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INDUCTION OF A HIGH SPIN FORM OF MICROSOMAL CYTOCHROME P-448 IN RAT LIVER BY 4-AMINOAZOBENZENE DERIVATIVES*

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Abstract—Male Sprague–Dawley rats were treated with 4-aminoazobenzene derivatives or other drug metabolizing enzyme inducers such as phenobarbital, 3-methylcholanthrene and isosafrole. The expression of hepatic microsomal cytochrome P-450 of the rats, principally that of a high spin form of cytochrome P-448 (cytochrome P-448H), was assessed by a bacterial mutation test and by immunological methods. The results of the mutation test with use of *Salmonella typhimurium* TA98 and 3 aromatic amine substrates showed that 2-methoxyl, 3-methoxyl and 2′,3-dimethoxyl derivatives of AAB and methyl derivatives of AAB such as *o*-aminoazotoluene, *N*-methyl-4-aminoazobenzene and *N,N*-dimethyl-4-aminoazobenzene have a large capacity for the selective induction of cytochrome P-448H. Activity of the cytochrome increased by 6 hr after an azo dye treatment, reached a maximum after 24 hr, and then declined. In contrast, 4′-methoxy-AAB has a small, and AAB has no, capacity for the cytochrome induction. The aminoazo dye-induced enzymes differ in their substrate specificities from those induced with 3-methylcholanthrene or phenobarbital, and the induced enzyme was identified to be cytochrome P-448H, as determined by an enzyme-linked immunosorbent assay and immunoblotting with use of anti-cytochrome P-448 monoclonal antibodies. These observations indicate that several methoxyl and methyl derivatives of 4-aminoazobenzene are potent and selective inducers of cytochrome P-448H in the rat.

Cytochrome P-450 isozymes in a NADPH-dependent monooxygenase system catalyze the metabolism of a variety of chemicals, but they differ in their substrate specificities and molecular characteristics. Conversions of a large fraction of chemical carcinogens to carcinogenically or mutagenically active metabolites are found to be principally mediated by cytochrome P-448 isozymes which are inducible with 3-methylcholanthrene (MC), polychlorinated biphenyl, 5,6-benzoflavone or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. These chemicals induce at least two forms of hepatic cytochrome P-448, low spin form of cytochrome P-448 (P-448L‡) having an apparent molecular weight of 55,000–56,000 dalton (identical with Levin's cytochrome P-450c) and high spin form of cytochrome P-448 (P-448H) having an apparent molecular weight of 52,000–54,000 dalton (identical with Levin's cytochrome P-450d) [1–5].

Kamataki *et al.* [1] and we [6] have studied the metabolic changes of several carcinogenic aromatic amines and demonstrated that these amines are converted to mutagenically or carcinogenically proximate metabolites via catalysis of cytochrome P-448H [1, 6]. Therefore, to investigate the role of the cytochrome in aromatic amine carcinogenesis, it is desirable to obtain selective cytochrome P-448H inducers. Isosafrole is known as such a cytochrome P-448H inducer, but the cytochrome P-448H induced in liver appears to be inactivated by the binding with the metabolites of isosafrole [4]. Our earlier studies have indicated that a hepatocarcinogenic 3-methoxy-4-aminoazobenzene (3-MeO-AAB) [7] is also a selective cytochrome P-448H inducer, as demonstrated by bacterial mutation test and immunological assays using anti-cytochrome P-448 monoclonal antibodies (MoAbs) [6].

In the present study, we examined the relationship between the chemical structure of AAB derivatives and their activity in inducing hepatic microsomal cytochrome P-448H. We herein report that several methyl and methoxyl derivatives of AAB are potent and selective inducers of microsomal cytochrome P-448H in rats, although their activities as the cytochrome P-448H inducer do not always correlate with their hepatocarcinogenic potencies.

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‡ Abbreviations used: cytochrome P-448H, high spin form of cytochrome P-448 (identical with Levin's cytochrome P-450d); cytochrome P-448L, low spin form of cytochrome P-448 (identical with Levin's cytochrome P-450c); PB, sodium phenobarbiturate; MC, 3-methylcholanthrene; AAB, 4-aminoazobenzene; Me-AAB, methyl-4-aminoazobenzene; MeO-AAB, methoxy-4-aminoazobenzene; MAB, *N*-methyl-4-aminoazobenzene; DAB, *N,N*-dimethyl-4-aminoazobenzene; OAT, *o*-aminoazotoluene; Trp P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate; Glu P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole acetate; SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; MoAb(s), monoclonal antibody (antibodies); Protein A-ELISA, protein A-enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline, pH 7.2; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Chemicals. 3-Amino-1-methyl-5*H*-pyrido(4,3-*b*)indole acetate (Trp P-2) and 2-amino-6-methyldipyrido(1,2-a:3′,2′-*d*)imidazole acetate (Glu P-1) were kindly donated by Drs T. Sugimura and M. Nagao, National Cancer Center Research Institute, Tokyo, Japan. SKF 525-A was a gift from Smith, Klein, & French Inc., Philadelphia, PA. 7,8-Benzoflavone, MC, isosafrole and *o*-aminoazotoluene (OAT) were obtained from Wako Pure Chemical Industries, Osaka. 4-Aminoazobenzene (AAB), *N*-methyl-4-aminoazobenzene (MAB), *N,N*-dimethyl-4-aminoazobenzene (DAB), 3′-methyl-DAB (3′-Me-DAB) and sodium phenobarbiturate (PB) were purchased from Tokyo Kasei Kogyo, Tokyo. 2-Methoxy-4-aminoazobenzene (2-MeO-AAB), 3-methoxy-4-aminoazobenzene (3-MeO-AAB), 4′-methoxy-4-aminoazobenzene (4′-MeO-AAB) and 2′,3-dimethoxy-4-aminoazobenzene (2′,3-diMeO-AAB) were synthesized in our laboratory as previously reported [7, 8–10]. All aminoazo dyes used were purified by alumina column chromatography followed by recrystallization from ethanol.

Preparation of liver microsomes. Male Sprague–Dawley rats of 7–9 weeks old were obtained from Shizuoka Agricultural Corporation for Laboratory Animals, Hamamatsu, Japan. The rats were treated intraperitoneally with an aminoazo dye (0.11 or 0.22 mmoles/kg), MC (0.11 mmoles/kg) or isosafrole (0.22 mmoles/kg) in corn oil. PB (0.22 mmoles/kg) was given as physiological saline solution. Animals were sacrificed, in general, 24 hr after the treatment.

Hepatic microsomes was prepared by differential centrifugation as described in a previous paper [11]. The contents of protein and cytochrome P-450 in the liver microsomes were assayed by the methods of Lowry *et al.* [12] and Omura and Sato [13], respectively.

Activity of drug metabolizing enzymes. Activities of aniline *p*-hydroxylase and aminopyrine *N*-demethylase were assayed by the methods of Cochin and Axelrod [14] and Imai *et al.* [15], respectively.

Mutation test. Trp P-2 (2 nmoles/plate), Glu P-1 (2 nmoles/plate) or 3-MeO-AAB (100 nmoles/plate) was incubated with microsomes for 20 min at 37° and then assayed for the mutagenicity against *Salmonella typhimurium* TA98 as described in a previous paper [11].

Immunochemical characterization of induced cytochrome P-450. Monoclonal antibodies (MoAbs) used for immunoassays were APH-3 and APH-8 anti-rat cytochrome P-448 MoAbs prepared in our laboratory [16]. APH-3 MoAb is selective to hepatic microsomal cytochrome P-448H of rats, whereas APH-8 MoAb is reactive with both cytochrome P-448H and cytochrome P-448L. Both MoAbs, however, react little with other cytochrome P-450 isozymes [16].

For enzyme-linked immunosorbent assay (ELISA), microsomal preparations (0.1 mg of microsomal protein/well) were fixed to wells of Costar No. 2590 polyvinyl chloride strips by incubating at 4° overnight and then successively treated with each 100 μl of the following solutions: (1) 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), (2) MoAb, (3) rabbit anti-mouse immunoglobulin (Zymed Laboratory Inc., San Francisco, CA) in 1% BSA-PBS, (4) horseradish peroxidase-conjugated protein A, and (5) citrate buffer solution (pH 4.0) containing 0.05% 2,2′-azino-di(3-ethylbenzthiazoline)-6-sulfonic acid (Sigma) and 0.01% H_2O_2 [16]. Optical density of the developed color was measured at 414 nm using an InterMed NJ-200 automatic ELISA reader.

Immunoblots of microsomal cytochrome P-450. The method has been described in detail in our previous report [16]. Briefly, hepatic microsomes were solubilized with sodium dodecyl sulfate (SDS) and developed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred from the gels to nitrocellulose sheets and then immunostained with use of APH-8 MoAb, mouse peroxidase anti-peroxidase (Jackson Immuno Research Lab., Avondale, PA), and 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma).

RESULTS

Change of enzyme activity by treatment of rats with AAB derivatives

Hepatic microsomes were prepared from untreated or aminoazo dye-treated rats, and their activities for the mutagenic activation of carcinogenic aromatic amines were examined. We selected Trp P-2, Glu P-1 and 3-MeO-AAB as substrates, because these 3 compounds are found to show different susceptibilities to microsomal cytochrome P-450 isozymes in their mutagenic activation [1, 6, 17]. In all tests, mutagenic activities of Trp P-2, Glu P-1 and 3-MeO-AAB were dependent upon the dose of microsomes used, and the increase of the number of revertant bacterial colonies were linear in a range of 0.05–0.2 mg of microsomal protein per plate (data not shown).

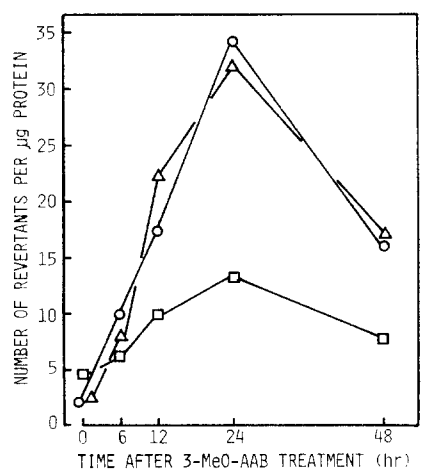

Fig. 1. Time-dependent changes of the activities of the hepatic microsomal aromatic amine activation enzyme(s) after 3-MeO-AAB treatment. The mutagenicity of Trp P-2, Glu P-1 or 3-MeO-AAB against TA98 bacteria was assayed in triplicate with use of microsomes (0.05–0.2 mg protein/plate) from the rats treated with 3-MeO-AAB (0.22 mmoles/kg) as described in Materials and Methods: ○, Trp P-2; △, Glu P-1; □, 3-MeO-AAB.

We first examined the relationship between the time after 3-MeO-AAB treatment and the change of microsomal enzyme activity for the mutagenic conversions of Trp P-2, Glu P-1 and 3-MeO-AAB (Fig. 1). The enzyme activities to all these 3 substrates were significantly increased by 6 hr after the injection of 3-MeO-AAB (0.22 mmoles/kg), reached a maximum after 24 hr, and declined thereafter. Similar changes were observed with the hepatic microsomes of rats treated with other AAB-derivatives such as OAT and 2',3-diMeO-AAB (data not shown).

We next examined the activities of various AAB derivatives for the induction of hepatic microsomal enzyme(s) which could catalyze the mutagenic activation of 3-MeO-AAB, Trp P-2 or Glu P-1 (Table 1). AAB did not induce such enzyme(s). By contrast, with the exception of 4'-MeO-AAB (less than 1.4-fold of the control), methoxyl derivatives of AAB such as 2-MeO-, 3-MeO- and 2',3-diMeO-AAB greatly induced the enzyme(s) active for the mutagenesis of Trp P-2 and Glu P-1 (12–18-fold of the control). As to the methyl derivatives, OAT and MAB were strongly (7–18-fold) and DAB was moderately (4–5-fold) active in terms of the enzyme induction. When 3-MeO-AAB was used as a substrate, the microsomal enzyme(s) induced with these aminoazo dyes were only weakly (2-MeO- and 3-MeO-AAB) or moderately (MAB and DAB) active for the mutagenic conversion.

As to the known drug metabolizing enzyme inducers, PB induced the enzymes which were potent to the 3-MeO-AAB mutagenesis (9-fold of the control) but weak to the Trp P-2 and Glu P-1 mutageneses (1.3- and 1.9-fold, respectively). In contrast, the MC-induced enzymes were strongly active to the mutageneses of both Trp P-2 (34-fold) and Glu P-1 (18-fold) and moderately active to the 3-MeO-AAB mutagenesis (4-fold). The microsomal enzyme(s) induced by treatment with isosafrole, a selective cytochrome P-448H inducer, was only slightly active to the mutageneses of Trp P-2 (1.7-fold) and Glu P-1 (1.6-fold). Although the AAB derivatives and the drug metabolizing enzyme inducers could induce the enzymes which mediated the mutagenic conversion of Trp P-2 and Glu P-1, the induced enzymes appeared to differ in their substrate specificities; the aminoazo dye-induced enzyme(s) showed similar activities to Trp P-2 and Glu P-1, but the MC-induced enzymes were more active to Trp P-2 than to Glu P-1 (see Table 1).

The form of aminoazo dye-induced enzyme(s) was examined by use of cytochrome P-450 inhibitors such as 7,8-benzoflavone (selective to cytochrome P-448) and SKF 525A (selective to cytochrome P-450) [18–20]. The activities of the 3-MeO-AAB-induced microsomes for the mutagenic conversions of Trp P-2 and Glu P-1 were almost completely inhibited (to less than 5% of the control) by addition of 7,8-benzoflavone (10 μM) to the assay mixture, but addition of 10 times larger amount of SKF 525-A resulted in only 30% inhibition, indicating that the induced enzyme(s) consist mainly of cytochrome P-448.

We further examined the effect of chemicals on the induction of aniline *p*-hydroxylase and amino-

Table 1. Effect of chemical treatment of rats on microsome-mediated mutagenic activation of aromatic amines

Treatment of rat		No. revertant colonies/μg microsomal protein		
Chemical	Dose (mmol/kg)	3-MeO-AAB	Trp P-2	Glu P-1
		Expt. 1***		
Vehicle (corn oil)		4.4	5.8	6.3
AAB	0.22	NT	5.4 (0.9)	6.6 (1.0)
2-MeO-AAB	0.22	7.1 (1.6)	79.4 (13.7)	98.2 (15.6)
3-MeO-AAB	0.11	NT	49.7 (8.6)	51.2 (8.1)
3-MeO-AAB	0.22	7.8 (1.8)	76.5 (13.2)	97.7 (15.5)
OAT	0.22	NT	95.9 (16.5)	112.3 (17.8)
Isosafrole	0.22	NT	9.9 (1.7)	10.1 (1.6)
PB	0.22	37.7 (8.6)	7.8 (1.3)	11.9 (1.9)
		Expt. 2***		
Vehicle (corn oil)		4.8	2.4	2.1
3-MeO-AAB	0.22	NT	27.5 (11.5)	31.2 (14.9)
4'-MeO-AAB	0.22	NT	3.4 (1.4)	2.8 (1.3)
2',3-diMeO-AAB	0.22	NT	42.8 (17.8)	36.2 (17.2)
MAB	0.22	15.4 (3.2)	17.5 (7.3)	19.9 (9.5)
DAB	0.22	11.8 (2.5)	12.3 (5.1)	9.4 (4.5)
3'-Me-DAB	0.22	NT	10.5 (4.4)	11.0 (5.2)
MC	0.11	19.1 (4.0)	80.5 (33.5)	38.7 (18.4)

Hepatic microsomes were prepared from rats treated with a chemical 24 hr before, and their activities for the mutagenic conversion of aromatic amines were assayed as described in Materials and Methods.

A dose–response curve of the mutation was made with use of 4 doses of the microsomes (0.05 to 0.2 mg/plate; linear dose-response range) and a substrate (3-MeO-AAB, 100 nmoles/plate; Trp P-2, 2 nmoles/plate; Glu P-1, 2 nmoles/plate). The values shown are means of triplicated samples. In all experiments, S.E.M. was less than 10% of the mean. In parenthesis is shown a ratio to the control (vehicle alone). NT, not tested.

pyrine *N*-demethylase in liver. Hepatic microsomes were obtained from the rats treated with an aminoazo dye (AAB, 2-MeO-AAB, 3-MeO-AAB or OAT), MC, isosafrole or PB and assayed for their total cytochrome P-450 content and enzyme activities. The total cytochrome P-450 content in the microsomes was slightly (1.4-fold of the control, $P < 0.01$) increased by the treatment with 3-MeO-AAB but not with other aminoazo dyes. Aniline *p*-hydroxylase was induced by the treatment with 2-MeO-AAB (2.1-fold of the control), 3-MeO-AAB (2.8-fold) or OAT (2.1-fold), but not with AAB. By contrast, all these aminoazo dyes did not induce aminopyrine *N*-demethylase. Both MC and PB increased the cytochrome P-450 content and the activities of aniline *p*-hydroxylase and aminopyrine *N*-demethylase, but isosafrole increased aniline *p*-hydroxylase activity only.

Immunochemical characterization of aminoazo dye-induced cytochrome P-450

Characteristics of induced microsomal cytochrome P-450 were determined by means of Protein A-ELISA and immunobolts with use of APH-3 and APH-8 anti-rat cytochrome P-448 MoAbs. We first examined the relationship between the period after *in vivo* treatment with 3-MeO-AAB (0.22 mmoles/kg) and the quantitative changes of the microsomal components reactive with these MoAbs (Fig. 2). The components reactive with either APH-3 or APH-8 MoAb increased by 6 hr after the 3-MeO-AAB treatment, increased further up to 24 hr, and then declined. In any time period, APH-3 MoAb showed greater reactivity with the microsomes than did APH-8 MoAb. The kinetics pattern observed in this experiment resembles that of the microsomal activities in the bacterial mutation test (see Fig. 1).

We then examined the reactivities of MoAbs with induced microsomal components. Microsomes were obtained 24 hr after chemical treatment of rats and

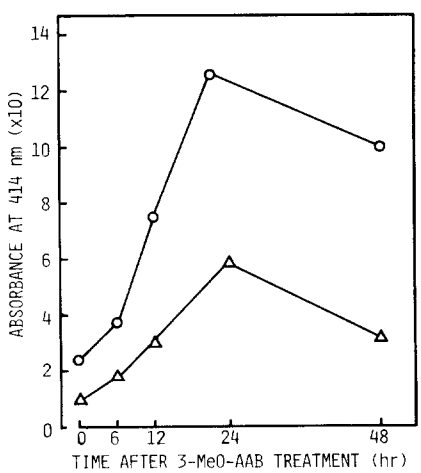

Fig. 2. Time-dependent changes of the reactivity of microsomal component(s) from the 3-MeO-AAB-treated rats with anti-rat cytochrome P-448 MoAbs. Hepatic microsomes were periodically obtained from the rats treated with 3-MeO-AAB (0.22 mmoles/kg). The reactivities of the microsomes with MoAb were assayed in triplicate by Protein A-ELISA as described in Materials and Methods: ○, APH-3 MoAb; △, APH-8 MoAb.

Table 2. Reactivities of hepatic microsomes with APH-3 and APH-8 anti-cytochrome P-448 monoclonal antibodies

Treatment of rat		Optical density at 414 nm	
Chemical	Dose (mmol/kg)	APH-3	APH-8
Vehicle (corn oil)		0.13	0.04
AAB	0.22	0.20 (1.5)	0.05 (1.3)
2-MeO-AAB	0.22	0.99 (7.6)	0.35 (8.8)
3-MeO-AAB	0.22	1.15 (8.8)	0.62 (15.5)
4′-MeO-AAB	0.22	0.24 (1.8)	0.09 (2.2)
2′,3-diMeO-AAB	0.22	1.64 (12.6)	0.62 (15.5)
OAT	0.22	1.06 (8.2)	0.55 (13.2)
MAB	0.22	0.71 (5.5)	0.31 (7.8)
DAB	0.22	0.51 (3.9)	0.22 (5.5)
3′-Me-DAB	0.22	0.40 (3.1)	0.13 (3.3)
Isosafrole	0.22	1.15 (8.8)	0.30 (7.5)
MC	0.11	1.19 (9.2)	1.10 (27.5)
PB	0.22	0.05 (0.4)	0.07 (1.8)

Microsomes were prepared from rats treated with a chemical 24 hr before and assayed for their reactivity with APH-3 and APH-8 monoclonal antibodies as described in Materials and Methods. In parenthesis is shown a ratio to the control (vehicle alone).

assayed for the reactivity with the APH-3 or APH-8 MoAb by means of Protein A-ELISA (Table 2). As compared to the microsomes from untreated rats, AAB and 4′-MeO-AAB produced a slight increase in the reactivity of the microsomes with the MoAbs (1.3–2.2-fold of the controls), 3′-Me-DAB increased moderately (about 3-fold), and the other aminoazo dyes (2-MeO-AAB, 3-MeO-AAB, 2′,3-diMeO-AAB, OAT, MAB and DAB) greatly increased the reactivity. As to the known drug metabolizing enzyme inducers, both MC and isosafrole, but not PB, increased the reactivity of the microsomes with the MoAbs.

We next determined the molecular characteristics of the induced cytochrome P-450 by means of immunoblots using the anti-cytochrome P-448 MoAbs. A fixed amount of microsomal protein was applied on a gel sheet, developed by SDS-PAGE, and then transferred to a nitrocellulose sheet. The transferred proteins were immunostained with APH-8 MoAb (Fig. 3). In this experiment, APH-3 MoAb (selective to cytochrome P-448H) could not be employed, because the epitope recognized by this antibody is unstable to SDS-treatment.

All microsomal preparations from the aminoazo dye-treated rats gave a single immunostained protein band showing an apparent molecular weight of 54,000 dalton, which corresponds to the molecular weight of cytochrome P-448H [1, 6]. As revealed from the intensity of the immunostained band, treatment of rats with AAB or 4′-MeO-AAB appeared to not or a little affect the microsomal cytochrome P-448H content, but the other aminoazo dyes (2-MeO-AAB, 3-MeO-AAB, 2′,3-diMeO-AAB, OAT, MAB, DAB and 3′-Me-DAB) produced a great increase in the content. The microsomal preparation from the isosafrole-treated rats also gave an intense protein band at a position of cytochrome P-448H, whereas those from either untreated or PB-

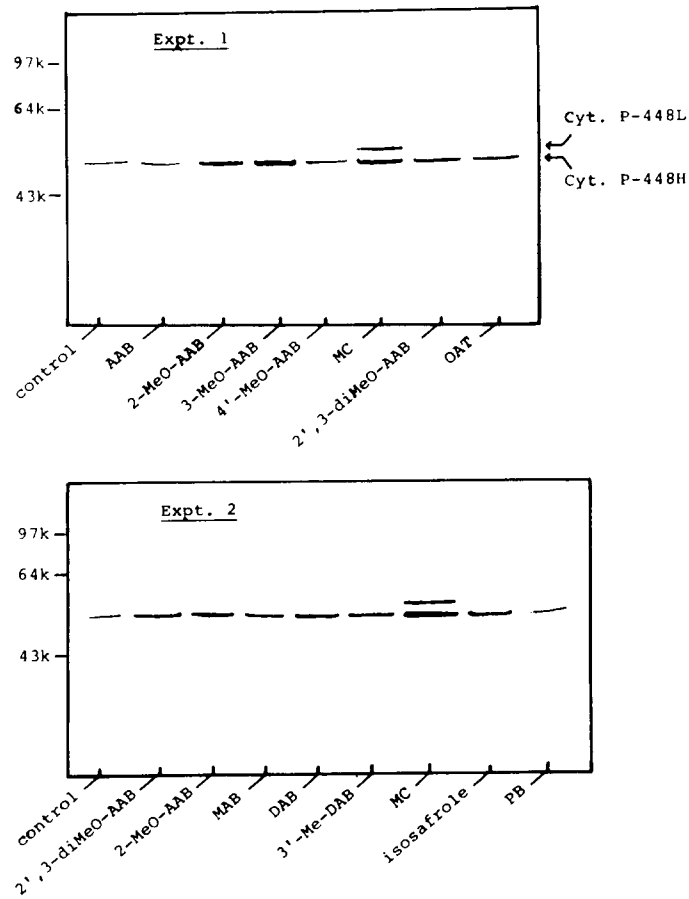

Fig. 3. Immuno-blot patterns of hepatic microsomes from the untreated rats and the rats treated with AAB derivatives or other cytochrome P-450 inducers. Hepatic microsomes were prepared from the untreated rats and the rats treated with MC (0.11 mmoles/kg) or other chemicals (0.22 mmoles/kg) 24 hr before. Immuno-blots were carried out with an aliquot (40 μg protein) of each microsomal preparation and APH-8 MoAb as described in Materials and Methods: k, kilo dalton.

treated rats gave only a very faint protein band at the position. Among the samples tested, only the MC-treated rat microsomes confered two protein bands at the positions of cytochrome P-448H and cytochrome P-448L (molecular weight of 56,000 dalton).

The form of the 3-MeO-AAB-induced cytochrome P-450 component was further characterized spectrophotometrically. The cytochrome P-450 fraction was sequentially purified by chromatographies with columns of ω-amino-*n*-octyl Sepharose 4B, Whatmann DE52 anion exchanger, and hydroxyapatite [1]. Specific content of cytochrome P-450 in the fraction was 5.8 nmoles per mg protein. Like the authentic cytochrome P-448H sample [1], the oxidized and CO-bound forms of the partially purified cytochrome P-450 component showed Soret peaks at 393 nm and 448 nm, respectively. This partially purified cytochrome P-448H was also identical with the authentic cytochrome P-448H sample in its immunological characteristics, as determined by Protein A-ELISA and immunoblots using APH-3 and APH-8 MoAbs (data not shown).

DISCUSSION

This work demonstrated that several methyl and methoxyl derivatives of AAB selectively induce microsomal cytochrome P-448H in rat liver, as indicated by both bacterial mutation test and immunological methods using anti-cytochrome P-448 MoAbs. AAB itself did not show a potency as the inducer, whereas methoxyl derivatives of AAB such as 2-MeO-AAB, 3-MeO-AAB and 2',3-diMeO-AAB and methyl derivatives such as OAT, MAB and DAB exhibited strong activity for the enzyme induction. The potency of the AAB derivatives as cytochrome P-448H inducers appeared not to correlate with their hepatocarcinogenic activity in rats [7, 21, 22], as exemplified by a fact that non-carcinogenic 2-MeO-AAB is as potent as strong carcinogenic 3-MeO-AAB in its activity for the cyto-

chrome P-448H induction. Although several AAB derivatives are demonstrated to be potent inducers of cytochrome P-448H, the total content of microsomal cytochrome P-450 was not or a little (the case of 3-MeO-AAB) influenced by the aminoazo dye treatment.

A relationship between the structure of azo dyes and their activities for the induction of cytochrome P-448 has been reported by Fujita *et al.* [23]. They indicated that only azo dyes having a bay-region in their molecules (phenanthrene type) could induce cytochrome P-448; however, subtypes of the induced cytochrome P-448 have not been identified. We confirmed by the immunoblotting assay that Sudan III with a bay region structure in the molecule could induce both cytochrome P-448L and cytochrome P-448H (unpublished data). As demonstrated in the present study, the AAB derivatives tested lack a bay region structure in the molecule, but they induced cytochrome P-448H. Therefore, it is probable that a bay region in azo dye molecules is responsible for the induction of cytochrome P-448L but not for the induction of cytochrome P-448H.

With regard to substrate specificity of induced cytochrome P-448, although both cytochrome P-448H and P-448L are found to mediate the mutagenic conversion of both Trp P-2 and Glu P-1, cytochrome P-448L seems to be much more efficient for the mutagenesis of Trp P-2 than for that of Glu P-1. By contrast, cytochrome P-448H is found to be equally efficient to both substrates [1]. This is reflected in the substrate specificity of the MC-induced enzymes which contain both cytochrome P-448L and P-448H and show stronger activity for the Trp P-2 mutagenesis than for the Glu P-1 mutagenesis. In contrast, the PB-induced enzymes are inefficient in both Trp P-2 and Glu P-1 mutageneses [6]. In this context, the results of the bacterial mutation tests with the aminoazo dye-induced microsomal enzymes also substantiate that the AAB derivatives are selective inducers for cytochrome P-448H, since they induce the microsomal enzyme similarly active for both Trp P-2 and Glu P-1 mutageneses (Table 1).

Isosafrole is known as a selective inducer for cytochrome P-448H. Indeed, as revealed from the results of immunological assays (Table 2 and Fig. 3), the amount of the isosafrole-induced cytochrome P-448 appeared to be as large as those induced with several AAB derivatives. However, the activity of the isosafrole-induced enzyme in the bacterial mutation test was much weaker than that of the aminoazo dye-induced enzyme (Table 1). This discrepancy may be attributable to the interaction of the induced microsomal cytochrome P-448H with metabolites of the inducer; the isosafrole-induced cytochrome P-448H is found to form a complex with metabolite(s) of isosafrole, leading to inactivation of the induced cytochrome [4], whereas the function of the cytochrome P-448H induced by AAB derivatives is seemingly not inactivated by the metabolites.

Considering the results obtained in the present experiments, methoxyl or methyl derivatives of AAB, especially noncarcinogenic 2-MeO-AAB, can be provided as selective cytochrome P-448H inducers in rats.

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