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CHANGES IN CYTOCHROME P450 MOLECULAR SPECIES IN RAT LIVER IN CHLOROFORM INTOXICATION*

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Abstract—The effect of $CHCl_3$ on the composition of hepatic microsomal cytochrome P450 species was compared with that of CCl_4 in rats pretreated with phenobarbital (PB) or 3-methylcholanthrene (3MC). The administration of $CHCl_3$ hardly affected cytochrome P450 content in non-treated rat liver, but caused a similar degree of depletion in the content as observed after CCl_4 administration in PB-pretreated rats. In the pretreatment with 3MC, the administration of $CHCl_3$ brought about a marked decrease in the content to 24% of control after 12 hr, while CCl_4 reduced the content only to one-half of control.

It was demonstrated by SDS–polyacrylamide gel electrophoresis and Whatman DE-52 anion-exchange chromatography that 3MC-induced P450 species decreased with $CHCl_3$, while it was affected little by CCl_4 treatment. The activity of benzo[*a*]pyrene hydroxylase was altered together with the change in the content of cytochrome P450 species. The administration of $CHCl_3$ to PB-pretreated rats caused the depletion in PB-induced P450. These findings indicate that cytochrome P450 species induced with 3MC as well as PB are highly susceptible to $CHCl_3$ intoxication, whereas the administration of CCl_4 depletes the PB-induced species without affecting the 3MC-induced species.

A number of studies have provided much evidence that the hepatotoxicity of $CHCl_3$ as well as CCl_4 is mediated through the metabolic activation [1–3]. Although $CHCl_3$ has been reported to be less hepatotoxic than CCl_4 [4, 5], a marked enhancement in the toxicity of $CHCl_3$ was observed in the response to the pretreatment with inducers of cytochrome P450 [6, 7]. While specific form(s) of cytochrome P450 induced with phenobarbital (PB)§ have been proposed to be sensitive to CCl_4 intoxication [8, 9] and to be involved in the hepatotoxicity of CCl_4 [10, 11], the mechanism for the bioactivation of $CHCl_3$ has not been resolved clearly enough.

In the present paper, *in vivo* effect of $CHCl_3$ on the composition of cytochrome P450 species induced with PB or 3MC was compared with that of CCl_4, and the change in the composition was demonstrated to be reflected in the drug-metabolizing activities.

MATERIALS AND METHODS

Reagents. [G-^{3}H]Benzo[*a*]pyrene was purchased from Amersham, U.K. and Emulgen 911 was generously supplied from Kao Atlas Co. Ltd, Japan. Cholic acid (guaranteed reagent) purchased from Nakarai Chemicals Ltd, Japan was recrystallized twice from 50% ethanol using activated charcoal and celite, and dried to constant weight *in vacuo*.

Animal treatment. Male Wistar rats (100–140 g) were pretreated by intraperitoneal injections of PB sodium salt in 0.9% NaCl solution (80 mg/kg/day) for 2 days or a single dose of 3MC in corn oil (50 mg/kg). Control animals were given the vehicle only. Before the administration of $CHCl_3$ or CCl_4, the animals were fasted overnight with water *ad libitum*. Twenty-four hours after the last injection of PB or 48 hr after the injection of 3MC, $CHCl_3$ or CCl_4 dissolved in an equal volume of corn oil were given intraperitoneally. Control rats received corn oil alone. As our primary objective was to compare the mechanism for metabolic activation of $CHCl_3$ with that of CCl_4, we selected a relatively high dose, with which we could observe an apparent difference between the two toxicants.

Preparation of microsomes. Livers were excised from decapitated rat immediately, washed with ice-cold saline, and homogenized in 4 vol. of 1.15% KCl. The homogenate was centrifuged at 12,000 *g* for 10 min and the supernatant was centrifuged again at the same condition. Then, the supernatant was centrifuged at 105,000 *g* for 60 min. The resulting pellet was suspended in 20 ml of 1.15% KCl and recentrifuged at 105,000 *g* for 30 min. The washed pellet was used as microsomal fraction.

Assay of drug-metabolizing component. Cytochrome P450 content was determined by the method of Omura and Sato [12]. Aminopyrine demethylase and aniline hydroxylase activities were determined according to the method of Ishidate *et al.* [13]. Benzo[*a*]pyrene hydroxylase was measured by the

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§ Abbreviations used: PB, phenobarbital; 3MC, 3-methylcholanthrene.

method of Van Cantfort *et al.* [14] and the activity was calculated according to the method of Hayakawa and Udenfriend [15].

Solubilization of liver microsomes. Cholic acid was dissolved in 0.1 M potassium phosphate buffer (pH 7.4) containing 30% glycerol and 1.0 mM EDTA to make a 10% (w/v) solution. The solution was added slowly to the microsomes suspended in the same buffer with stirring at 4° to make a final concentration of 1% cholic acid and 10 mg/ml microsomal protein. Ten milliliters of the microsomal suspension was stirred for 15 min and then sonicated at a power setting 50 with Artek dismembrator using microtip probe for 30 sec. The suspension was centrifuged at 105,000 *g* for 60 min and the fraction recovered in the supernatant was designated as "solubilized microsomes".

Analysis of cytochrome P450 molecular species. The chromatography was carried out according to the method of Ryan *et al.* [16] with a slight modification. The solubilized microsomes containing 50 mg protein was dialyzed in Visking tube (20/32) overnight against 500 ml of 0.01 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM dithiothreitol, 0.2% Emulgen 911 and 0.5% sodium cholate. The dialyzed samples were adjusted to 15 ml with the phosphate buffer to apply to Whatman DE-52 column (2 × 8 cm) equilibrated previously with 80 ml of the buffer. The column was washed with 20 ml of the buffer, and then eluted with a linear gradient of NaCl (0.0–0.5 M) in 100 ml of the buffer solution. The flow rate was maintained at 10–12 ml/hr and 1.8 ml fractions were collected. Each fraction was monitored by absorbance at 417 nm to detect hemoproteins, and the cytochrome P450 content was determined as described above.

Other methods. Protein concentration was determined by the method of Lowry *et al.* [17], using bovine serum albumin as standard. SDS–polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [18]. The stacking gel contained 3.9% acrylamide and the separating gel contained 10% acrylamide.

The values are expressed as mean ± standard deviation (S.D.) and statistical comparisons were made with Student's *t*-test.

RESULTS

The administration of 1.0 ml/kg $CHCl_3$ did not show any lethal effect and a significant change in cytochrome P450 content in non-pretreated rats (Fig. 1A), while the same dose of $CHCl_3$ caused most of the rats pretreated with PB or 3MC to die by 6 hr after the injection. Therefore, the dose of $CHCl_3$ was reduced to 0.5 ml/kg thereafter and the same dose of CCl_4 was used to compare the effect with that of $CHCl_3$. A marked decrease in the content of cytochrome P450 in the liver from PB-pretreated rats occurred in a similar manner with $CHCl_3$ and CCl_4 (Fig. 1B). In the 3MC-pretreated group, on the other hand, the content decreased to one-half by 3 hr with either $CHCl_3$ or CCl_4. The level was retained thereafter in the CCl_4 intoxication, whereas the content in the $CHCl_3$-intoxicated rat liver continued to decrease gradually and reached 24% of control after 12 hr (Fig. 1C).

The drug-metabolizing activities were measured 3 hr after the treatment with the hepatotoxins (Fig. 2). The administration of CCl_4 reduced aminopyrine demethylase and aniline hydroxylase activities without affecting benzo[*a*]pyrene hydroxylase activity in the liver from rats, either non-treated or pretreated with PB or 3MC. Although the administration of $CHCl_3$ did not impair aminopyrine demethylase, aniline hydroxylase, and benzo[*a*]pyrene hydroxylase activities in non-pretreated rats, these three activities decreased with $CHCl_3$ in PB- or 3MC-pretreated animals. It was noticeable that $CHCl_3$ reduced significantly benzo[*a*]pyrene hydroxylase activity induced by 3MC-pretreatment, while CCl_4 exhibited no significant effect on the activity. Although the loss of cytochrome P450 content in 3MC-pretreated rats was a similar degree 3 hr after the administration of $CHCl_3$ and CCl_4 (Fig. 1C), the drug-metabolizing activities were shown to be altered in a different way. Figure 3 shows a time course of the effect of the two hepatotoxins on drug-metabolizing activities. Aminopyrine demethylase and aniline hydroxylase activities reduced in a similar manner after the administration of $CHCl_3$ or CCl_4. Benzo[*a*]-pyrene hydroxylase activity, on the other hand, decreased gradually to 35% of control 12 hr after

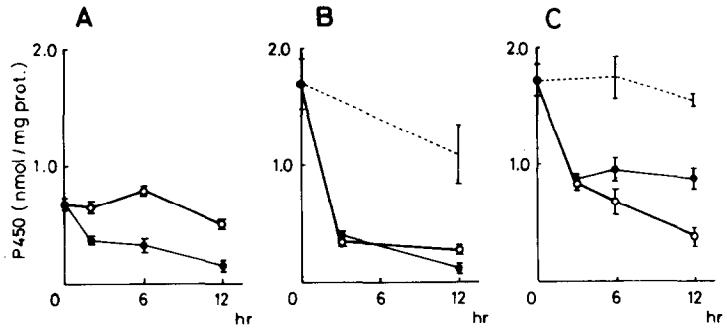

Fig. 1. Effects of chloroform or carbon tetrachloride administration on cytochrome P450 content in rat liver. Rats were pretreated with saline (A), PB (B) and 3MC (C). $CHCl_3$ or CCl_4 (1.0 ml/kg (A), 0.5 ml/kg (B and C)) were injected intraperitoneally. Experimental details are described in the text. Dotted line indicates the respective controls and open symbol and closed symbol indicate the values from $CHCl_3$- and CCl_4-treated rats, respectively. Each point represents the mean ± S.D. of three rats.

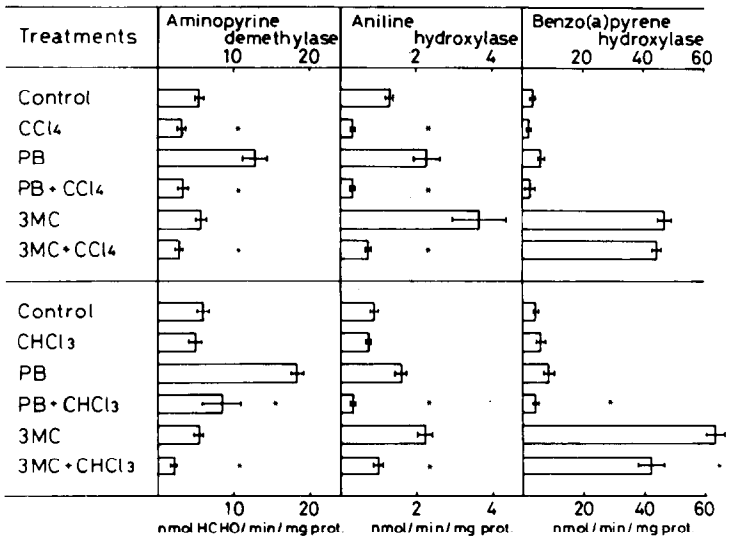

Fig. 2. Effects of chloroform or carbon tetrachloride administration on microsomal drug-metabolizing activities. Rats were pretreated with PB or 3MC and injected 0.5 ml/kg of $CHCl_3$ or CCl_4. Drug-metabolizing activities were determined 3 hr after the administration. Experimental details are shown in the text. Each point represents the mean ± S.D. of three rats. * Significantly different from control ($p < 0.05$).

$CHCl_3$ intoxication, while it hardly declined with CCl_4 until 12 hr.

The changes in the composition of cytochrome P450 species in microsomes were exhibited in a CO-reduced difference spectrum (data not shown). The peak was located at 448 nm in the 3MC-treated rat liver microsomes, representing the spectral property of 3MC-induced P450. After the administration of $CHCl_3$, the peak shifted from 448 to 450 nm by the depletion of 3MC-induced species. In the case of the administration of CCl_4, only the height decreased and the peak still remained at 448 nm because of the conservation of 3MC-induced P450.

SDS–polyacrylamide gel electrophoresis also provided another evidence for the different effect of $CHCl_3$, and CCl_4 on 3MC-induced species of cytochrome P450 (Fig. 4). The induction of two polypeptide bands of 54 kDa and 56 kDa was observed in the liver microsomes by the treatment with 3MC. The band of 56 kDa was faint 12 hr after the treatment with $CHCl_3$, whereas it was retained with little change by the administration of CCl_4.

The microsomal cytochrome P450 was separated by DE-52 anion-exchange chromatography. Prior to application to a column, solubilization of the microsomes with cholic acid was attempted. Table 1 shows the recovery of microsomal protein and cytochrome P450 in the soluble portion. While microsomal protein and cytochrome P450 isolated from PB- or 3MC-pretreated animals were almost completely solubilized, the recovery was decreased considerably by the administration of the hepatotoxins. The damage caused by the administration of $CHCl_3$ was especially noted.

The chromatography of solubilized microsomes prepared from the liver of control rat gave two major peaks (I, II) and a minor peak (III) of cytochrome P450 by the elution at NaCl concentrations of 0, 100

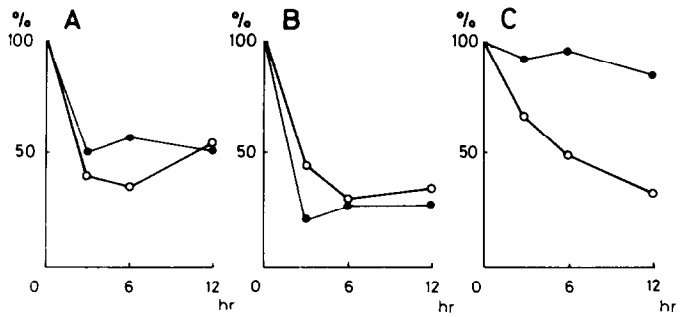

Fig. 3. Time course of drug-metabolizing activities of liver microsomes from 3MC-pretreated rats. Rats were pretreated with 3MC and administered $CHCl_3$ or CCl_4 (0.5 ml/kg) as described in the text. Drug-metabolizing activities: aminopyrine demethylase (A), aniline hydroxylase (B) and benzo[*a*]pyrene hydroxylase (C) were determined after the administration of $CHCl_3$ or CCl_4. Mean values obtained from three rats were represented as percentage of control. Open and closed symbols indicate the values from $CHCl_3$ and CCl_4 administered rats, respectively.

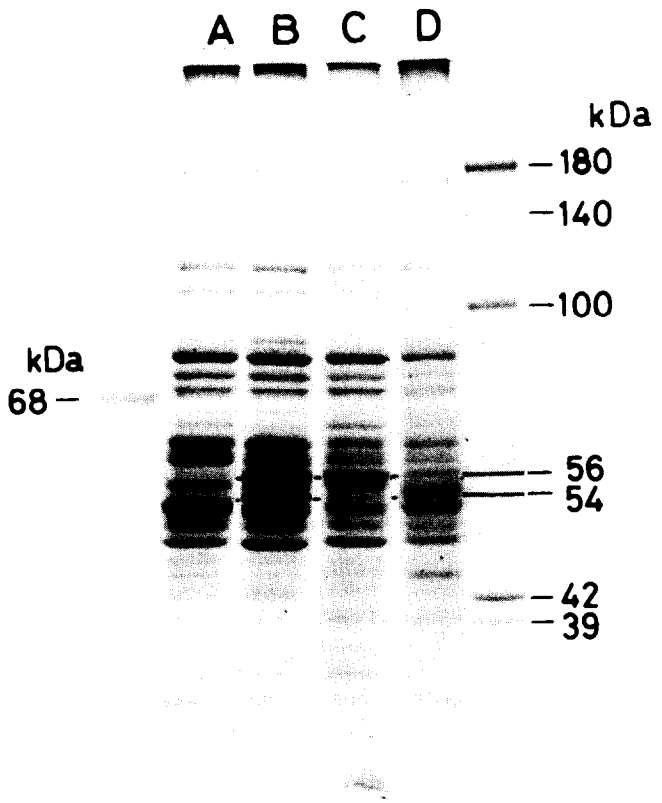

Fig. 4. SDS–polyacrylamide gel electrophoresis of liver microsomes. Rat liver microsomes were suspended in 0.125 M Tris–HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.001% bromophenol blue and heated in boiling water for 2 min. Forty micrograms of protein was applied to each lane. Lane (A) and (B) are microsomes from non-treated and 3MC-pretreated rats, lane (C) and (D) are microsomes prepared from 3MC-pretreated rat 12 hr after the administration of 0.5 ml/kg CCl_4 and $CHCl_3$, respectively. Molecular mass was determined by comparison with known standards consisting of subunits of RNA polymerase B (39, 42, 100, 140 and 180 kDa) and bovine serum albumin (68 kDa).

Table 1. Recovery in the cholate solubilization of microsomal protein and cytochrome P450

Treatment	Microsomal protein (%)	Cytochrome P450 (%)
3MC	97 ± 6	101 ± 3
3MC + $CHCl_3$	55 ± 3	69 ± 6
3MC + CCl_4	85 ± 5	88 ± 4
PB	91 ± 1	98 ± 5
PB + $CHCl_3$	48 ± 4	68 ± 4
PB + CCl_4	73 ± 1	90 ± 1

The liver microsomes were prepared from 3MC or PB pretreated rats 12 hr after the administration of $CHCl_3$ or CCl_4 and treated with cholate solution as described in the text. The values represent the percentages of the solubilized and total amounts and the mean ± S.D. of three rats.

and 180 mM, respectively (Fig. 5A). Treatment with 3MC or PB resulted in a marked increase in peak III which was the major component in both microsomes (Fig. 5B, C). Peak III induced with 3MC and that induced with PB corresponded to the polypeptides of 56 kDa and 52 kDa estimated by SDS–polyacrylamide gel electrophoresis, and showed peaks situated at 447 and 452 nm in a CO-reduced difference spectrum, respectively. Peak I or peak II exhibited the absorption maximum at 449 or 450 nm. The fourth peak of hemoprotein, which did not exhibit a CO-reduced spectrum, is regarded as cytochrome b_5 from its spectral property. In the liver microsomes from 3MC-pretreated rat, peak III occupied approximately 67% of hepatic microsomal cytochrome P450. By the administration of CCl_4, the ratio of peak III increased to 76% in spite of a slight decrease in the amount, suggesting that 3MC-induced P450 was more resistant than other P450 species against CCl_4 intoxication (Fig. 5D). In contrast, $CHCl_3$ administration brought about a great decrease in peak III induced with 3MC. (Fig. 5F).

In PB-pretreated rat liver, an increase in peak II and III was observed and peak III occupied 52% of total P450. By the administration of $CHCl_3$ or CCl_4, all peaks were greatly decreased. Especially, peak III almost completely disappeared in CCl_4 intoxication (Fig. 5E, G).

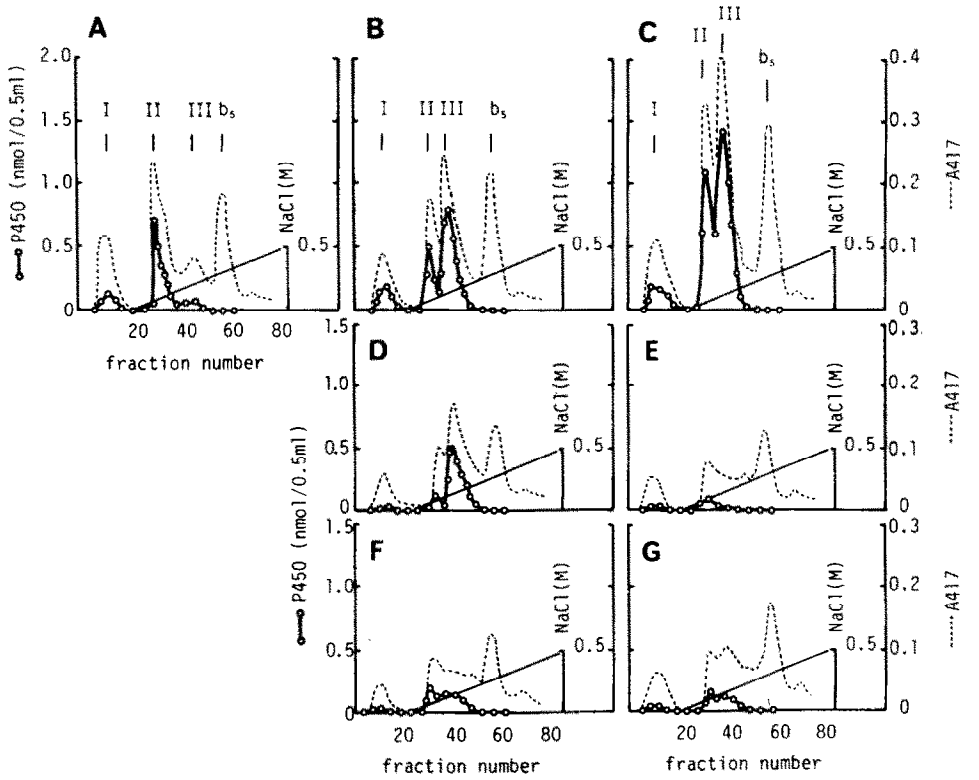

Fig. 5. Whatman DE-52 anion-exchange chromatography of rat liver microsomes. Rats were pretreated with 3MC or PB and given 0.5 ml/kg of CCl_4 or $CHCl_3$. The liver microsomes were prepared from non-treated rat (A), 3MC-treated rat (B), PB-treated rat (C), 3MC-treated rat 12 hr after the administration of CCl_4 (D) and $CHCl_3$ (F), and PB-treated rat 12 hr after the administration of CCl_4 (E) and $CHCl_3$ (G). Fifty milligrams of solubilized microsomes was dialyzed and applied to a column. Experimental details are described in the text. Solid and dotted lines represent cytochrome P450 and hemoprotein contents, respectively.

DISCUSSION

Active metabolites of halogenomethanes produced by cytochrome P-450-dependent reaction have been shown to interact with macromolecules of physiological importance in the liver [3, 9, 11, 19, 20], probably resulting in a loss of cytochrome P450 by release from the microsomal membrane or by formation of large aggregates which could not be solubilized even by treatment with detergent. In the present study, hepatic content of cytochrome P450 was shown to be decreased to 24% of the control 12 hr after the administration of $CHCl_3$ to 3MC-pretreated rats. By SDS–polyacrylamide gel electrophoresis and Whatman DE-52 column chromatography, 3MC-induced species of cytochrome P450 was demonstrated to be deleted distinctly by $CHCl_3$ treatment. Since this species occupied over one-half of total P450 species after induction with 3MC, the decrease in cytochrome P450 was presumed to be mainly owing to the reduction of this species. Cytochrome P450 content in the liver from PB-pretreated rats was also decreased by the administration of $CHCl_3$ as well as CCl_4. The change was due to the deletion in the PB-induced species as shown by the column chromatography.

The alteration in drug-metabolizing activities was compatible with change in the content of 3MC- or PB-induced species of cytochrome P450. Aminopyrine demethylase activity induced by PB-pretreatment and aniline hydroxylase activity induced by PB- and 3MC-pretreatment were impaired by the administration of either $CHCl_3$ or CCl_4. The activity of benzo[*a*]pyrene hydroxylase induced with 3MC [21] was injured considerably by the intake of $CHCl_3$, but affected little by CCl_4.

Sadano and Omura [22] proposed that the turnover of 3MC-induced species of cytochrome P450 was more rapid than that of other drug-metabolizing components in the microsomes, and estimated the half-life to be approximately 15 hr. Although it is possible to assume that the decrease in 3MC-induced P450 was due to non-specific damage of microsomal membrane and the subsequent inhibition of protein synthesis caused by active metabolites of $CHCl_3$, the finding that benzo[*a*]pyrene hydroxylase activity was reduced to 35% of control 12 hr after administration could not be explained only by the turnover rate of the cytochrome. Therefore, the membrane damage was presumed to be initiated from cytochrome P450.

Noguchi *et al.* [9] demonstrated that treatment with CCl_4 resulted in an early loss of PB-induced species of cytochrome P450 in rat liver, while β-naphthoflavone-induced 56 kDa form was not

affected. The present experiment also showed that 56 kDa form of cytochrome P450 induced with 3MC was affected little by the treatment with CCl_4. Noguchi *et al.* [10] further proposed that CCl_4 can be converted to the active metabolites by PB-induced species of cytochrome P450 and the participation of β-naphthoflavone-induced species is distinctly excluded. The present results demonstrated clearly that the 3MC-induced species is highly susceptive to $CHCl_3$ intoxication. Assuming that the active metabolites of $CHCl_3$ denature cytochrome P450 species which is involved in the activation process as was postulated for CCl_4, it is suggested that $CHCl_3$ is activated with both PB- and 3MC-induced species of cytochrome P450. It has been shown that the hepatotoxicity of CCl_4 is enhanced by PB-pretreatment [23, 24], while that of $CHCl_3$ is potentiated by either PB- or 3MC-pretreatment [7]. The different effect of the pretreatment on the toxicities of the two hepatotoxins could be explained by the present findings. The precise mechanism for the manifestation of hepatotoxicity of $CHCl_3$ is now under investigation.

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