Biochemical Pharmacology, Vol. 34, No. 13, pp. 2281–2286, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

CYTOCHROME P-450-DEPENDENT NICOTINE OXIDATION BY LIVER MICROSOMES OF GUINEA PIGS

IMMUNOCHEMICAL EVIDENCE WITH ANTIBODY AGAINST PHENOBARBITAL-INDUCIBLE CYTOCHROME P-450

HITOSHI NAKAYAMA,* TOSHIKATSU NAKASHIMA and YUTAKA KUROGOCHI
Department of Pharmacology, Nara Medical University, Kashihara 634, Japan

(*Received* 17 *July* 1984; *accepted* 30 *October* 1984)

Abstract—When guinea pigs were treated with phenobarbital (PB), the specific activity of liver microsomal nicotine oxidase increased by 42%. PB-inducible cytochrome P-450 (PB-P-450) was purified to homogeneity from liver microsomes of PB-treated guinea pigs. Purified PB-P-450 catalyzed nicotine oxidation when reconstituted with NADPH-P-450 reductase and phospholipid system. Antibody prepared against the purified PB-P-450 formed single precipitation lines with both purified PB-P-450 and microsomal components in livers of PB-treated guinea pigs, and both precipitation lines fused. The antibody against PB-P-450 strongly inhibited nicotine oxidation in the reconstituted system. The antibody also inhibited liver microsomal nicotine oxidase activities in PB-treated and untreated guinea pigs by about 30% and less than 5% respectively. About 45% of total P-450 in liver microsomes of PB-treated guinea pigs was precipitated by the antibody. These results show that PB-P-450 participates in liver microsomal nicotine oxidation in PB-treated guinea pigs but not in untreated control animals.

Nicotine is a major constituent of tobacco and tobacco smoke. Many studies concerned with the metabolites and the tissue and the subcellular distribution of nicotine metabolism have shown that nicotine is metabolized mainly by liver microsomes [1–6]. However, it remains obscure as to the reaction mechanisms of microsomal nicotine oxidation and the effects of nicotine treatment of animals on nicotine and other drug-metabolizing enzyme activities since the enzymes participating in microsomal nicotine oxidation have not been precisely characterized. Hill *et al.* [7] suggested that P-450† and mixed-function amine oxidase catalyzed nicotine oxidation in liver microsomes. Unavailability of inhibitors to distinguish between P-450 and mixed-function amine oxidase [8] has delayed confirmation of their suggestion. In addition, the purification of P-450 and mixed-function amine oxidase was difficult at that time. P-450 is known to play an important role in drug metabolism and to be composed of several distinct molecular forms [9]. To study the role of P-450 in microsomal nicotine oxidation, it is necessary to characterize nicotine oxidation with purified P-450. Recently, we showed that PB-P-450 purified from rat liver microsomes catalyzed nicotine oxidation in a reconstituted system [10]. In this report, participation of PB-P-450 in microsomal nicotine oxidation in guinea pigs is quantitatively investigated using antibody against PB-P-450.

* Author to whom all correspondence should be addressed.

† Abbreviations: P-450, cytochrome P-450; dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine; IgG, immunoglobulin G; PB, phenobarbital; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; and PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

Treatment of guinea pigs with PB. Male Hartley guinea pigs (220–270 g) were obtained from the Kiwa Laboratory Animal Co. (in Wakayama prefecture, Japan) and received food and tap water *ad lib.* PB was injected each day into guinea pigs intraperitoneally at 60 mg/kg for 5 days. Animals were killed between 8:30 and 10:00 a.m. The livers were minced and homogenized in 4 vol. of 10 mM potassium phosphate buffer (pH 7.4) containing 1.15% KCl and 1 mM EDTA with six to eight strokes of a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 10,000 *g* for 40 min, and then the supernatant fraction was centrifuged at 105,000 *g* for 1 hr. The pellet was washed by suspending it in the homogenizing buffer and recentrifuging. The resultant microsomal pellet was resuspended in a small volume of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA.

Purification of PB-P-450. The washed microsomal pellet was suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 30% glycerol and 1 mM EDTA to give 7–10 mg protein/ml of the buffer and then solubilized with sodium cholate (3 mg detergent/mg protein). After the solution was centrifuged at 65,000 *g* for 1.5 hr, the supernatant fraction was applied to an *n*-octylamino-Sepharose column. Octylamino-Sepharose column chromatography was carried out as described by Imai and Sato [11]. The subsequent DE-52 column chromatography was carried out at room temperature as described by Guengerich and Martin [12]. The eluent from the DE-52 column was applied to a hydroxylapatite column which had been equilibrated with 30 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol and 0.1% Emulgen 913. After

the column was washed with 60 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol, 0.2% sodium cholate and 0.1% Emulgen 913, P-450 was eluted with a linear gradient of 60–110 mM phosphate buffer (pH 7.2) also containing 20% glycerol, 0.2% sodium cholate and 0.1% Emulgen 913. The major P-450 peak contained PB-P-450. The early fractions of the peak contained electrophoretically homogeneous PB-P-450 whereas the later fractions contained PB-P-450 with a few minor components. To remove contaminants and detergents, the later fractions were diluted to 2 vol. with 20% glycerol and then applied to a second hydroxylapatite column. After the column was washed with 60 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol until the absorbance at 275 nm in the eluent was 0.01, PB-P-450 was eluted from the column with 250 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol. When the eluent containing PB-P-450 from a second hydroxylapatite column was analyzed by SDS-PAGE, the preparation electrophoresed as a major protein band with a faint band. The faint band was most likely due to the dimerization of a monomeric PB-P-450 since the faint band appeared after removal of detergents, and the molecular weights were estimated to be about twice that of the major PB-P-450 band by SDS-PAGE analysis. NADPH-P-450 reductase was partially purified from PB-treated guinea pigs by the method of Yasukochi and Masters [13]. The preparation was free of P-450 and b_5 and had a specific activity of 33 μmoles of cytochrome *c* reduced/min/mg protein.

Preparation of antibody against PB-P-450. Purified P-450 (0.8 mg/ml) was mixed vigorously with an equal volume of Freund's complete adjuvant. On days 1 and 15, male Japanese White rabbits were subcutaneously injected on the back with 0.4 mg of PB-P-450 in Freund's complete adjuvant. The rabbits were boosted by intravenous injection of 0.4 mg P-450 in 0.9% saline on day 30. Rabbits were bled 8 days after the last injection. The sera with high titers were pooled, and the IgG fraction was prepared by the method of Thomas *et al.* [14]. Control IgG was prepared by the same method using blood obtained from non-immunized rabbits.

Immunochemical procedures. When inhibition of liver microsomal and reconstituted nicotine oxidase activity by anti-PB-P-450 IgG was studied, various amounts of the antibody were added to the assay mixture, and the maximum inhibition of the reaction was determined. Microsomes were preincubated with the IgG in 100 mM phosphate buffer (pH 7.4) for 10 min at room temperature. In the reconstituted system, P-450 was first mixed with the antibody in 100 mM phosphate buffer (pH 7.4) containing 20% glycerol for 10 min, NADPH-P-450 reductase and phospholipid were added, followed by an additional 5-min incubation at room temperature. After this preincubation, $MgCl_2$, NADPH and phosphate buffer were added to the mixture. Reactions were started by the addition of nicotine.

Immunoprecipitation was used to determine the amounts of PB-P-450 in liver microsomes of PB-treated guinea pigs. Washed microsomes were solubilized with sodium cholate as described above and, then, centrifuged at 105,000 *g* for 1 hr. The supernatant fraction was incubated with anti-PB-P-450 or control IgG in 5 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.4 mM PMSF and 0.23% sodium cholate. Each mixture contained 1.54 nmoles of P-450. The incubation was carried out at room temperature for 30 min and, then, at 4° for 2 days. After the mixture was centrifuged at 5000 *g* for 20 min, the total amount of P-450 remaining in the supernatant fraction was determined.

Ouchterlony diffusion plates contained 0.9% agarose, 50 mM potassium phosphate buffer (pH 7.4), 0.9% NaCl, 0.02% sodium azide and 0.2% Emulgen 913. The diameter of the wells was 2.5 mm. After filling the wells with appropriate sample, the plates were incubated at 4° for at least 2 days.

Other analytical procedures. Nicotine oxidase activity was determined at 37° by the methods of Hucker *et al.* [3] and Tsujimoto *et al.* [15] except for the standard assay mixture. The standard assay mixture contained 100 mM potassium phosphate buffer (pH 7.4), 0.5 mM nicotine, 0.5 mM NADPH, 6 mM $MgCl_2$, and enzymes in a total volume of 1 or 2 ml. The reaction rates were linear for at least 30 min. NADPH-P-450 reductase was assayed by its ability to catalyze cytochrome *c* reduction in 100 mM phosphate buffer (pH 7.7) at 30° [16]. P-450 contents were determined by the method of Omura and Sato [17]. SDS-PAGE was carried out according to the procedure of Laemmli [18]. Protein was determined by the method of Lowry *et al.* [19].

Materials. Octylamino-Sepharose was prepared by the method of Guengerich and Martin [12]. Hydroxylapatite and 2′,5′-ADP-Sepharose were purchased from Bio-Rad Laboratories and Pharmacia Fine Chemicals respectively. Cholic acid (Wako Pure Chemical, Japan) was recrystallized from 50% (v/v) ethanol. Emulgen 913 was supplied by the Kao Atlas Co., Japan. All other chemicals were of the highest purity commercially available.

RESULTS

Treatment of guinea pigs with PB. PB treatment increased the specific content of P-450 by 40% (Table 1). The specific activity of nicotine oxidase also increased by 42% but the activity per nmole of P-450 remained unchanged. These results suggest that PB-P-450 and constitutive enzymes are active in liver microsomal nicotine oxidation.

Purification of PB-P-450. Purification was done repeatedly, and highly purified PB-P-450 was obtained in all cases. The specific content of the purified enzyme was 17 to 18.5 nmoles/mg protein with a recovery of 7–12% of total microsomal P-450. The purified PB-P-450 in the presence of detergents showed a single protein band and was estimated to have minimum molecular weights of 51,000 by comparison with the standards (Fig. 1a). It was found that the absolute spectrum of the oxidized PB-P-450 was a Soret peak at 417 nm, and the difference spectrum of the CO complex of the reduced enzyme was a peak at 450 nm (data not shown). This purified PB-P-450 is probably identical to a form isolated by Kitada *et al.* on the basis of criteria such as spectral

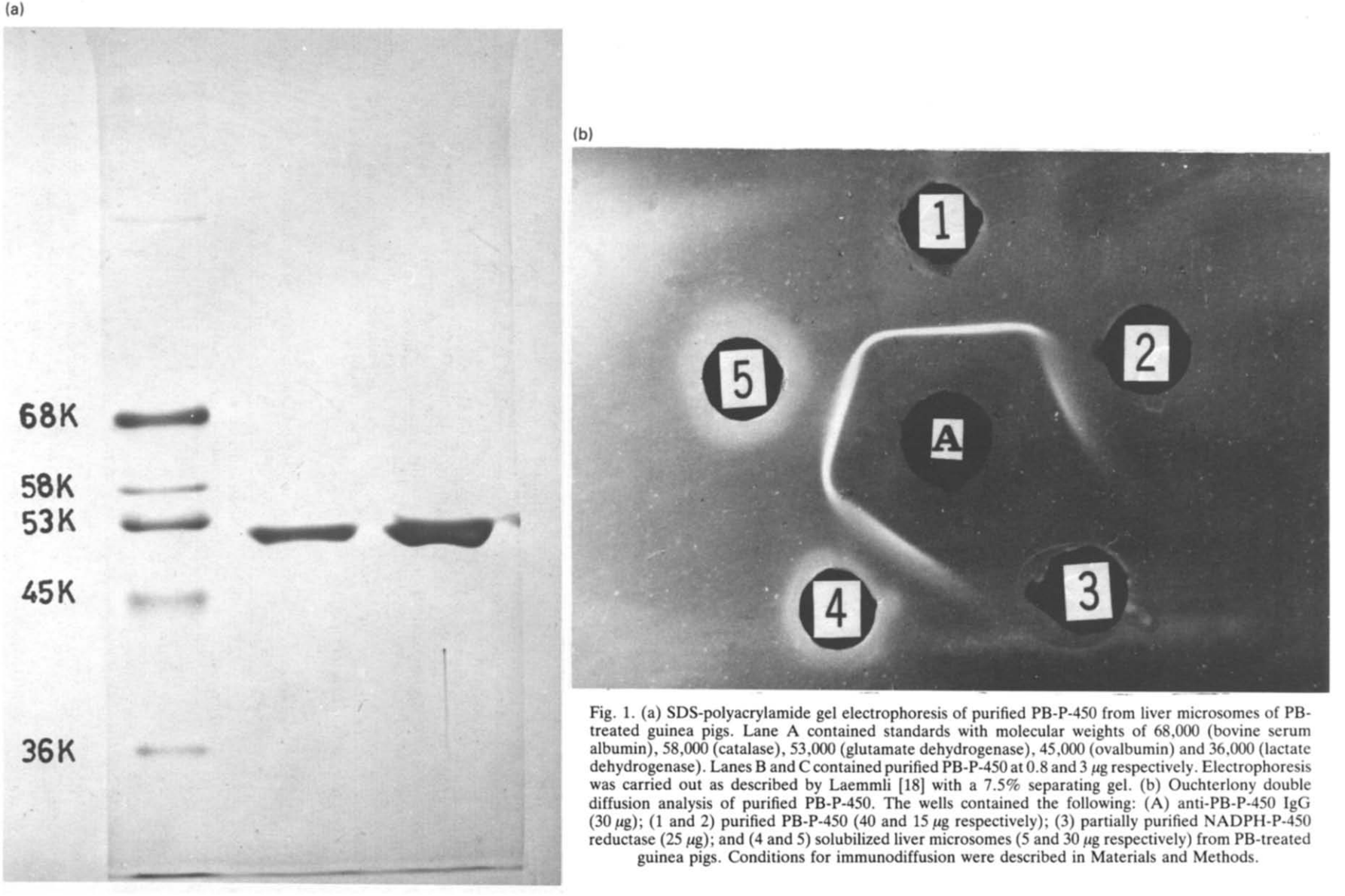

Fig. 1. (a) SDS-polyacrylamide gel electrophoresis of purified PB-P-450 from liver microsomes of PB-treated guinea pigs. Lane A contained standards with molecular weights of 68,000 (bovine serum albumin), 58,000 (catalase), 53,000 (glutamate dehydrogenase), 45,000 (ovalbumin) and 36,000 (lactate dehydrogenase). Lanes B and C contained purified PB-P-450 at 0.8 and 3 μg respectively. Electrophoresis was carried out as described by Laemmli [18] with a 7.5% separating gel. (b) Ouchterlony double diffusion analysis of purified PB-P-450. The wells contained the following: (A) anti-PB-P-450 IgG (30 μg); (1 and 2) purified PB-P-450 (40 and 15 μg respectively); (3) partially purified NADPH-P-450 reductase (25 μg); and (4 and 5) solubilized liver microsomes (5 and 30 μg respectively) from PB-treated guinea pigs. Conditions for immunodiffusion were described in Materials and Methods.

Table 1. Effect of PB treatment on P-450 content and nicotine oxidase activity in liver microsomes of guinea pigs*

Treatment	P-450 (nmoles/mg protein)	Nicotine oxidase activity (nmoles nicotine/min/mg protein)	Nicotine oxidase activity (nmoles nicotine/min/nmoles P-450)
Control	1.17 ± 0.07	4.02 ± 0.22	3.55 ± 0.38
PB	1.63 ± 0.07	5.69 ± 0.32	3.55 ± 0.29

* PB was injected intraperitoneally each day into guinea pigs at 60 mg/kg for 5 days. Each value represents the mean ± S.E.M. (N = 6).

properties [20] and minimum molecular weights*. Figure 1b shows the reactivity of anti-PB-P-450 IgG with purified PB-P-450 and with liver microsomal components from PB-treated guinea pigs. Anti-PB-P-450 IgG reacted with the purified PB-P-450 and with the microsomal components, and formed a single fused precipitation line while the antibody did not cross-react against a partially purified NADPH-P-450 reductase preparation. On longer incubation, no other precipitation lines appeared.

Effects of anti-PB-P-450 IgG on nicotine oxidation catalyzed by the reconstituted system. Table 2 shows requirements for nicotine oxidation in the reconstituted system. PB-P-450, NADPH-P-450 reductase and NADPH were required absolutely. Figure 2 shows the effects of anti-PB-P-450 IgG on nicotine oxidation in the reconstituted system. Anti-PB-P-450 IgG inhibited nicotine oxidation up to more than 95% at a concentration of 14.7 mg of anti-PB-P-450 IgG/nmole of PB-P-450. Control IgG inhibited the enzyme activity by less than 5% under these assay conditions. These results show that anti-PB-P-450 IgG is useful in investigating microsomal nicotine oxidation catalyzed by PB-P-450.

Effects of anti-PB-P-450 IgG on nicotine oxidation catalyzed by liver microsomes. Anti-PB-P-450 IgG maximally inhibited nicotine oxidase activity in liver microsomes of PB-treated guinea pigs by 28% at a concentration of 7 mg of anti-PB-P-450 IgG/nmole of P-450 (Fig. 3). On the other hand, the antibody inhibited nicotine oxidase activity in liver microsomes of untreated guinea pigs by less than 5%. The amounts of anti-PB-P-450 IgG required to obtain maximum inhibition of nicotine oxidation in the microsomal system were one-third to one-half of those in the reconstituted system, suggesting that

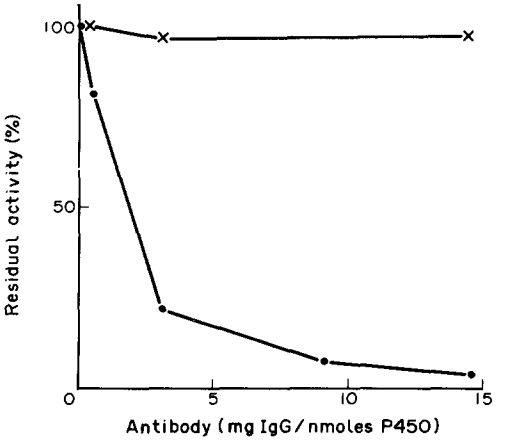

Fig. 2. Effects of anti-PB-P-450 IgG on nicotine oxidation catalyzed by the reconstituted system. PB-P-450 was preincubated with anti-PB-P-450 IgG or control IgG as described in Materials and Methods. The reaction mixture contained the following components in a total volume of 1 ml: 100 mM potassium phosphate buffer (pH 7.4), 0.5 mM nicotine, 0.5 mM NADPH, 6 mM $MgCl_2$, 30 μg dilauroyl-GPC, 1.37 units NADPH-P-450 reductase, 0.46 nmole PB-P-450, and various amounts of IgG. The control activity was assayed in the absence of anti-PB-P-450 IgG and control IgG, and 0.39 nmole of nicotine reacted per min. The activities in the presence of anti-PB-P-450 IgG (●) and control IgG (×) are shown in the figure as percentage of control.

* K. Kitada, personal communication.

Table 2. Requirements for nicotine oxidation catalyzed by the reconstituted system

System	Nicotine oxidation (nmoles nicotine/min/ml)	% Maximal activity
Complete*	0.33	100
−P-450	0	0
−Reductase	0	0
−NADPH	0	0
$-MgCl_2$	0.24	73

* The complete system contained 0.1 M potassium phosphate buffer (pH 7.4), 0.5 mM nicotine, 0.5 mM NADPH, 6 mM $MgCl_2$, 30 μg dilauroyl-GPC, 0.46 nmoles PB-P-450, and 1.37 μmoles/min NADPH-P-450 reductase in a total volume of 1 ml.

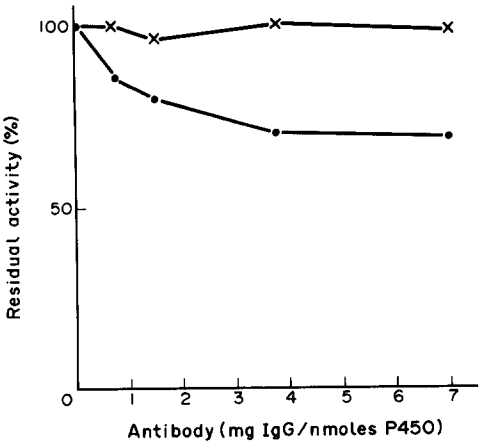

Fig. 3. Effect of anti-PB-P-450 IgG on microsomal nicotine oxidase activities in livers of PB-treated and untreated guinea pigs. Anti-PB-P-450 (or control) IgG was preincubated with liver microsomes from PB-treated or untreated guinea pigs as described in Materials and Methods. The reaction mixture contained the following components in a total volume of 2 ml; 100 mM potassium phosphate buffer (pH 7.4), 0.5 mM nicotine, 0.5 mM NADPH, 6 mM $MgCl_2$, various amounts of IgG, and microsomes from livers of PB-treated or untreated guinea pigs. The liver microsomal P-450 contents from PB-treated and untreated guinea pigs were 1.43 and 0.60 nmoles respectively. The activities in the presence of anti-PB-P-450 IgG are shown in the figure as percentages of the activities assayed with control IgG. Key: (●) PB-treated guinea pigs, and (×) untreated guinea pigs.

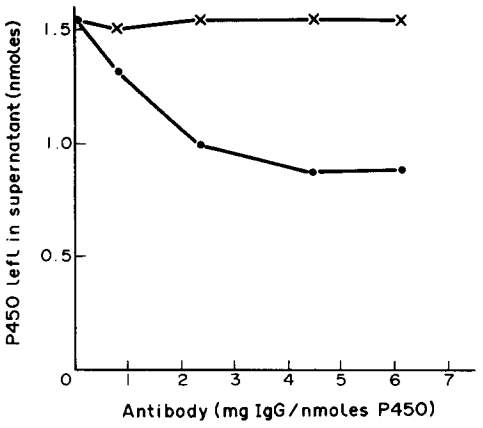

Fig. 4. Quantitative immunoprecipitation of PB-P-450 in liver microsomes of PB-treated guinea pigs by anti-PB-P-450 IgG. Quantitative immunoprecipitation was carried out in the presence of anti-PB-P-450 IgG (●) or control IgG (×) as described in Materials and Methods. Each sample contained 1.54 nmoles of P-450.

one-third to one-half of the total P-450 in liver microsomes of PB-treated guinea pigs is immunochemically identical to PB-P-450.

Quantitative immunoprecipitation of liver microsomal P-450 in PB-treated guinea pigs by anti-PB-P-450 IgG. Various amounts of anti-PB-P-450 IgG were added to the liver microsomal solution from PB-treated guinea pigs, and P-450 remaining in the solution after incubation and centrifugation was determined (Fig. 4). About 55% of total microsomal P-450 was found in the supernatant fraction, indicating that 45% of total microsomal P-450 is immunochemically identical to PB-P-450. Under these incubation conditions, no conversion of P-450 to P-420 was found. The observed pattern of the immunoprecipitation was similar to that of the inhibition of microsomal nicotine oxidation by anti-PB-P-450 IgG.

DISCUSSION

Antibodies against the components of the microsomal electron transport system have been used in studies of drug metabolism [14, 21–23]. Using antibody against PB-P-450 from liver microsomes of PB-treated guinea pigs, the present report confirmed quantitatively that PB-P-450 catalyzed nicotine oxidation in liver microsomes of PB-treated guinea pigs as well as in a reconstituted system. Several different immunoquantitative techniques have been applied to PB-P-450 in rat liver microsomes [23–26]. We also determined the high amounts of PB-P-450 in liver microsomes of PB-treated guinea pigs by a simple immunoprecipitation technique, though the ratio of PB-P-450 to total microsomal P-450 in PB-treated guinea pigs was lower than that in PB-treated rats [23–26]. On the other hand, PB-P-450 in liver microsomes of untreated rats was reported to be low [23–26]. When liver microsomes from untreated guinea pigs were analyzed by SDS-PAGE, the protein staining-band corresponding to PB-P-450 was found to be faint on the gels (data not shown). PB-P-450 seems scarcely to participate in nicotine oxidation in liver microsomes of untreated guinea pigs. P-450 isozymes are shown to exhibit different broad and overlapping substrate specificities, suggesting that constitutive forms of P-450 may participate in microsomal nicotine oxidation. Liver microsomal preparations have been reported to catalyze nicotine oxidation to hydroxynicotine and nicotine-1′-oxide [3, 7, 27, 28]. However, hydroxynicotine, which had been assumed to be a metabolite of nicotine oxidation catalyzed by P-450, has not been identified definitively, since the compound is unstable [3]. On the other hand, hepatic microsomal mixed-function amine oxidase has been reported to catalyze N-oxidation of a variety of *tert*-alkyl or arylamines [8]. Both constitutive forms of P-450 and mixed-function amine oxidase probably catalyze nicotine oxidation in liver microsomes of PB-treated and untreated guinea pigs. Antibodies against the components of the microsomal electron transport system appear to be useful in investigating the metabolites of microsomal nicotine oxidation.

Acknowledgement—This work was supported in part by a grant from the Japan Tobacco and Salt Public Corp.

REFERENCES

1. A. W. Miller and P. S. Larson, *J. Pharmac. exp. Ther.* **109**, 218 (1953).
2. H. McKennis, Jr., L. B. Turnbull and E. R. Bowman, *J. Am. chem. Soc.* **79**, 6342 (1957).

3. H. B. Hucker, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **129**, 94 (1960).
4. E. Hansson and G. G. Schmiterlow, *J. Pharmac. exp. Ther.* **137**, 91 (1962).
5. N. M. Papadopous and J. A. Kintzios, *J. Pharmac. exp. Ther.* **140**, 269 (1963).
6. E. Hansson, P. C. Hoffmann and C. G. Schmiterlow, *Acta physiol. scand.* **61**, 380 (1964).
7. D. L. Hill, W. R. Laster, Jr. and R. F. Struck, *Cancer Res.* **32**, 658 (1972).
8. D. M. Ziegler and L. L. Poulsen, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 52, p. 142. Academic Press, New York (1978).
9. A. Y. H. Lu and S. B. West, *Pharmac. Rev.* **31**, 277 (1979).
10. H. Nakayama, T. Nakashima and Y. Kurogochi, *Biochem. biophys. Res. Commun.* **108**, 200 (1982).
11. Y. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **60**, 8 (1974).
12. F. P. Guengerich and M. V. Martin, *Archs Biochem. Biophys.* **205**, 365 (1980).
13. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
14. P. E. Thomas, A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek and W. Levin, *J. biol. Chem.* **251**, 1385 (1976).
15. A. Tsujimoto, T. Nakashima, S. Tanino, T. Dohl and Y. Kurogochi, *Toxic. appl. Pharmac.* **32**, 21 (1975).
16. H. W. Strobel and J. D. Dignam, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 52, p. 89. Academic Press, New York (1978).
17. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
18. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. M. Kitada, C. Yamazaki, K. Hirota and H. Kitagawa, *Biochem. biophys. Res. Commun.* **93**, 1020 (1980).
21. B. S. S. Masters, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 52, p. 240. Academic Press, New York (1978).
22. M. Noshiro and T. Omura, *J. Biochem., Tokyo* **83**, 61 (1978).
23. N. Harada and T. Omura, *J. Biochem., Tokyo* **89**, 237 (1981).
24. P. E. Thomas, D. Korzeniowski, D. Ryan and W. Levin, *Archs Biochem. Biophys.* **192**, 524 (1979).
25. C. B. Pickett, R. L. Jeter, J. Morin and A. Y. H. Lu, *J. biol. Chem.* **256**, 8815 (1981).
26. F. P. Guengerich, P. Wang and N. K. Davidson, *Biochemistry* **21**, 1698 (1982).
27. J. Booth and E. Boyland, *Biochem. Pharmac.* **20**, 407 (1971).
28. P. J. Murphy, *J. biol. Chem.* **248**, 2796 (1973).