[CANCER RESEARCH 40, 1652-1657, May 1980]
0008-5472/80/0040-0000$02.00

Immunochemical Study on the Role of Different Types of Microsomal Cytochrome P-450 in Mutagenesis by Chemical Carcinogens

Kaname Kawajiri,[1] Hiromichi Yonekawa, Nobuhiro Harada, Mitsuhide Noshiro, Tsuneo Omura, and Yusaku Tagashira

Department of Biochemistry, Saitama Cancer Center Research Institute, Ina-machi, Kitaadachi-gun, Saitama, 362 [K. K., H. Y., Y. T.], and Department of Biology, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka, 812 [N. H., M. N., T. O.], Japan

ABSTRACT

Rabbit antisera were prepared against two molecular species of cytochrome P-450, PB-P-450 (major cytochrome P-450 component of the liver microsomes of phenobarbital-treated rats) and MC-P-448 (major cytochrome P-450 component of the liver microsomes of 3-methylcholanthrene-treated rats), purified from the liver microsomes of phenobarbital-treated and 3-methylcholanthrene-treated rats, respectively, and utilized in examining the role of these two molecular species of cytochrome P-450 in the metabolic activation of chemical carcinogens in the Ames test system. The specificity of the antibodies used in this study was examined and confirmed by Ouchterlony double-diffusion tests and by inhibition studies on microsomal benzo(*a*)pyrene hydroxylase, 7-ethoxycoumarin *O*-deethylase, and benzphetamine *N*-demethylase activities.

Studies on the effect of the antibodies on the mutagenicities of benzo(*a*)pyrene, 7,12-dimethylbenzanthracene, 3-methylcholanthrene, 2-acetylaminofluorene, 2-naphthylamine, *o*-aminoazotoluene, *N*-nitrosodimethylamine, and aflatoxin B_1 were carried out. The mutagenicities of benzo(*a*)pyrene and 7,12-dimethylbenzanthracene were inhibited by 82 and 85% by antibody against MC-P-448 (anti-MC-P-448 immunoglobulin), respectively, and were not inhibited by antibody against PB-P-450 (anti-PB-P-450 immunoglobulin) at all. The mutagenicities of 2-naphthylamine, *o*-aminoazotoluene, and 2-acetylaminofluorene were inhibited by 80, 70, and 60% by anti-MC-P-448 immunoglobulin and also inhibited by 25, 30, and 35% by anti-PB-P-450 immunoglobulin. The two antibodies had identical inhibitory action on the mutagenicities of *N*-nitrosodimethylamine and 3-methylcholanthrene, which were inhibited by 50 and 60%, respectively. The mutagenicity of aflatoxin B_1 was inhibited by 30 and 75% by anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin, respectively.

It is concluded that the contribution of two types of cytochrome P-450, PB-P-450 and MC-P-448, to the metabolic activation is different among various species of chemical carcinogens.

INTRODUCTION

Most chemical carcinogens and mutagens require metabolic activation before they exert their deleterious effects on the organism (14, 22). A satisfactory correlation between carcinogenicity and mutagenicity has been established for various chemical compounds with the Ames test system in the presence of activating enzymatic systems (20, 21, 23). These metabolic activations are carried out primarily by the monooxygenase system of microsomes (14, 22).

The microsomal monooxygenase system is composed of NADPH-cytochrome P-450 reductase (EC 1.6.2.4) and cytochrome P-450.[2] Evidence suggesting the existence of multiple forms of cytochrome P-450 in liver microsomes has accumulated in recent years (4, 10, 33, 36, 37). These different forms of cytochrome P-450 have different substrate specificities, and they participate in various pathways of activation and detoxification of numerous xenobiotics in liver cells (3, 14).

However, little is known about the contribution of each form of cytochrome P-450 to the activation of chemical carcinogens. In this study, we prepared antibodies to 2 forms of cytochrome P-450, PB-P-450[3] and MC-P-448, and utilized the antibodies in elucidating the role of these 2 molecular species of cytochrome P-450 in the metabolic activation of chemical carcinogens in the Ames test system.

We found that the contribution of 2 forms of cytochrome P-450, PB-P-450 and MC-P-448, to the metabolic activation is different among various chemical carcinogens.

MATERIALS AND METHODS

Preparation of S-9 Fraction and Microsomes from Rat Liver. Male Sprague-Dawley rats weighing 200 to 300 g were used. PCB (Kanechlor KC500) was injected i.p. into animals once daily at 100 mg/kg of body weight, and the injection was repeated over 3 successive days. After the final injection, the animals were fasted overnight and then sacrificed. The livers were excised, perfused thoroughly with ice-cold sterile 0.15 M KCl, and then homogenized in 0.15 M KCl with the aid of a Potter glass-Teflon homogenizer to give a 25% homogenate (2). The S-9 fraction was the supernatant fraction of homogenate obtained by centrifugation at 9,000 × *g* for 10 min. The S-9 fraction was centrifuged at 105,000 × *g* for 60 min to separate microsomes and the soluble fraction, and the precipitated microsomes were washed once with 0.15 M KCl-10 mM EDTA. The recovery of protein in the microsomal fraction was about 32% of the total of the S-9 fraction. Preparations of liver microsomes from rats pretreated with 3-methylcholanthrene (25 mg/kg of body weight) or with phenobarbital (80 mg/kg of body weight) were similarly carried out. 3-Methylcholanthrene was injected into the animals 3 times at 1-day intervals. Phe-

[1] To whom requests for reprints should be addressed.

Received October 16, 1979; accepted January 15, 1980.

[2] The term cytochrome P-450 is used to designate all forms of liver microsomal cytochrome P-450.

[3] The abbreviations used are: PB-P-450, major cytochrome P-450 component of the liver microsomes of phenobarbital-treated rats; MC-P-448, major cytochrome P-450 component of the liver microsomes of 3-methylcholanthrene-treated rats; PCB; polychlorinated biphenyls; anti-PB-P-450, antibody to PB-P-450; anti-MC-P-448, antibody to MC-P-448.

nobarbital treatment was continued over 5 successive days.

Preparation of Antibodies against 2 Types of Microsomal Cytochrome P-450. PB-P-450 and MC-P-448 were purified from the liver microsomes of rats pretreated with phenobarbital and 3-methylcholanthrene, respectively, according to the procedure of Imai and Sato (15), with some modifications, details of which will be reported elsewhere.[4] The specific contents of cytochrome P-450 in purified PB-P-450 and MC-P-448 preparations were 16.8 and 17.0 nmol per mg of protein, respectively, and both preparations were homogeneous, as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Rabbit antisera against these 2 types of cytochrome P-450 were prepared, as described previously (28), by using purified PB-P-450 and MC-P-448 as antigens. Control serum was obtained from a nonimmunized rabbit. The immunoglobulin fractions were prepared from the serum preparations by ammonium sulfate fractionation. Antibodies against PB-P-450 and MC-P-448 were further purified by an immunoadsorption method (35).

Mutagenesis Assay. *Salmonella typhimurium* strain TA 98 and TA 100 were kindly provided by Dr. K. Takeishi of our institute. Before the addition of chemical carcinogens, the S-9 fraction was preincubated with antibodies at 25° for 20 min. The final concentration of immunoglobulin was adjusted to the same value by the addition of control immunoglobulin. The mutagenicity was assayed as described by Nagao *et al.* (24), except that 4 mM NADPH was used instead of a NADPH-generating system (29). The S-9 protein concentration used for each mutagen was on the linear portion of the curve for inducing mutations.

Analytical Procedures. The microsomal content of cytochrome P-450 was determined according to the method of Omura and Sato (30), using an extinction coefficient of 91 $cm^{-1} \cdot mM^{-1}$. NADPH-cytochrome *c* reductase was assayed as described previously (31). Aryl hydrocarbon hydroxylase was measured by the method of Nebert and Gelboin (26). Assay of 7-ethoxycoumarin *O*-deethylation was performed as described by Ullrich and Weber (41). Benzphetamine *N*-demethylation was determined by a colorimetric method measuring the formation of formaldehyde (25). Assay of microsomal drug oxidations in the presence of antibodies was performed as described by Noshiro and Omura (28). Protein was determined by the method of Lowry *et al.* (19), using bovine serum albumin as a standard.

Reagents and Biochemicals. NADPH and NADH were obtained from Oriental Yeast Co., Tokyo, Japan. Kanechlor KC500 was obtained from Gasukuro Kogyo Co., Tokyo, Japan. Benzo(*a*)pyrene and cytochrome *c* were obtained from Sigma Chemical Co., St. Louis, Mo. 7,12-Dimethylbenzanthracene, 3-methylcholanthrene, *o*-aminoazotoluene, and *N*-nitrosodimethylamine were obtained from Wako Pure Chemical Industries, Osaka, Japan. 2-Acetylaminofluorene was obtained from Nakarai Chemicals, Kyoto, Japan. Aflatoxin B_1 was obtained from Makor Chemicals, Jerusalem, Israel. The following chemical compounds were generous gifts: 2-naphthylamine from Dr. H. Nohira (Saitama University, Urawa, Japan), 7-ethoxycoumarin from Dr. V. Ullrich (Saarland University, Hamburg-Saar, Federal Republic of Germany), and benzphetamine from Dr. Y. Hirokata (Kyushu University, Fukuoka, Japan). Other chemicals were of reagent grade.

RESULTS

Characterization of the Liver Microsomes of PCB-treated Rats. Since the most widely used system for the metabolic activation of chemical carcinogens in the bacterial mutation system is the S-9 fraction of the livers of PCB-treated rats, we investigated the drug oxidation activities of the microsomal fraction prepared from the S-9 fraction. Table 1 shows the drug oxidation activities of the microsomes obtained from untreated and PCB-treated rats. NADPH-cytochrome *c* reductase and cytochrome P-450, the components of the microsomal monooxygenase, were both induced 2.4- and 3.2-fold, respectively, by PCB treatment. Aryl hydrocarbon hydroxylase, 7-ethoxycoumarin *O*-deethylase, and benzphetamine *N*-demethylase were also induced by the treatment by 8.5-, 12.3-, and 4.6-fold, respectively. Since aryl hydrocarbon hydroxylase and benzphetamine *N*-demethylase are mediated by MC-P-448 and PB-P-450 (33), respectively, these 2 types of cytochrome P-450 were both induced by PCB treatment.

Fig. 1 shows the results of Ouchterlony double-diffusion tests between monospecific antibodies and solubilized liver microsomes from the rats treated with different inducers. Monospecific anti-PB-P-450 immunoglobulin reacted with partially purified cytochrome P-450 from phenobarbital-treated rats and microsomes of phenobarbital-treated and PCB-treated rats to form a single fused precipitation line (Fig. 1*A*), whereas it did not react with partially purified MC-P-448 or with microsomes of 3-methylcholanthrene-treated rats. On the other hand, anti-MC-P-448 immunoglobulin reacted with partially purified MC-P-448, microsomes of 3-methylcholanthrene-treated rats, and also with microsomes of PCB-treated rats, forming a single fused precipitin line (Fig. 1*B*). Anti-MC-P-448 immunoglobulin did not react with PB-P-450 and microsomes of phenobarbital-treated rats. It was concluded that anti-PB-P-450 immunoglobulin and anti-MC-P-448 immunoglobulin were specific against PB-P-450 and MC-P-448, respectively, and that the cytochrome P-450 induced by the PCB treatment were molecularly identical with PB-P-450 and MC-P-448, obtained from phenobarbital- and 3-methylcholanthrene-treated rats, respectively.

Effects of Antibodies on Microsomal Drug Oxidations. Chart 1*A* shows the effect of the antibodies against 2 types of microsomal cytochrome P-450 on the activity of NADPH-supported benzo(*a*)pyrene hydroxylase of the liver microsomes of PCB-treated rats. Anti-MC-P-448 immunoglobulin inhibited the reaction by about 90% at a concentration of 4 mg antibody per nmol of microsomal cytochrome P-450, while anti-PB-P-450 immunoglobulin did not inhibit the reaction at all. 7-Ethoxycoumarin *O*-deethylase was inhibited by both antibody preparations. Anti-MC-P-448 immunoglobulin inhibited the reaction by 75%, whereas the inhibition by anti-PB-P-450 immunoglobulin was only 15% at a concentration of 7 mg of the antibody per nmol of cytochrome P-450 (Chart 1*B*). The reaction was completely inhibited when both anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin were included together in the reaction mixture (data not shown). Chart 1*C* shows the inhibitory effect of the antibodies on microsomal benzphetamine *N*-

[4] N. Harada, M. Noshiro, and T. Omura, manuscript in preparation.

Table 1

Drug-oxidation activities of liver microsomes from untreated and PCB-treated rats

Three untreated and 3 PCB-treated rats were used in preparing microsomes from their pooled livers.

Microsomes	NADPH-cytochrome *c* reductase (μmol reduced/min/mg)[a]	Cytochrome P-450 (nmol/mg)	Aryl hydrocarbon hydroxylase (nmol/min/mg)	7-Ethoxycoumarin *O*-deethylase (nmol/min/mg)	Benzphetamine *N*-demethylase (nmol/min/mg)
Untreated	0.09 ± 0.01[b]	0.92 ± 0.1	0.35 ± 0.04	0.69 ± 0.05	2.15 ± 0.2
PCB treated	0.22 ± 0.02	2.94 ± 0.2	2.98 ± 0.1	8.50 ± 0.4	9.94 ± 0.7

[a] mg of microsomal protein.
[b] Mean ± S.D. of 3 experiments.

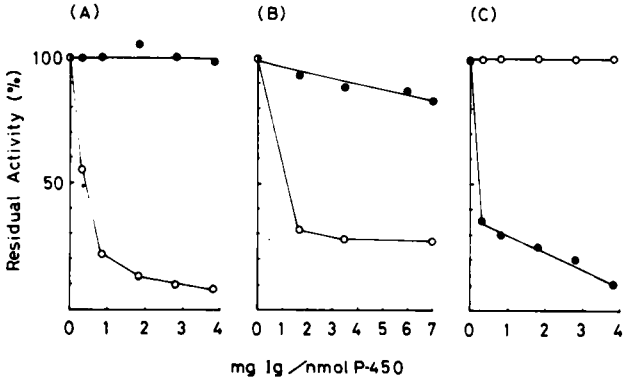

Chart 1. Effects of antibodies on microsomal drug oxidations. Liver microsomes of rats treated with PCB were preincubated with antibodies at 25° for 10 min. All reaction mixtures were made up to the same final concentration of immunoglobulin with control immunoglobulin. Activities in the presence of anti-PB-P-450 immunoglobulin (●) or anti-MC-P-448 immunoglobulin (○) are shown as the percentages of the activities assayed with control immunoglobulin. *A*, benzo(*a*)pyrene hydroxylation; *B*, 7-ethoxycoumarin *O*-deethylation; *C*, benzphetamine *N*-demethylation. The amounts of microsomes used in the assay of benzo(*a*)pyrene hydroxylase, 7-ethoxycoumarin *O*-deethylase, and benzphetamine *N*-demethylase were 50, 25, and 500 μg of protein, respectively.

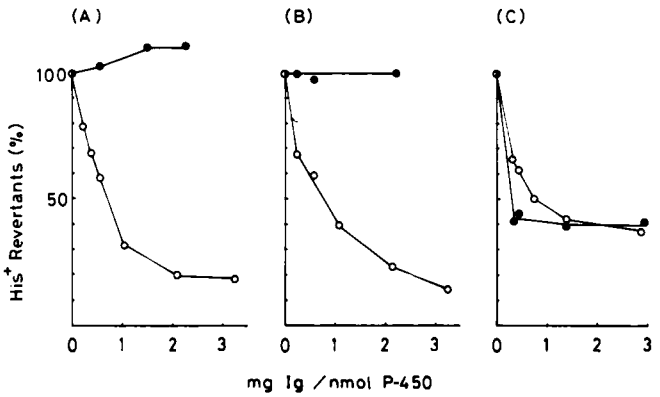

Chart 2. Effects of antibodies on mutagenicity of polycyclic aromatic hydrocarbons. Various amounts of antibodies were incubated with fixed amounts of the S-9 fraction of PCB-treated rats for 20 min at 25°. Appropriate amounts of control immunoglobulin were added to the assay mixtures so that the final concentrations of immunoglobulin were the same. The *his*+ revertants in the presence of anti-PB-P-450 immunoglobulin (●) or anti-MC-P-448 immunoglobulin (○) are shown as the percentages of the *his*+ revertants assayed with control immunoglobulin. *A*, benzo(*a*)pyrene, 5 μg, was incubated with 3.1 nmol of cytochrome P-450 in the S-9 fraction per plate in the presence of antibodies. There were 914 strain TA 98 *his*+ revertants in the presence of control immunoglobulin. *B*, 7,12-dimethylbenzanthracene, 20 μg, was incubated with 3.0 nmol of cytochrome P-450 in the S-9 fraction per plate in the presence of antibodies. The number of *his*+ revertants in the presence of control immunoglobulin was 302 by the use of strain TA 100. *C*, 3-methylcholanthrene, 50 μg, was incubated with 2.3 nmol of cytochrome P-450 in the S-9 fraction per plate in the presence of antibodies. There were 376 strain TA 98 number of *his*+ revertants in the presence of control immunoglobulin.

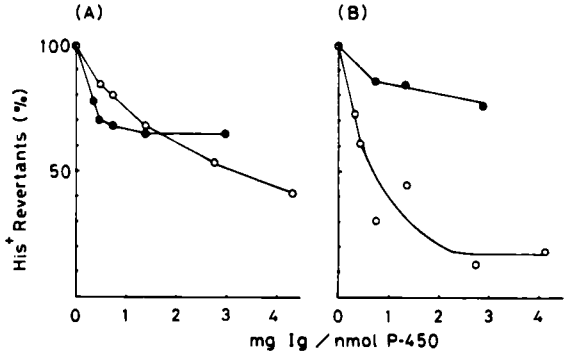

Chart 3. Effects of antibodies on the mutagenicity of aromatic amines. Experimental conditions were as described in the legend to Chart 2. *A*, 2-acetylaminofluorene, 10 μg, was incubated with 2.3 nmol of cytochrome P-450 in the S-9 fraction per plate in the presence of antibodies. There were 4420 strain TA 98 *his*+ revertants in the presence of control immunoglobulin. *B*, 2-naphthylamine, 100 μg, was incubated with 3.2 nmol of cytochrome P-450 in the S-9 fraction in the presence of antibodies. There were 148 strain TA 98 *his*+ revertants in the presence of control immunoglobulin. ●, anti-PB-P-450 immunoglobulin; ○, anti-MC-P-448 immunoglobulin.

demethylase activity. Anti-PB-P-450 immunoglobulin inhibited the activity by 90% at a concentration of 4 mg of the antibody per nmol of cytochrome P-450, whereas anti-MC-P-448 immunoglobulin did not inhibit the reaction at all. These results confirm that anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin specifically inhibit the drug oxidation reactions catalyzed by corresponding types of cytochrome P-450 in microsomes.

Effects of Antibodies on the Mutagenic Activity of Chemical Carcinogens. Chart 2 shows the effect of the antibodies on the mutagenic activities of polycyclic aromatic hydrocarbons. The mutagenic activities of benzo(*a*)pyrene and 7,12-dimethylbenzanthracene were inhibited by 82 and 85%, respectively, at a concentration of 3.2 mg of anti-MC-P-448 immunoglobulin per nmol of cytochrome P-450. Anti-PB-P-450 immunoglobulin did not inhibit the mutagenicity of these 2 carcinogens (Chart 2, *A* and *B*). Chart 2*C* shows the inhibitory effect of the antibodies on the mutagenicity of 3-methylcholanthrene. Both anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin inhibited the mutagenicity of 3-methylcholanthrene by about 60% at a concentration of 3 mg of the antibodies per nmol of cytochrome P-450.

Chart 3 shows the effects of antibodies on the mutagenicity of aromatic amines. The mutagenicity of 2-acetylaminofluorene was inhibited by about 60% by anti-MC-P-448 immunoglobulin at a concentration of 4.3 mg of the antibody per nmol of cytochrome P-450. It was also inhibited by anti-PB-P-450 immunoglobulin by 35% at a concentration of 3 mg of the antibody per nmol of cytochrome P-450 (Chart 3*A*). Anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin inhibited the mutagenic activity of 2-naphthylamine by about 80 and 25%, respectively, at a concentration of 3 mg of antibodies

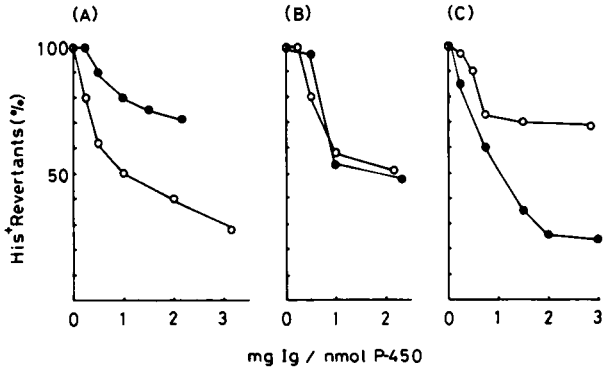

Chart 4. Effects of antibodies on mutagenicities of *o*-aminoazotoluene, *N*-nitrosodimethylamine, and aflatoxin B_1. Experimental conditions were as described in the legend to Chart 2. *A*, *o*-aminoazotoluene, 50 μg, was incubated with 3.1 nmol of cytochrome P-450 in the S-9 fraction per plate in the presence of antibodies. There were 1450 strain TA 98 *his*$^+$ revertants in the presence of control immunoglobulin. *B*, *N*-nitrosodimethylamine, 5 mg, was incubated with 3.0 nmol of cytochrome P-450 in the S-9 fraction per plate in the presence of antibodies. There were 296 strain TA 100 *his*$^+$ revertants in the presence of control immunoglobulin. *C*, aflatoxin B_1, 0.1 μg, was incubated with 2.3 nmol of cytochrome P-450 in the S-9 fraction per plate in the presence of antibodies. There were 3306 strain TA 98 *his*$^+$ revertants in the presence of control immunoglobulin. ●, anti-PB-P-450 immunoglobulin; ○, anti-MC-P-448 immunoglobulin.

per nmol of cytochrome P-450 (Chart 3*B*).

Chart 4*A* shows the inhibitory effect of the antibodies on the mutagenic activity of *o*-aminoazotoluene. The mutagenicity was inhibited by 70 and 30% by anti-MC-P-448 immunoglobulin (3.2 mg/nmol of cytochrome P-450) and anti-PB-P-450 immunoglobulin (2.2 mg/nmol of cytochrome P-450), respectively.

The primary metabolism of *N*-nitrosodimethylamine *N*-demethylation is carried out by cytochrome P-450-dependent monooxygenase (6). The inhibition of the mutagenicity of *N*-nitrosodimethylamine by 2 antibodies was almost identical. Fifty % inhibition of the mutagenicity was obtained by both anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin at a concentration of 2.2 mg of the antibodies per nmol of cytochrome P-450 (Chart 4*B*).

Chart 4*C* shows the effect of the antibodies on the mutagenicity of aflatoxin B_1. In the presence of excess amounts of antibodies, the mutagenic activity was inhibited by 30 and 75% by anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin, respectively.

DISCUSSION

In this study, we prepared and used 2 kinds of antibodies, each of which specifically reacted with PB-P-450 or with MC-P-448, in order to elucidate the role of these 2 molecular species of cytochrome P-450 in the metabolic activation of chemical carcinogens catalyzed by liver microsomes of PCB-treated rats. Although many investigators have studied the mechanism of metabolic activation of chemical carcinogens by liver microsomes using inducers or chemical inhibitors of cytochrome P-450, such approaches seem to be restricted by the limited specificity of the inducers and inhibitors. Recently, several reports demonstrated directly the role of specific forms of cytochrome P-450 in the metabolic activation of chemical carcinogens by the use of reconstituted systems of microsomal monooxygenase using purified cytochrome P-450 preparations (7, 27, 44–47). The reconstituted systems were very useful in assessing the participation of each one of the multiple molecular species of cytochrome P-450 in the metabolic activation of chemical carcinogens. However, since various forms of cytochrome P-450 coexist in the microsomal membrane (35), studies with reconstituted systems do not seem suited for the evaluation of the relative importance of each cytochrome P-450 species in the activation of chemical carcinogens by intact microsomes. The use of antibodies against specific forms of cytochrome P-450 in the bacterial mutation system enabled us to determine the relative importance of specific forms of cytochrome P-450 in the complete Ames test system (47).

The specificity of the antibodies used in our experiments was confirmed by Ouchterlony double-diffusion tests (Fig. 1) and by their inhibition effects on 3 microsomal drug oxidation activities (Chart 1). Our results, showing that benzo(*a*)pyrene hydroxylation and benzphetamine *N*-demethylation were completely inhibited by anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin, respectively, are compatible with the substrate specificities of these 2 types of cytochrome P-450, as studied by the use of reconstituted monooxygenase systems, using purified cytochrome P-450 preparations (33).

Benzo(*a*)pyrene has been intensively investigated in relation to its metabolism, its mutagenesis, and its carcinogenesis. Recent reports suggested that specific forms of cytochrome P-450 preferentially activate benzo(*a*)pyrene in reconstituted systems using highly purified cytochrome P-450 preparations from rat (45) and rabbit (7, 27) liver microsomes. Wood *et al.* (45) clearly demonstrated the role of cytochrome P-448 in benzo(*a*)pyrene-induced mutagenesis by using purified enzyme preparations prepared from rat liver microsomes. On the other hand, Deutsch *et al.* (7) suggested from their studies on reconstituted systems, using purified cytochrome P-450 preparations from rabbit liver microsomes, that 2 forms of cytochrome P-450 are involved in the activation of benzo(*a*)pyrene to the benzo(*a*)pyrene-7,8-diol-9,10-epoxide. $P\text{-}450_{LM2}$ was more active than $P\text{-}450_{LM4}$ in the oxidation of benzo(*a*)pyrene, but the reverse was true when the substrate was benzo(*a*)-pyrene-trans-7,8-diol, which is believed to be the immediate precursor of the ultimate carcinogen, the corresponding 9,10-epoxide. The difference in their observations may be explained by the species difference in the metabolism of benzo(*a*)pyrene. Our immunochemical study showed that the mutagenicity of benzo(*a*)pyrene was completely inhibited by anti-MC-P-448 immunoglobulin and was not inhibited by anti-PB-P-450 immunoglobulin. This observation suggests that MC-P-448, the major component of cytochrome P-450 in the liver microsomes of 3-methylcholanthrene-treated rats, preferentially activates benzo(*a*)pyrene in the microsomes of PCB-treated rats and is compatible with the results of Wood *et al.* (45). The effect of our antibodies on the mutagenicity of 7,12-dimethylbenzanthracene was identical with that of benzo(*a*)pyrene (Chart 2, *A* and *B*). However, the effect of our antibodies on the mutagenicity of 3-methylcholanthrene was different from that of benzo(*a*)pyrene and 7,12-dimethylbenzanthracene, although all of them belong to the same class of polycyclic aromatic hydrocarbons. Judging from the inhibitory action of the antibodies, both species of cytochrome P-450 activate 3-methylcholanthrene to mutagenic forms. Since several metabolites of 3-methylcholanthrene possess high mutagenic activity (44), different metabolites might have been formed by PB-P-450 and MC-P-448, respectively.

It has been known that the first step of metabolic activation of 2-acetylaminofluorene and 2-naphthylamine is the *N*-hydroxylation by microsomal monooxygenase (5, 32, 39). Since the inducing effects by 3-methylcholanthrene on the formation of *N*-hydroxy-2-acetylaminofluorene (38, 40), on the binding of 2-acetylaminofluorene to macromolecules (13), and on the mutagenicity of this carcinogen (8) were larger than those of phenobarbital, MC-P-448 must predominantly activate this carcinogen in microsomes. The results of our present study clearly demonstrated that both MC-P-448 and PB-P-450 participate in the mutagenicity of 2-acetylaminofluorene, although the contribution of the former is larger than that of the latter (Chart 3*A*). We also observed that the binding of 2-acetylaminofluorene to the nuclear DNA catalyzed by nuclear monooxygenase (17, 18) or by microsomes was mediated by these 2 species of cytochrome P-450 (data not shown).

o-Aminoazotoluene is one of the typical carcinogens among azo dyes. Although little is known about the metabolic activation of this carcinogen, it is likely that *N*-hydroxylation of *o*-aminoazotoluene by microsomes is the first activation step (34). Of the 2 species of cytochrome P-450 examined in this study, MC-P-448 mainly activated *o*-aminoazotoluene in the bacterial mutation system. Similar results were obtained with 2-acetylaminofluorene and 2-naphthylamine. However, Kadlubar *et al.* (16) reported that *N*-hydroxylation of *N*-methyl-4-aminoazobenzene was catalyzed by the microsomal flavoprotein mixed-function amine oxidase but not by the cytochrome P-450-dependent monooxygenase system. The effects of antibodies on the mutagenicity of *N*-methyl-4-aminoazobenzene and other azo dyes are currently under investigation in our laboratory.

Aflatoxin B_1, produced by the mold *Aspergillus flavus*, is one of the important environmental hepatocarcinogens (42). Aflatoxin B_1 is activated to aflatoxin B_1 2,3-oxide, which is possibly involved in the carcinogenesis, mutagenesis, and toxicity of this carcinogen (9, 12). Other microsomal metabolites of this carcinogen are less than 5% as active as aflatoxin B_1 (43). Recently, Gurtoo *et al.* (11) suggested, based on their genetic analysis, that the metabolism of aflatoxin B_1 to aflatoxin B_1 2,3-oxide and to aflatoxin M_1 is preferentially mediated by cytochrome P-450- and P-448-dependent reactions, respectively. On the other hand, Alexandrov and Frayssinet (1) concluded that the metabolism of aflatoxin B_1 to DNA-binding metabolites was catalyzed by aryl hydrocarbon hydroxylase. The results of our present study, using specific antibodies to 2 forms of cytochrome P-450, showed that PB-P-450 preferentially catalyzed the conversion of aflatoxin B_1 to mutagenic active forms.

A summary of the inhibition profiles of various chemical carcinogens by the antibodies against 2 types of cytochrome P-450 is shown in Chart 5. Judging from this chart, we can divide chemical carcinogens into 4 classes according to the dependency on the 2 forms of cytochrome P-450: (*a*) activated selectively by MC-P-448; (*b*) activated predominantly by MC-P-448; (*c*) activated equally by both MC-P-448 and PB-P-450; and (*d*) activated predominantly by PB-P-450. We showed in this study that the use of antibodies against specific forms of cytochrome P-450 in the Ames test system enabled us to determine the relative importance of specific forms of cytochrome P-450 in the mutagenesis by chemical carcinogens.

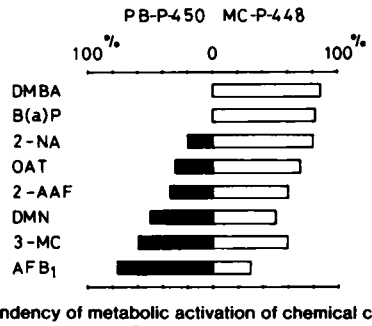

Chart 5. Dependency of metabolic activation of chemical carcinogens on the 2 types of cytochrome P-450. Summarizes the effects of antibodies on the mutagenicity of 8 chemical carcinogens. The percentages of the inhibition of mutagenicity in the presence of excess amounts of anti-MC-P-448 immunoglobulin (□) and anti-PB-P-450 immunoglobulin (■) are indicated. These values indicate the dependency of the mutagenicity on the corresponding molecular species of cytochrome P-450. *DMBA*, 7,12-dimethylbenzanthracene; *B(a)P*, benzo(*a*)pyrene; *2-NA*, 2-naphthylamine; *OAT*, *o*-aminoazotoluene; *2-AAF*, 2-acetylaminofluorene; *DMN*, *N*-nitrosodimethylamine; *3-MC*, 3-methylcholanthrene; *AFB*$_1$, aflatoxin B_1.

REFERENCES

1. Alexandrov, K., and Frayssinet, C. Microsome-dependent binding of benzo(*a*)pyrene and aflatoxin B_1 to DNA, and benzo(*a*)pyrene binding to aflatoxin-conjugated DNA. Cancer Res., *34:* 3289–3295, 1974.
2. Ames, B. N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat. Res., *31:* 347–364, 1975.
3. Conney, A. H. Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev., *19:* 317–366, 1967.
4. Coon, M. J., Chiang, Y. L., and French, J. Chemical characterization of the enzymes involved in drug metabolism. *In*: R. W. Estabrook and L. Lindenlaub (eds.), The Induction of drug metabolism, pp. 201–211. Stuttgart: F. K. Schattauer-Verlag, 1979.
5. Cramer, J. W., Miller, J. A., and Miller, E. C. *N*-Hydroxylation: a new metabolic activation observed in the rat with the carcinogen 2-acetylaminofluorene. J. Biol. Chem., *235:* 885–888, 1960.
6. Czygan, P., Greim, H., Garro, A. J., Hutterer, F., Schaffner, F., Popper, H., Rosenthal, O., and Cooper, D. Y. Microsomal metabolism of dimethylnitrosoamine and cytochrome P-450 dependency of its activation to a mutagen. Cancer Res., *33:* 2983–2986, 1973.
7. Deutsch, J., Leutz, J. C., Yang, S. K., Gelboin, H. V., Chiang, Y. L., Vatsis, K. P., and Coon, M. J. Regio- and stereoselectivity of various forms of purified cytochrome P-450 in the metabolism of benzo(*a*)pyrene and (−)*trans*-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene as shown by product formation and binding to DNA. Proc. Natl. Acad. Sci. U. S. A., *74:* 3123–3127, 1978.
8. Felton, J. S., and Nebert, D. W. Mutagenesis of certain activated carcinogens *in vitro* associated with genetically mediated increase in monooxygenase activity and cytochrome P_1-450. J. Biol. Chem., *250:* 6769–6778, 1975.
9. Garner, R. C., Miller, E. C., and Miller, J. A. Liver microsomal metabolism of aflatoxin B_1 to a reactive derivative toxic to *Salmonella typhimurium* TA 1530. Cancer Res., *32:* 2058–2066, 1972.
10. Guengerich, F. P. Separation and purification of multiple forms of microsomal cytochrome P-450. J. Biol. Chem., *253:* 7931–7939, 1978.
11. Gurtoo, H. L., Dahms, R. P., Kanter, P., and Vaught, J. B. Association and dissociation of the Ah locus with the metabolism of aflatoxin B_1 by mouse liver. J. Biol. Chem., *253:* 3952–3961, 1978.
12. Gurtoo, H. L., and Dave, C. V. *In vitro* metabolic conversion of aflatoxins and benzo(*a*)pyrene to nucleic acid-binding metabolites. Cancer Res., *35:* 382–389, 1975.
13. Hara, E., Yonekawa, H., Kawajiri, K., Masuda A., and Tagashira, Y. *In vitro* binding of 2-acetaminofluorene to RNA and protein with rat liver microsomes. Gann, *69:* 533–538, 1978.
14. Heidelberger, C. Chemical carcinogenesis. Annu. Rev. Biochem., *44:* 79–121, 1975.
15. Imai, Y., and Sato, R. A gel-electrophoretically homogeneous preparation of cytochrome P-450 from liver microsomes of phenobarbital-pretreated rabbits. Biochem. Biophys. Res. Commun., *60:* 8–14, 1974.
16. Kadlubar, F. F., Miller, J. A., and Miller, E. C. Microsomal *N*-oxidation of the hepatocarcinogen *N*-methyl-4-aminoazobenzene and the reactivity of *N*-hydroxy-*N*-methyl-4-aminoazobenzene. Cancer Res., *36:* 1196–1206, 1976.
17. Kawajiri, K., Yonekawa, H., Hara, E., and Tagashira, Y. Biochemical basis for the resistance of guinea pigs to carcinogenesis by 2-acetylaminofluorene. Biochem. Biophys. Res. Commun., *85:* 959–965, 1978.
18. Kawajiri, K., Yonekawa, H., Hara, E., and Tagashira, Y. Activation of 2-acetylaminofluorene in the nuclei of rat liver. Cancer Res., *39:* 1089–1093, 1979.

19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265–275, 1951.
20. McCann, J., and Ames, B. N. Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. U. S. A., *73:* 950–954, 1976.
21. McCann, J., Choi, E., Yamasaki, E., and Ames, B. N. Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. U. S. A., *72:* 5135–5139, 1975.
22. Miller, E. C., and Miller, J. A. Biochemical mechanisms of chemical carcinogenesis. *In*: H. Busch (ed.), The Molecular Biology of Cancer, pp. 377–402. New York: Academic Press, Inc., 1974.
23. Nagao, M., Sugimura, T., and Matsushima, T. Environmental mutagens and carcinogens. Annu. Rev. Genet., *12:* 117–159, 1978.
24. Nagao, M., Suzuki, E., Yasuo, K., Yahagi, T., Seino, Y., Sugimura, T., and Okada, M. Mutagenicity of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, a bladder carcinogen, and related compounds. Cancer Res., *37:* 399–407, 1977.
25. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem. J., *55:* 416–421, 1953.
26. Nebert, D. W., and Gelboin, H. V. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. J. Biol. Chem., *243:* 6242–6249, 1968.
27. Norman, R. L., Muller-Eberhard, U., and Johnson, E. F. The role of cytochrome P-450 forms in 2-aminoanthracene and benzo(*a*)pyrene mutagenesis. Biochem. Biophys. Res. Commun., *89:* 195–201, 1979.
28. Noshiro, M., and Omura, T. Immunochemical study on the electron pathway from NADH to cytochrome P-450 of liver microsomes. J. Biochem., *83:* 61–77, 1978.
29. Okuno, S., Takeishi, K., and Seno, T. Differential effect of a microsomal deacetylase inhibitor on the mutagenicity in *Salmonella typhimurium* of 2-acetylaminofluorene by liver homogenates of guinea pigs, mice and rats. Cancer Lett., *6:* 1–5, 1979.
30. Omura, T., and Sato, R. The carbon monoxide-binding pigment of liver microsomes. J. Biol. Chem., *239:* 2370–2378, 1964.
31. Omura, T., and Takesue, S. A new method for simultaneous purification of cytochrome b_5 and NADPH-cytochrome *c* reductase from rat liver microsomes. J. Biochem., *67:* 249–257, 1970.
32. Radomski, J. L., Brill, E., Deichmann, W. B., and Glass, E. M. Carcinogenicity testing of *N*-hydroxy and other oxidation and decomposition products of 1- and 2-naphthylamine. Cancer Res., *31:* 1461–1467, 1971.
33. Ryan, D. E., Thomas, P. E., Karzeniowski, D., and Levin, W. Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. J. Biol. Chem., *254:* 1365–1374, 1979.
34. Terayama, H. Aminoazo carcinogenesis. Methods and biochemical problems. Methods Cancer Res., *1:* 399–449, 1967.
35. Thomas, P. E., Korzeniowski, D., Ryan, D., and Levin, W. Preparation of monospecific antibodies against two forms of rat liver cytochrome P-450 and quantitation of these antigens in microsomes. Arch. Biochem. Biophys., *192:* 524–532, 1979.
36. Thomas, P. E., Lu, A. H. Y., Ryan, D., West, S. B., Kawalek, J., and Levin, W. Multiple forms of rat liver cytochrome P-450. Immunochemical evidence with antibody against cytochrome P-448. J. Biol. Chem., *251:* 1385–1391, 1976.
37. Thorgeirsson, S. S., Atlas, S. A., Boobis, A. R., and Felton, J. S. Species differences in the substrate specificity of hepatic cytochrome P-448 from polycyclic hydrocarbon-treated animals, Biochem. Pharmacol., *28:* 217–226, 1979.
38. Thorgeirsson, S. S., Felton, J. S., and Nebert, D. W. Genetic differences in the aromatic hydrocarbon-inducible *N*-hydroxylation of 2-acetylaminofluorene and acetaminophen-produced hepatotoxicity in mice. Mol. Pharmacol., *11:* 159–165, 1975.
39. Thorgeirsson, S. S., Jollow, D. J., Sasame, H. A., Green, I., and Mitchell, J. R. The role of cytochrome P-450 in *N*-hydroxylation of 2-acetylaminofluorene. Mol. Pharmacol., *9:* 398–404, 1973.
40. Thorgeirsson, S. S., and Nelson, W. L. Separation and quantitative determination of 2-acetylaminofluorene and its hydroxylated metabolites by high pressure liquid chromatography. Anal. Biochem., *75:* 122–128, 1976.
41. Ullrich, V., and Weber, P. The *o*-dealkylation of 7-ethoxycoumarin by liver microsomes. A direct fluorometric test. Hoppe-Seyler's Z. Physiol. Chem., *353:* 1171–1177, 1972.
42. Wogan, G. N. Aflatoxin carcinogenesis. Methods Cancer Res., *7:* 309–344, 1973.
43. Wong, J. J., and Hsieh, D. P. H. Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. Proc. Natl. Acad. Sci. U. S. A., *73:* 2241–2244, 1976.
44. Wood, A. W., Chang, R. L., Levin, W., Thomas, P. E., Ryan, D., Stoming, T. A., Thakker, D. R., Jerina, D. M., and Conney, A. H. Metabolic activation of 3-methylcholanthrene and its metabolites to products mutagenic to bacterial and mammalian cells. Cancer Res., *38:* 3398–3404, 1978.
45. Wood, A. W., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M., and Conney, A. H. Metabolism of benzo(*a*)pyrene and benzo(*a*)pyrene derivatives to mutagenic products by highly purified hepatic microsomal enzymes. J. Biol. Chem., *251:* 4882–4890, 1976.
46. Wood, A. W., Levin, W., Thakker, D. R., Yagi, H., Chang, R. L., Ryan, D. E., Thomas, P E., Dansette, P. M., Whittaker, N., Turujman, S., Lehr, R. E., Kumar, S., Jerina, D. M., and Conney, A. H. Biological activity of benzo(*e*)pyrene. J. Biol. Chem., *254:* 4408–4415, 1979.
47. Wood, A. W., Levin, W., Thomas, P. E., Ryan, D., Karle, J. M., Yagi, H., Jerina, D. M., and Conney, A. H. Metabolic activation of dibenzo(*a*, *h*)anthracene and its dihydrodiols to bacterial mutagens. Cancer Res., *38:* 1967–1973, 1978.

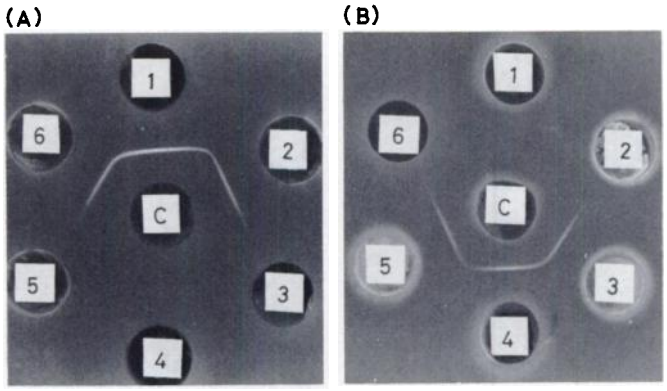

Fig. 1. Ouchterlony immunodiffusion tests of antibodies. The gel plate contained 1.2% agar (Difco Co.), 0.9% NaCl, 0.02% NaN_3, and 0.5% sodium cholate in 50 mM phosphate buffer (pH 7.5). The diameter of the wells was 4 mm, and the well-to-well distance was 12 mm. *A*, center well (*C*) was filled with anti-PB-P-450 Ig. *Wells 1* and *4* contained partially purified PB-P-450 and MC-P-448 from liver microsomes of rats treated with phenobarbital and 3-methylcholanthrene, respectively. *Wells 2, 5,* and *6* were filled with detergent-solubilized liver microsomes of rats treated with PCB, 3-methylcholanthrene, and phenobarbital, respectively. *Well 3*, buffer only. *B*, center well (*C*) was filled with anti-MC-P-448 Ig. *Wells 1* and *4* contained partially purified PB-P-450 and MC-P-448, respectively. *Wells 2, 3,* and *5* were detergent-solubilized microsomes from rats treated with phenobarbital, PCB, and 3-methylcholanthrene, respectively. *Well 6*, buffer only. Microsomes were solubilized with 0.5% cholate in the presence of 20% glycerol.