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IMMUNOCHEMICAL CHARACTERIZATION OF A CYTOCHROME P450 ISOZYME AND A PROTEIN PURIFIED FROM LIVER MICROSOMES OF MALE GUINEA PIGS AND THEIR ROLES IN THE OXIDATIVE METABOLISM OF Δ^9-TETRAHYDROCANNABINOL BY GUINEA PIG LIVER MICROSOMES

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Abstract—A protein (designated as protein-B) was purified from liver microsomes of adult male guinea pigs by an affinity chromatography with ω-aminooctyl Sepharose 4B, followed by HPLC using DEAE-5PW and hydroxyapatite columns which had been used to purify a cytochrome P450 (P450) isozyme (P450-A) from the same subcellular fraction (Narimatsu *et al.*, *Biochem Biophys Res Commun* **172**: 607–613, 1990). Protein-B had a molecular mass of 49 kDa in SDS–PAGE, but did not show absorbance at 417 nm for heme. Further, it did not show any oxidative activities towards aniline (AN), *d*-benzphetamine (*d*-BP), *p*-nitroanisole (*p*-NA) or Δ^9-tetrahydrocannabinol (Δ^9-THC) in a reconstituted system including dilauroylphosphatidylcholine, NADPH-P450 reductase, and cytochrome b_5. However, antiserum against protein-B raised in rabbits suppressed liver microsomal oxidative activities towards *d*-BP and *p*-NA dose-dependently. The antibody decreased Δ^9-THC oxidation activity most effectively, but did not decrease AN hydroxylation activity. Antiserum against P450-A suppressed all the activities towards these four substrates, especially towards Δ^9-THC, in liver microsomes of male guinea pigs. Moreover, reconstitution with hemin made it possible for protein-B to produce some oxidative activity toward Δ^9-THC. These results suggest that protein-B is also a cytochrome P450 isozyme which has lost a heme moiety during purification steps. Both P450-A and protein-B could have a role as cytochrome P450 isozymes in the oxidative metabolism of drugs, especially that of Δ^9-THC by the liver microsomes of adult male guinea pigs.

Oxidative metabolism of Δ^9-tetrahydrocannabinol (Δ^9-THC§), a major psychoactive constituent of marihuana, has been studied in various mammals both *in vitro* and *in vivo* [1, 2]. *In vitro* studies using hepatic subcellular fractions such as 9000 *g* supernatant or microsomal fraction have suggested that oxidation of Δ^9-THC was catalysed mainly by the hepatic mixed function monooxygenase system including cytochrome P450 (P450) isozymes [3, 4]. However, only a little direct evidence supporting the participation of P450 enzymes in the oxidative metabolism of Δ^9-THC has been reported so far [5, 6].

Among various experimental animal species, the guinea pig is unique from the point of view of cannabinoid metabolism. Namely, similarly to the human, the guinea pig has rather high activity for epoxy metabolite formation from Δ^9-THC and Δ^8-THC [7], and does not biosynthesize vitamin C. Although a number of P450 isozymes have been purified from various mammals and their functions in xenobiotic metabolism examined [8], only a few isozymes have been purified so far from the liver microsomes of guinea pigs [9–11]. Recently, we have purified a P450 isozyme (designated as P450-A) from the liver microsomes of adult male guinea pigs [12]. The isozyme showed some oxidative activity towards Δ^9-THC in a reconstituted system [12]. In the present study, we purified P450-A and another protein from the liver microsomes of adult male guinea pigs, and examined their roles in microsomal Δ^9-THC oxidation.

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§ Abbreviations: P450, cytochrome P450; THC, tetrahydrocannabinol; AN, aniline; *d*-BP, *d*-benzphetamine; *p*-NA, *p*-nitroanisole; DLPC, dilauroylphosphatidylcholine.

MATERIALS AND METHODS

Chemicals. Glucose-6-phosphate, NADP and NADPH were obtained from Boehringer Mannheim GmbH (Duren, F.R.G.); glucose-6-phosphate dehydrogenase, dilauroylphosphatidylcholine (DLPC), fast blue BB salt, molecular mass markers for SDS–PAGE (Dalton Mark VII-L), cytochrome *c* and hemin were from the Sigma Chemical Co. (St Louis, MO, U.S.A.); cholic acid and dithiothreitol

were from the Wako Pure Chemical Co. (Osaka, Japan); (trimethylsilyl)trifluoroacetamide was from the Tokyo Kasei Kogyo Co. (Tokyo, Japan); Sepharose 4B and 2′,5′-ADP-Sepharose 4B were from Pharmacia Fine Chemicals (Uppsala, Sweden); Emulgen 911 was from the Kao-Atlas Co. (Tokyo, Japan). ω-Aminooctyl-Sepharose 4B was prepared by the method of Guengerich and Martin [13]. Δ^9-THC was isolated from cannabis leaves [14]. 8α-Hydroxy-(8α-OH-) and 8β-OH-Δ^9-THCs were prepared by the published method [15]. 11-OH-, 1′-OH-, 2′-OH-, 3′-OH-, 4′-OH- and 5′-OH-Δ^9-THCs were kindly supplied by the National Institutes on Drug Abuse (Bethesda, MD, U.S.A.).

Animals. Adult male Hartley guinea pigs (8 weeks old, 350–410 g body weight) were purchased from the Hokuriku Experimental Animals Lab. (Kanazawa, Japan); adult female New Zealand white rabbits (12 weeks old, 2.4–2.6 kg body weight) were from the Sankyo Lab. (Toyama, Japan). The animals were housed in air-conditioned rooms (22–24°) and allowed to take food and water *ad lib.*

HPLC. Instrument, a Hitachi 655A-11 liquid chromatograph equipped with a 655A variable wavelength UV monitor, an L-3000 photo diode array detector, and D-2000 and D-2500 chromato-integrators. Column, DEAE-5PW (21.5 mm i.d. × 150 mm), hydroxylapatite (7.5 mm i.d. × 75 mm) and hydroxylapatite minicolumn (7.5 mm i.d. × 10 mm), all of which were obtained from the Toso Co. (Tokyo, Japan). Detection wavelength, 244 nm (for protein) and 417 nm (for heme).

Purification of proteins. Liver microsomes of guinea pigs (N = 10) were solubilized with sodium cholate and applied to an ω-aminooctyl-Sepharose 4B column as described previously [12]. Fractions containing P450 isozymes were combined and subjected to HPLC using an anion-exchange column (DEAE-5PW). Proteins were eluted at a flow rate of 2 mL/min with a linear gradient of sodium acetate from 0 to 200 mM over 90 min in 20 mM Tris-acetate buffer (pH 7.5) containing 20% glycerol and 0.4% Emulgen 911. Fractions eluted from 40 to 45 min were combined, and a P450 isozyme (designated as P450-A) was purified as reported previously (sp. content, 14.31 nmol/mg of protein) [12].

SDS–PAGE showed that fractions eluted from 31 to 37 min contained considerable amounts of protein, although absorbance at 417 nm for heme was very low. The effluents were then combined and concentrated by ultrafiltration. The fraction was subjected to HPLC using a longer hydroxylapatite column. Proteins were eluted from the column by a linear gradient from 10 to 350 mM of potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.2% (w/v) sodium cholate and 0.4% (w/v) Emulgen 911. Fractions eluted from 22 to 26 min were combined, and the detergent was removed by a hydroxylapatite minicolumn as described previously [12] to obtain a protein (arbitrarily designated as protein-B), which was homogeneous on SDS–PAGE. NH_2-terminal amino acid sequence was analysed by the method reported by Funae and Imaoka [16].

Preparation of antibodies. The purified proteins, P450-A and protein-B, were suspended separately in Freund's complete adjuvant, and injected intradermally in to the backs of rabbits (10 μg, once a week for 4 weeks). The same dose (10 μg) of each protein was suspended in physiological saline and injected i.v. into the animals at week 5. From week 6, blood was collected weekly, and antisera were obtained by centrifugation. Ouchterlony immunodiffusion analysis was performed according to the method of Thomas *et al.* [17]. Western blot analysis was carried out as described previously [18]. Cytochrome b_5 (23.3 nmol/mg protein) and NADPH-P450 reductase (32.3 U/mg protein) from the liver microsomes of male guinea pigs were purified to homogeneity using SDS–PAGE, by the method of Omura and Takesue [19] and Yasukochi and Masters [20], respectively.

Reconstitution of protein-B with hemin. Protein-B was reconstituted with hemin by the method of Uvarov *et al.* [21] with some modifications. Namely, protein-B (25 μg) and DLPC (100 μg) in 114 μL of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol were sonicated for 30 sec at room temperature. Hemin (10 mM in ethanol, 6 μL) was added to the protein solution for 20 min with stirring (final concentration of 0.5 mM), and incubated at 37° for 30 min. After adding 5 mL of the buffer described above and standing at 4° for 12 hr, the solution was concentrated with a collodion bag and an aspirator to 0.5 mL. This sample was diluted again with 5 mL of the buffer, and concentrated to obtain a final preparation (0.5 mL). Oxidation activity of the sample toward Δ^9-THC was examined in a reconstituted system employed for the purified P450-A as follows: the reconstitution system contained protein-B (10 μg), NADPH-P450 reductase (0.20 U), cytochrome b_5 (30 pmol), DLPC (15 μg), Δ^9-THC (10 μg), NADPH (1 μmol) and potassium phosphate buffer (pH 7.4, 100 mM) in a final volume of 0.5 mL. The reaction medium was incubated at 37° for 60 min, and metabolites formed were extracted with ethyl acetate (5 mL). The organic layer was evaporated *in vacuo* and the residue was dissolved in 40 μL of acetonitrile. A half (20 μL) of the sample solution was subjected to silica gel TLC, and the remaining half was subjected to GC and GC/MS after the conversion to TMS derivatives under the condition reported previously [6]. The conditions in GC and GC/MS analyses were as follows. For GC: instrument, a Shimadzu GC-16A gas chromatograph equipped with a hydrogen flame ionization detector and a Shimadzu C-R5A Chromatopac data processor; column, 5% SE-30 on Chromosorb W (60–80 mesh, 3 mm i.d. × 2 m); column temperature, 250°; detector temperature, 275°; carrier gas, N_2. For GC/MS: instrument, a JEOL JMS DX-300 mass spectrometer, a JEOL GCG 06 gas chromatograph and a JMA DA-5000 mass data system; column, 5% SE-30 on Chromosorb W (60–80 mesh, 3 mm i.d. × 2 m); column temperature, 250°; carrier gas, He 40 mL/min; ionization energy, 70 eV; ionization current, 300 μA.

Enzyme assays. Microsomal oxidations of aniline (AN) [22], *p*-nitroanisole (*p*-NA) [23], *d*-benzphetamine (*d*-BP) [24] and Δ^9-THC [25] were

determined by the published methods. Oxidation activities of purified proteins towards the same substrates were assessed by the methods reported previously [12]. In immunochemical studies, microsomes and antiserum against P450-A or protein-B were preincubated at 25° for 30 min. Each of the substrates and an NADPH-generating system were added, and incubation proceeded at 37° for appropriate times. Enzyme activities were assayed as described above. Protein concentrations and P450 content were determined by the method of Lowry *et al.* [26], and Omura and Sato [27], respectively.

RESULTS AND DISCUSSION

In a previous study, we purified a P450 isozyme (P450-A) from the liver microsomes of adult male guinea pigs and found that it had some oxidation activities towards *d*-BP, *p*-NA and AN in the reconstituted system [12]. The isozyme showed hydroxylation activity towards Δ^9-THC at the 8β, 2′ and 3′ positions [12]. It is unclear, however, to what extent P450-A and other P450 isozymes contribute to the oxidation reactions towards these substrates in the liver microsomes of guinea pigs. As a first step in the present study, we isolated another protein that might be a major protein in microsomes as judged from an SDS–PAGE profile. As a second step, antibodies against these two proteins were then raised in rabbits, and immunochemical studies were performed to elucidate their roles in the oxidation reactions of the above substrates in the liver microsomes of male guinea pigs.

Microsomal proteins solubilized with sodium cholate were subjected to hydrophobic affinity chromatography with ω-aminooctyl-Sepharose 4B, followed by HPLC with an anion-exchange column (DEAE-5PW). As shown in Fig. 1, P450-A had been purified from fractions shaping Peak 3 as described previously [12]. Although absorbance at 417 nm was rather low, SDS–PAGE showed that fractions eluted from 31 to 37 min contained a single and thick protein band, the molecular mass of which was about 50 kDa. The fractions were combined, concentrated and subjected to HPLC with a longer hydroxylapatite column for further purification. Judging from an elution profile of proteins from the hydroxylapatite column, which was monitored at a wavelength of 244 nm, and from an SDS–PAGE profile, fractions eluted from 13 to 24 min were found to contain a single band (data not shown). The combined fraction was applied to a minicolumn of hydroxylapatite for removal of the detergent in order to obtain a final preparation (designated as protein-B). An SDS–PAGE profile of the purified protein-B is shown in Fig. 2. Purification steps are summarized in Table 1. On the basis of protein amounts, the yield of protein-B from the microsomes was 0.12%. The molecular mass was calculated to be 49 kDa on the basis of mobilities of marker proteins. Figure 3 illustrates the NH_2-terminal amino acid sequence of protein-B and also the sequences of P450-A [12] and GP-53K [10], both of which were purified from the liver microsomes of guinea pigs for comparison. The latter was the only P450 isozyme whose NH_2-terminal sequence had been reported

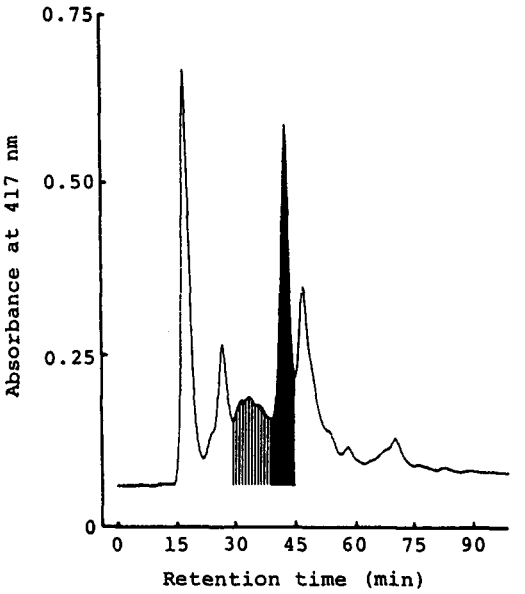

Fig. 1. An elution profile of cytochrome P450 from a DEAE-5PW column in HPLC. Proteins (about 15 mg) were injected and eluted at a flow rate of 2 mL/min with a linear gradient of sodium acetate from 0 to 200 mM over 90 min in 20 mM Tris-acetate buffer (pH 7.5) containing 20% glycerol and 0.4% Emulgen 911. P450-A was purified from fractions shaping Peak 3 (black area) as reported previously [12]. On the basis of an SDS–PAGE profile, a hatched area (Peak 2) was combined and subjected to a next purification step.

before that of P450-A [12]. Following our publication of P450-A purification, two other groups also reported purification of P450 isozymes from liver microsomes of guinea pigs [28, 29]. NH_2-terminal amino acid sequences of $P450_{GP1}$ by Oguri *et al.* [28] and of P450 GP-1 by Taniguchi *et al.* [29] are the same as that of P450-A [12]. The NH_2-terminal sequences of protein-B and P450-A were only 26% homologous. Ouchterlony immunodiffusion analysis showed that protein-B and P450-A were immunochemically different proteins, since spurs produced by antisera against protein-B and P450-A apparently crossed each other (Fig. 4). Furthermore, antisera against P450-A and protein-B recognized a single protein band of 52 and 49 kDa, respectively, in the liver microsomal fraction of the guinea pig in Western blot analysis (Fig. 5). These results also demonstrate that P450-A (50 kDa) and protein-B (49 kDa) do not immunochemically cross-react with each other.

When microsomes were preincubated with antiserum against P450-A, microsomal oxidation activities towards AN, *p*-NA and *d*-BP were suppressed in a concentration-dependent manner (Fig. 6). At an antiserum/microsomal protein ratio of 8.7, oxidation activities were suppressed by 35, 38 and 27% for *p*-NA, AN and *d*-BP, respectively, as compared with those of control experiments using preimmune serum. Antiserum against protein-B suppressed microsomal oxidations towards *p*-NA

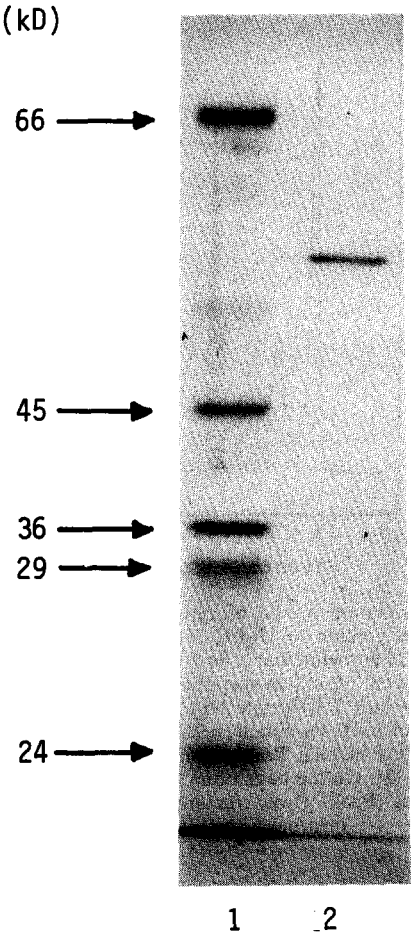

Fig. 2. SDS–PAGE (10%) of purified protein-B. The cathode is at the top of this figure. Proteins were stained with Coomassie brilliant blue. Lanes 1 and 2 contained standard marker proteins and protein-B (1 μg), respectively. Standard proteins consisted of bovine serum albumin molecular mass, 66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa) (3 μg each).

Table 1. Purification of protein-B from the liver microsomes of male guinea pigs

Purification step	Total protein (mg)	Yield (%)
Solubilized microsomes	1956	100
ω-Aminooctyl-Sepharose 4B	103	5.3
HPLC		
DEAE-5PW	25.3	1.3
hydroxylapatite	2.37	0.12

and *d*-BP concentration-dependently, but did not affect AN hydroxylation under similar conditions (Fig. 7). As compared with the control, activities of *p*-NA *O*-demethylase and *d*-BP *N*-demethylase were suppressed by 39 and 43%, respectively, in microsomes preincubated with the antiserum against protein-B.

Effects of the antisera on microsomal Δ^9-THC oxidation are summarized in Fig. 8. The antiserum against P450-A suppressed Δ^9-THC oxidations at the 11-, 8α-, 2′- and 3′-positions concentration-dependently (Fig. 7A). It should be noted that the antibody against P450-A suppressed 11-OH-Δ^9-THC formation. In our previous studies, P450-A did not mediate 11-OH- Δ^9-THC formation from Δ^9-THC under the conditions employed [12]. It is possible that this isozyme does not exhibit 11-OH-Δ^9-THC-forming activity under artificial conditions. It has been reported that testosterone 6β-hydroxylation activities of P450 isozymes purified from rat liver microsomes were increased by changing the lipid component from DLPC to microsomal lipids in the reconstituted system [30, 31]. Therefore, the lipid component of the reconstituted system in the present study was changed from DLPC to microsomal lipids extracted with a mixture of chloroform and methanol (2:1, v/v). However, the system did not show any detectable 11-OH-Δ^9-THC formation activity (data not shown). Taken together, it is likely that some P450 isozyme(s) being immunochemically closed to P450-A mainly catalyses 11-OH-Δ^9-THC formation from Δ^9-THC in the liver microsomes of the guinea pig.

Preincubation of microsomes with the antiserum raised against protein-B caused a concentration-dependent suppression of Δ^9-THC oxidation at the 8α-, 8β-, 2′- and 3′-positions (Fig. 7B). At an antiserum/microsomal protein ratio of 13.2, 39, 44, 42 and 44% of the microsomal activity towards the 8α-, 8β-, 2′- and 3′-OH-Δ^9-THC formations, respectively, was decreased by preincubation with the antibody, as compared with the control activities shown by microsomes preincubated with preimmune serum. In this case, no detectable inhibition of microsomal 11-OH-Δ^9-THC formation by the antiserum was observed.

These results suggest that this purified protein-B might be a P450 isozyme which has lost a heme moiety during the purification steps. We thus attempted to reconstitute protein-B with hemin according to the method of Uvarov *et al.* [21], and examined Δ^9-THC metabolism in a reconstituted system containing protein-B, NADPH-P450 reductase, cytochrome b_5, DLPC and NADPH. Only two spots colored with fast blue BB solution were observed on the TLC plate, one of which was the substrate (R_f 0.64) and the other small one was an unidentified metabolite (R_f 0.36). A co-developed R_f value of 11-OH-Δ^9-THC was 0.33. On the basis of our data for authentic samples reported previously [25], the R_f value of this metabolite is similar to those of 3′-OH-Δ^9-THC, 1′-OH-Δ^9-THC and 8α-OH-Δ^9-THC, which are oxidative Δ^9-THC metabolites formed in rat liver microsomes. In addition, none of the control experiments employing either the combination of protein-B and ethanol,

	1							10										20	Homology (%)
Protein -B	M	D	L	E	I	L	L	A	H	V	L	G	F	L	I	Y	X	F V H	
P450-A	M	E	L	S	L	L	L	F	L	A	L	L	L	G	L	L	L	L F	26
GP-53K	M	T	S	A	M	E	L	L	L	T	A	T	I	F	W	L	V	L W V	11

Fig. 3. Comparison of NH_2-terminal amino acid sequences of P450 isozymes purified from liver microsomes of guinea pigs. Homology of protein-B with the other isozymes purified from liver microsomes of guinea pigs reported so far is shown by the enclosed area.

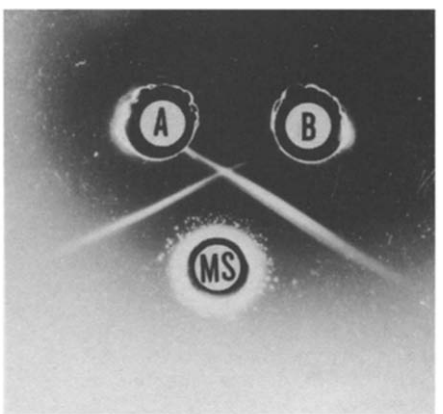

Fig. 4. Ouchterlony double diffusion analysis. A well designated as MS contained guinea pig liver microsomes (14.5 mg/mL) solubilized with 0.2% Emulgen 911 and 0.4% sodium cholate. Wells designated as A and B contained antisera against P450-A and protein-B, respectively.

instead of hemin, or the combination of hemin and the buffer, instead of protein-B, produced any metabolites of Δ^9-THC detectable in the TLC analysis. The metabolite, however, could not be identified by GC/MS because of its low yield. These results obtained in the reconstitution experiments further suggest the possibility that protein-B is an apoprotein of P450 enzyme in the liver microsomes of male guinea pigs.

As described above, the Western blot analysis using the anti-serum against P450-A revealed a sharp and thick protein band corresponding to P450-A (Fig. 5). Another Western blot using known amounts of purified P450 and its antibody tentatively showed that P450-A and its immunochemically related P450 isozyme(s) corresponded to 18–22% of liver microsomal P450 protein.

Considering these results and the fact that three groups including this laboratory purified possibly the same P450 isozyme from the liver microsomes of guinea pigs [12, 28, 29] almost at the same time, it is feasible that P450-A may be a major P450 isozyme in the liver microsomes of adult male guinea pigs. Similarly to these results, the Western blot analysis using known amounts of protein-B and its antibody

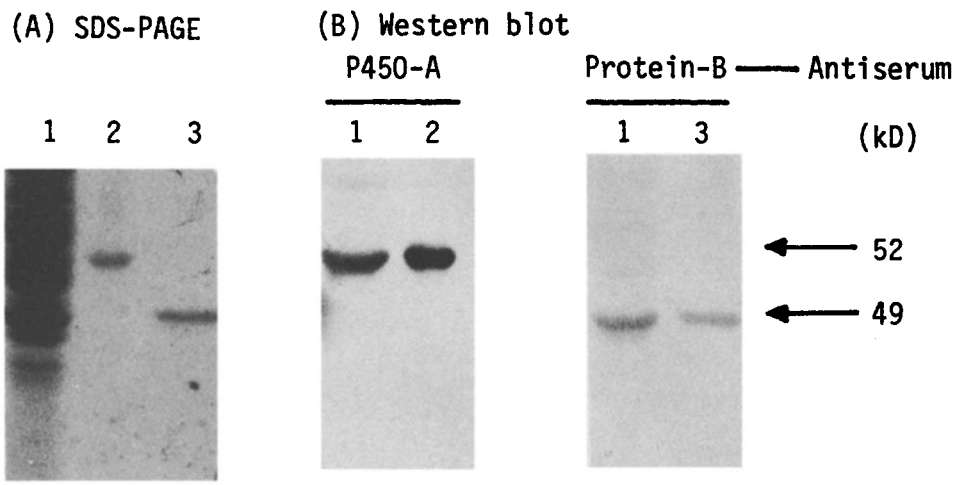

Fig. 5. SDS–PAGE (9%) (A) and Western blot analyses of P450-A and protein-B (B). Microsomes, P450-A and/or protein-B were subjected to SDS–PAGE, and were electrophoretically transferred to Clear Blot Membrane-p. The cathode is at the top. Lines 1, 2 and 3 contain microsomes (20 μg), P450-A (0.15 μg) and protein-B (0.15 μg). (A) Proteins were stained with Coomassie brilliant blue. (B) The protein of P450-A or protein-B was probed with the antiserum against the isozyme and alkaline phosphatase–conjugated goat second antibody.

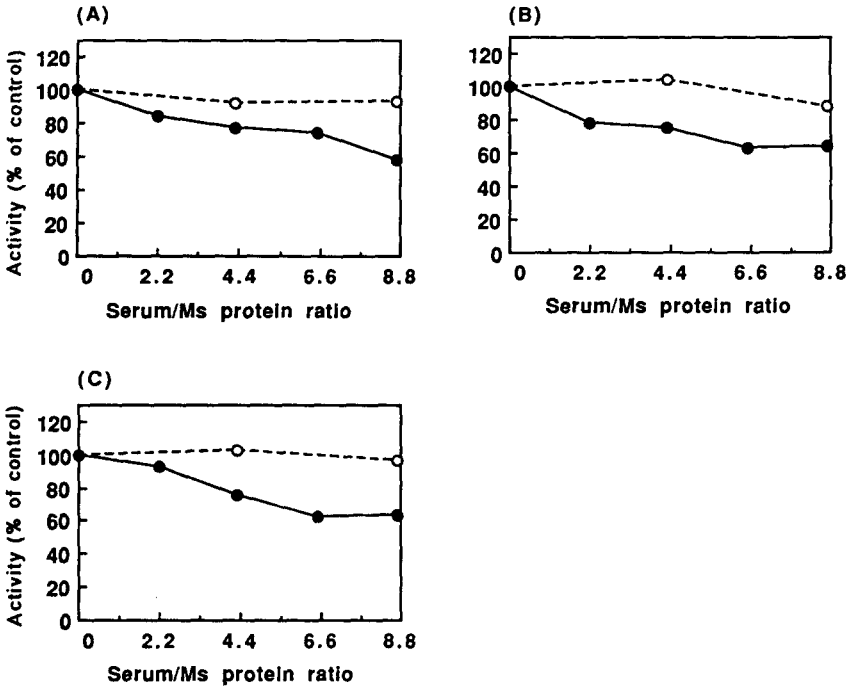

Fig. 6. Effects of antiserum against P450-A on oxidative activity towards AN (A), *d*-BP (B) and *p*-nitroanisole *p*-NA (C) by liver microsomes of male guinea pigs. Liver microsomes of male guinea pigs were preincubated with antiserum or preimmune serum at 25° for 30 min. An NADPH-generating system and each substrate were then added, and the reaction medium was incubated at 37° for 10 min. Activity of the microsomal fraction without added serum (100% as the control) towards AN, *d*-BP and *p*-NA was 4.01, 7.99 and 2.84 nmol/min/mg of protein, respectively. Closed circle, antiserum added; open circle, preimmune serum added.

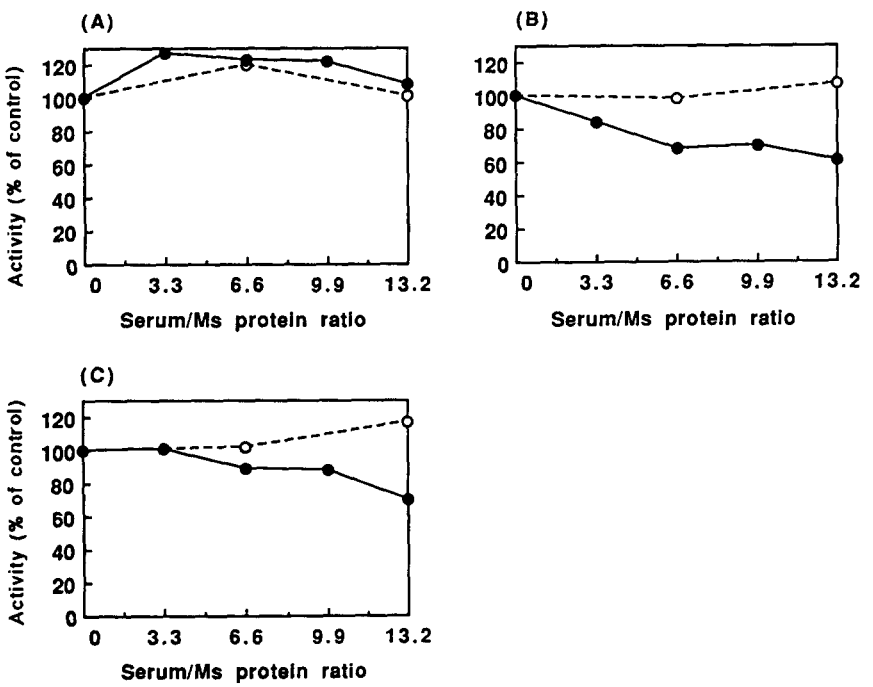

Fig. 7. Effects of antiserum against protein-B on oxidative activity towards AN (A), *d*-BP (B) and *p*-NA (C) by liver microsomes of male guinea pigs. Experimental conditions were the same as those described in the legend to Fig. 6.

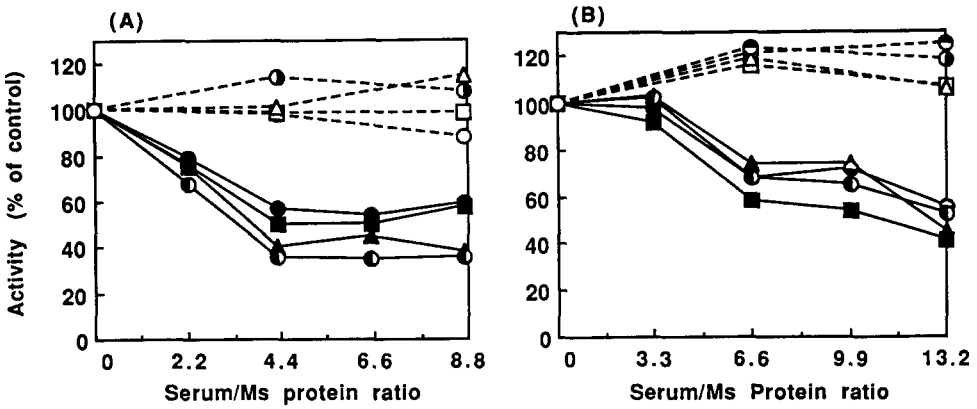

Fig. 8. Effects of antisera against P450-A (A) and protein-B (B) on oxidative activities towards Δ^9-THC by liver microsomes of male guinea pigs. Experimental conditions were the same as those described in the legend to Fig. 6. Activities of the microsomal fraction without added serum (100% as the control) towards Δ^9-THC were 0.185 nmol/min/mg of protein (11-OH-Δ^9-THC) (●, ○), 0.051 (8α-OH-Δ^9-THC) (■, □), 0.048 (3′-OH-Δ^9-THC) (◐, ◑), 0.16 (8β-OH-Δ^9-THC) (◒, ◓), and 0.09 (2′-OH-Δ^9-THC) (▲, △). Broken lines represent the control values obtained by preincubation with preimmune serum. Only metabolites whose formation was affected by preincubation with antibody are depicted in this figure.

suggested that protein-B and its immunochemically related protein(s) corresponded to about 2–3% of total microsomal protein. This may mean that both of the proteins (P450-A and protein-B) are major P450 isozymes in the liver microsomes of adult male guinea pigs, provided that protein-B is actually a P450 isozyme also. The results obtained here suggest further the possibility that protein-B was originally a P450 as a holoenzyme in microsomes and released a heme moiety during the purification steps.

As reported previously, the most abundant oxidative metabolite of Δ^9-THC is 11-OH-Δ^9-THC in the liver microsomes of adult male guinea pigs [7]. Considering the results obtained here, neither P450-A nor protein-B is likely to be responsible for 11-OH-Δ^9-THC formation by liver microsomes. We are now attempting to purify other P450 isozymes from the liver microsomes of guinea pigs as well as to characterize further the proteins obtained in the present study.

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