmol/g liver/min		
Control	11.7 ± 2.2§	18.4 ± 4.9	10.1 ± 0.3	11.2 ± 1.0
Fasted	16.0 ± 1.8¶	30.0 ± 7.9¶	23.5 ± 3.3¶	22.4 ± 3.5¶
PB-fed	11.0 ± 0.5	18.7 ± 5.1	8.5 ± 0.7	44.5 ± 8.4¶
PB-fasted	18.1 ± 2.4¶	40.2 ± 6.4¶	23.0 ± 1.4¶	63.8 ± 14.1¶
Ethanol	50.9 ± 11.5¶	140.1 ± 27.9¶	94.8 ± 10.0¶	94.2 ± 6.7¶
		nmol/mg protein/min		
Control	0.43 ± 0.06	0.68 ± 0.15	0.37 ± 0.03	0.41 ± 0.04
Fasted	0.62 ± 0.12¶	1.32 ± 0.26¶	0.91 ± 0.10¶	0.87 ± 0.11¶
PB-fed	0.32 ± 0.04¶	0.54 ± 0.09	0.25 ± 0.01¶	1.32 ± 0.33¶
PB-fasted	0.48 ± 0.07	1.07 ± 0.14¶	0.62 ± 0.05¶	1.71 ± 0.45¶
Ethanol	1.67 ± 0.51¶	4.51 ± 0.61¶	2.91 ± 0.35¶	2.90 ± 0.36¶
		nmol/nmol cytochrome P-450/min		
Control	0.69 ± 0.10	1.08 ± 0.27	0.60 ± 0.05	0.66 ± 0.06
Fasted	0.83 ± 0.20	1.76 ± 0.30¶	1.22 ± 0.08¶	1.16 ± 0.09¶
PB-fed	0.25 ± 0.01¶	0.42 ± 0.11¶	0.19 ± 0.02¶	1.01 ± 0.26¶
PB-fasted	0.31 ± 0.04¶	0.69 ± 0.10¶	0.40 ± 0.03¶	1.10 ± 0.28¶
Ethanol	1.42 ± 0.44¶	3.86 ± 0.63¶	2.47 ± 0.31¶	2.47 ± 0.35¶

* See the legend* in Table 1.

† Benzene metabolism was assessed by measuring the rate of substrate disappearance.

‡ Benzene metabolism was assessed by measuring the rate of product (phenol plus hydroquinone) formation.

§ The values represent the mean ± SD for 5 rats.

¶ Significantly different ($P < 0.05$) from control.

other and may be additive when combined (Table 2).

Table 3 shows the rates of phenol and hydroquinone formation determined at benzene concentrations of 0.23 and 6.26 mM. Undoubtedly, phenol was a major metabolite of benzene in all groups of rats. Only a small amount of hydroquinone was noted in the liver of control rats. Hydroquinone seemed to be formed preferentially in the liver of food-deprived or ethanol-treated rats especially at a lower benzene concentration (0.23 mM). The ratio of hydroquinone to phenol decreased with increase in the concentration (6.26 mM). However, no difference was noted in the rate of total product formation (phenol plus hydroquinone) between the two benzene concentrations, suggesting that the reaction with enzymes from fasted and ethanol-treated rats may be saturated at about 0.23 mM. On the other hand, the PB-induced enhancement of benzene metabolism at 6.26 mM was reflected in the formation rate of both products. This also suggests that PB-induced isozymes may have a different catalytic property toward phenol hydroxylation from ethanol- or food deprivation-induced isozymes.

Kinetic study of benzene metabolism

Benzene metabolism was assessed at several concentrations by measuring the rate of substrate disappearance, and the reciprocal of velocity was plotted against the reciprocal of concentration (Fig. 1). Table 4 shows the V_{max} (maximal velocity) and K_m (Michaelis constant) obtained from these double reciprocal plots with the least-square method. At substrate concentrations ranging from 0.0055 to 0.200 mM, two definitely different K_m values, 0.008 (0.007–0.009 mM) and 0.06 mM (0.056–0.063 mM), were obtained with enzymes from control, PB-treated (fed), and ethanol-treated rats. Ethanol treatment markedly increased the V_{max} of both reactions related to these two K_m values. However, PB-treatment affected neither of the V_{max}. In contrast, the enzymes from fasted rats, with or without PB-treatment, seemed to have only one K_m value of 0.06 mM (0.059–0.061 mM), which was coincident with the larger one of K_ms obtained with the control, ethanol-treated or PB-treated fed rats. The smaller K_m enzyme had disappeared by overnight fasting, which suggests that fasting may break down or degrade the low K_m-associated isozyme.

When the benzene concentration was raised above 0.2 mM, the rate of metabolism assessed with substrate disappearance measurement was unreliable. Therefore, the rate was determined by measuring the amount of phenol and hydroquinone formed during the incubation period using substrate concentrations from 0.05 to 6.26 mM, and the rates were plotted against the concentrations double-reciprocally (Figs 2 and 3). Within this range, enzymes from control, fasted and ethanol-treated rats all had the same K_m value of around 0.08 mM (0.077–0.083 mM), a value almost in agreement with the higher K_m obtained by the substrate disappearance measurement. Food deprivation and ethanol administration clearly increased the V_{max} of reaction catalyzed by this enzyme. It should be stressed here that V_{max} determined by measuring the rate of product formation was always lower than that by measuring the rate of substrate disappearance. It is partly because the major metabolite of benzene, phenol, is also a good substrate for the cytochrome P-450-related reaction [2, 13, 17].

On the other hand, the double-reciprocal plots of

Table 3. Phenol and hydroquinone formed at substrate concentrations of 0.23 mM and 6.26 mM

Treatment*	nmol/mg protein/min		
	Phenol	Hydroquinone	Total
		0.23 mM	
Control	0.32 ± 0.04 (86.4)†	0.05 ± 0.01 (13.6)	0.37 ± 0.03
Fasted	0.53 ± 0.06 (58.9)‡	0.37 ± 0.07 (41.1)‡	0.90 ± 0.10‡
PB-fed	0.20 ± 0.01 (80.0)‡¶	0.05 ± 0.01 (20.0)¶	0.25 ± 0.01‡¶
PB-fasted	0.26 ± 0.02 (54.2)‡¶	0.22 ± 0.03 (45.8)‡¶	0.48 ± 0.05‡¶
Ethanol	1.87 ± 0.44 (64.3)‡¶	1.04 ± 0.44 (35.7)‡¶	2.91 ± 0.35‡¶
		6.26 mM	
Control	0.38 ± 0.04 (92.7)	0.03 ± 0.01 (7.3)	0.41 ± 0.04
Fasted	0.74 ± 0.10 (86.0)‡	0.13 ± 0.03 (14.0)‡	0.86 ± 0.11‡
PB-fed	1.03 ± 0.28 (78.0)‡	0.29 ± 0.05 (22.0)‡¶	1.32 ± 0.33‡¶
PB-fasted	1.05 ± 0.20 (61.8)‡¶	0.65 ± 0.19 (38.2)‡¶	1.70 ± 0.45‡¶
Ethanol	2.65 ± 0.46 (91.4)‡¶	0.25 ± 0.11 (8.6)‡	2.90 ± 0.36‡¶

* See the legend * in Table 1.

† Mean ± SD for 5 rats. Figures in the parentheses show the mean percentage of total metabolites (phenol plus hydroquinone).

‡ Significantly different ($P < 0.05$) from control.

¶ Significantly different ($P < 0.05$) from fasted.

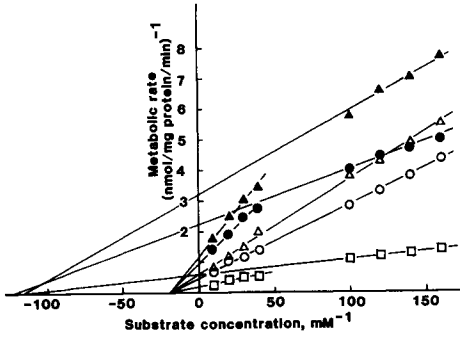

Fig. 1. Double-reciprocal plots of metabolic rate versus substrate concentration (substrate disappearance measurements). ●: Control, rats were maintained on the basal diet until death; ○: Fasted, rats on the basal diet were deprived of food on the day before killing; ▲: PB-fed, rats maintained on the basal diet were treated with PB for 4 days; △: PB-fasted, rats treated with PB for 4 days were deprived of food on the day before killing; □: Ethanol, rats were maintained on the ethanol diet until death. Each point represents the mean for 5 rats.

benzene metabolism assessed with the PB-induced enzymes showed a completely different pattern from the others (Figs 2 and 3). There seemed to exist another isozyme with a K_m value of 4.44–4.65 mM. Thus, the enzymes from both fed and fasted PB-treated groups had two K_m values within the concentration range of 0.05 mM and 6.26 mM. The smaller value fell in the same category as the one obtained with control, fasted, and ethanol-treated rats. The reaction with enzymes from these PB-nontreated rats was almost saturated around 0.4 mM of benzene. In the case of PB-induced enzymes, however, benzene metabolism was elevated abruptly at a benzene concentration of 1 mM, and saturation was not seen even at the concentration of 6.26 mM.

These findings suggest that, as far as the kinetic property is concerned, at least three forms of enzymes are involved in the *in vitro* metabolism of benzene, each having a definitely different K_m value of 0.01, 0.07 or 4.5 mM. Ethanol and overnight fasting preferentially induce the enzymes with a lower K_m, whereas PB the high-K_m-associated enzymes.

DISCUSSION

Post and Snyder [18] first demonstrated the existence of two different forms of benzene hydroxylases each having distinct catalytic properties toward benzene metabolism. One form, induced by benzene treatment, had the highest activity at a substrate concentration of 0.8 mM or below, and the other having affinity for benzene at 0.8 mM or higher, was mainly induced by PB-treatment. The present study shows that the liver of normally-fed rats originally contains two forms of isozymes for benzene metabolism with a K_m value of 0.01 mM or 0.07 mM. The isozyme with 0.01 mM K_m disappeared after one-day food deprivation, whereas the deprivation enhanced the activity of the 0.07 mM K_m isozyme. Ethanol treatment markedly increased the activity of both the normally-existing enzymes. PB-treatment of rats added another form of enzyme the presence of which was indistinct in normal rats. This PB-induced isozyme had an extremely high K_m of 4.5 mM, and was almost inactive at the lower benzene concentration where naturally-occurring isozymes showed a high activity.

The reports on the effect of PB-treatment on the metabolism of benzene are conflicting. Many investigators reported that the treatment enhanced benzene hydroxylation *in vitro* [8–11] and the others noted no influence on the metabolism [1, 2]. Part of this discrepancy may derive from the fact that

Table 4. Michaelis constant (K_m) and maximum velocity (V_{max}) for benzene metabolism*

Treatment†		Substrate disappearance	Product formation
Control	K_m	0.01 mM	
	V_{max}	0.43 nmol/mg protein/min	
	K_m	0.06 mM	0.08 mM
	V_{max}	1.00 nmol/mg protein/min	0.41 nmol/mg protein/min
Fasted	K_m	0.06 mM	0.08 mM
	V_{max}	1.67 nmol/mg protein/min	1.25 nmol/mg protein/min
PB-fed	K_m	0.01 mM	
	V_{max}	0.32 nmol/mg protein/min	
	K_m	0.06 mM	0.08 mM
	V_{max}	0.91 nmol/mg protein/min	0.37 nmol/mg protein/min
	K_m		4.44 mM
	V_{max}		1.67 nmol/mg protein/min
PB-fasted	K_m	0.07 mM	0.08 mM
	V_{max}	1.43 nmol/mg protein/min	0.77 nmol/mg protein/min
	K_m		4.65 mM
	V_{max}		3.13 nmol/mg protein/min
Ethanol	K_m	0.01 mM	
	V_{max}	1.72 nmol/mg protein/min	
	K_m	0.06 mM	0.08 mM
	V_{max}	5.00 nmol/mg protein/min	4.55 nmol/mg protein/min

* Cf. Figs 1–3.
† See the legend * in Table 1.

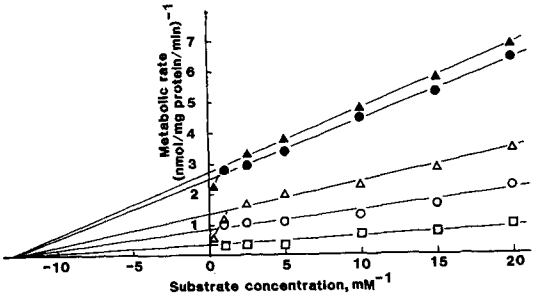

Fig. 2. Double-reciprocal plots of metabolic rate versus substrate concentration (product formation measurements). ●: Control, rats were maintained on the basal diet until death; ○: Fasted, rats on the basal diet were deprived of food on the day before killing; ▲: PB-fed, rats maintained on the basal diet were treated with PB for 4 days; △: PB-fasted, rats treated with PB for 4 days were deprived of food on the day before killing; □: Ethanol, rats were maintained on the ethanol diet until death. Each point represents the mean for 5 rats.

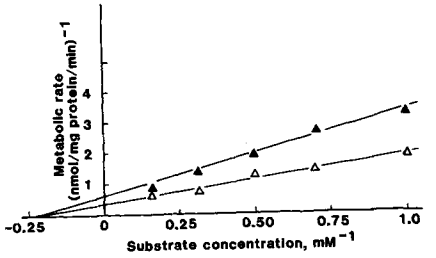

Fig. 3. Double-reciprocal plots of metabolic rate versus substrate concentration (product formation measurements). ▲: PB-fed, rats maintained on the basal diet were treated with PB for 4 days; △: PB-fasted, rats treated with PB for 4 days were deprived of food on the day before killing. Each point represents the mean for 5 rats.

substrate concentrations employed in the *in vitro* metabolism assay are different; for example, those who insisted on the negative effect used low benzene concentrations of 0.035–0.113 mM, whereas the concentrations in papers reporting the positive effect were much higher (2.0–10.0 mM). As clearly indicated in the present study, the major form of PB-induced isozymes has a low affinity for this hydrocarbon, i.e. PB-treatment manifests its enhancing effect only at higher substrate concentrations. This conclusion is further supported by the *in vivo* and *in vitro* metabolism study of Gut [10] who, demonstrating PB-induced enhancement of benzene metabolism *in vitro* (substrate concentration, perhaps above 6 mM), was unable to notice any effect on the metabolism *in vivo* as evidenced by no significant changes in the urinary excretion of phenol following i.p. injection of benzene at a dose of 3–3.5 mmol/kg. The maximum benzene concentration attained by this dose was as low as 0.4 mM. On the other hand, Timbrell and Mitchell [19] reported the PB-induced enhancement of benzene metabolism *in vivo* by using a dose three times larger than that used by Gut [10].

In our own experiment [2] where rats were exposed to 500 ppm of benzene for 2 hr, ethanol treatment evidently increased the *in vivo* metabolism as evidenced by accelerated disappearance of benzene from the blood as well as by elevated urinary excretion of phenol. However, PB-treatment had little or no influence on the metabolism. The highest concentration of benzene in the blood achieved immediately after exposure ranged from 0.1 to

0.2 mM. In addition, ethanol ingestion aggravated haemopoietic toxicity of benzene, whereas PB-treatment tended to protect animals from the toxicity. A low-K_m type of isozyme induced by ethanol was supposed to be preferentially active to a PB-induced high-K_m type at this lower concentration. Considering the actual level of human benzene exposure, the low-K_m type of isozymes may be more important than the high-K_m isozyme.

An isozyme of cytochrome P-450 with physicochemical and catalytic properties distinct from that induced by PB or 3-methylcholanthrene has been isolated and purified from the liver of ethanol-treated rabbits [20]. This newly-isolated isozyme has been shown to have a high activity toward aniline [20] and nitrosamine [21]. We have demonstrated, using a substrate concentration from 0.026 to 0.127 mM, that both food deprivation [3, 4] and ethanol consumption [5–7] enhances *in vitro* metabolism in rat liver of a variety of volatile hydrocarbons including benzene. This suggests, by analogy with benzene, that the microsomal enzymes induced by both treatments may also have a low-K_m value for such chemicals as are characterized by volatility, lipophilicity and low molecular weight. Similarly, food deprivation may stimulate the synthesis of a low-K_m form of cytochrome P-450 having a specifically high activity for these chemicals as suggested by Tu and Yang [22] with aniline and nitrosamine.

Most investigators assessed benzene metabolism by measuring the rate of phenol formation [8, 10, 11, 23]. However, as phenol is a much better substrate for the cytochrome P-450 reaction than benzene [2, 13], phenol generated from benzene in the reaction system readily disappears to be further metabolized into hydroquinone or catechol [11, 24]. In addition, a considerable part of these metabolites are covalently bound to microsomal proteins [25]. Therefore, the rate of benzene metabolism assessed by phenol measurement does not necessarily indicate the actual rate of the metabolism: the rate of phenol formation is always lower than the rate of benzene disappearance, as clearly indicated by the results shown in Tables 2 and 3. To assess the metabolism exactly, all the metabolites of benzene, i.e. phenol, hydroquinone, benzoquinone, etc. must be determined. This is tedious and sometimes impractical. In the case of benzene metabolism which is mainly catalyzed by a low-K_m isozyme, measurement of the rate of substrate disappearance may be preferable to the measurement of product formation.

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