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PARTICIPATION OF THE MICROSOMAL ELECTRON TRANSPORT SYSTEM IN MUTAGENIC ACTIVATION OF 4-DIMETHYLAMINOAZOBENZENE, 4-METHYLAMINOAZOBENZENE AND THEIR 3'-METHYL-DERIVATIVES *

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For the examination of the participation of the microsomal electron transport system in mutagenic activation by 4-dimethylaminoazobenzene (DAB), 4-methylaminoazobenzene (MAB) and their 3'-methyl-derivatives (3'-methyl-DAB and 3'-methyl-MAB), monospecific antibodies to NADPH-cytochrome P-450 reductase, 3-methylcholanthrene-inducible major P-450 (MC-P-448) and phenobarbital-inducible major P-450 (PB-P-450) were used. In Ames' assay system, the antibody to NADPH-cytochrome P-450 reductase inhibited the mutagenicities of DAB, MAB, 3'-methyl-DAB and 3'-methyl-MAB by 94, 94, 90 and 87%, respectively. The antibody to MC-P-448 inhibited their mutagenicities by more than 90%, while the antibody to PB-P-450 inhibited the mutagenicities less than 20%. These results indicate that the microsomal electron transport system, especially MC-P-448, is involved in activation of these dyes.

INTRODUCTION

Many chemical carcinogens are metabolically activated to forms that have harmful effects on organisms (1). This metabolic activation is an obligatory initiation step in chemical carcinogenesis and the microsomal electron transport system including cytochrome P-450s plays the most important role in oxidation of chemicals (2). In rats, at least five molecular species of cytochrome P-450 have been distinguished biochemically and immunologically (3-8). We previously reported that many chemical carcinogens are mainly catalyzed to

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ABBREVIATIONS: MC-P-448, 3-methylcholanthrene-inducible major cytochrome P-450; PB-P-450, phenobarbital-inducible major cytochrome P-450; PCB, polychlorinated biphenyls; Ig, immunoglobulin; DAB, 4-dimethylaminoazobenzene; MAB, 4-methylaminoazobenzene; AB, 4-aminoazobenzene.

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active mutagens by two species of cytochrome P-450, MC-P-448 and PB-P-450, and that the contributions of these two species of proteins to mutagenic activation differ with different species of chemical carcinogens (9, 10).

DAB is a potent hepatocarcinogen (11) and Kadlubar et al. reported that it is metabolically activated by two different microsomal enzyme systems: mixed function amine oxidase and the electron transport system (12). Tarpley et al. suggested that the former is more important than the latter in activation of DAB in vivo (13).

Most chemical carcinogens are mutagens (14-17), and DAB and its derivatives also have mutagenicity. For determination of the ultimate form(s) in mutagenesis, many metabolites of DAB have been synthesized and their mutagenicities have been examined. From the results, N-hydroxy-derivatives have been suggested to be ultimate mutagens (18-21). However, little is known about the enzyme(s) that participates in their mutagenic activation.

In this work, we studied the enzyme(s) involved in mutagenic activation of DAB, MAB and their 3'-methyl-derivatives using the antibodies to three components of the electron transport system. The results indicated that the electron transport system is involved in mutagenic activation of amino azo dyes.

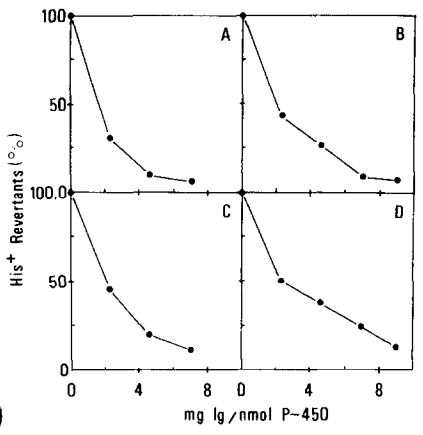
MATERIALS AND METHODS

1) Materials: Male Sprague Dawley rats weighing 200-250 g were used. DAB, MAB and 3'-methyl-DAB were purchased from Woko Chemical Co., Tokyo. 3'-methyl-MAB was synthesized as described previously (22). PCB (Kanechlor KC-500) was obtained from Gasukuro Kogyo Co., Tokyo. NADPH and NADH were from Oriental Yeast Co., Tokyo. ATP and norharman were obtained from Boehringer-Mannheim Yamanouchi, Tokyo and Aldrich Chemical Co., Milwaukee, respectively. Other chemicals were standard products of reagent grade.

2) Analytical procedures: The total microsomal content of cytochrome P-450 was determined by the method of Omura and Sato (23). Microsomal drug oxidation in the presence of antibodies was assayed as described previously (9). Protein contents were determined by the method of Lowry et al. (24) with bovine serum albumin as a standard.

3) Preparation of the S-9 fraction and microsomes from PCB-treated rat liver: The S-9 fraction and microsomes were prepared as described previously (9). The specific content of total cytochrome P-450 in the microsomes was 2.7 nmoles/mg protein. The contents of MC-P-448 and PB-P-450 were determined to be 36% and 45%, respectively, of the total P-450 by immunoprecipitation (3).

4) Preparation and purification of antibodies to components of the microsomal electron transport system: NADPH-cytochrome P-450 reductase was purified from the liver microsomes of rats pretreated with PB as described by Omura and Takesue (25). MC-P-448 and PB-P-450 were prepared from liver microsomes of rats pretreated with MC and PB, respectively, as described by Harada and Omura (3). Antibodies to these proteins and control Ig were prepared and purified as described previously (9). Each antibody was confined to be monospecific to the



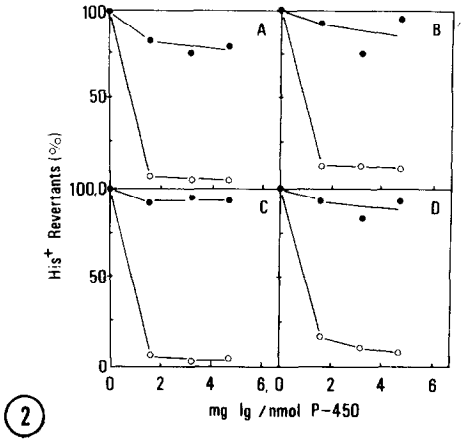


Figure 1. Effect of antibody to NADPH-cytochrome P-450 reductase on the mutagenic activities of DAB (A), MAB (B), 3'-methyl-DAB (C) and 3'-methyl-MAB (D). 12.6 μg of DAB or 3'-methyl-DAB, or 6.25 μg of MAB, 3'-methyl-MAB was incubated with 1.8 nmoles of total cytochrome P-450 in the S-9 fraction of liver from PCB-treated rats in the presence of antibody. The His^+ revertants in the presence of antibody are shown in the figures as percentages of the revertants with control Ig, the numbers of which were 387 with DAB; 596 with MAB; 431 with 3'-methyl-DAB; and 572 with 3'-methyl-MAB.

Figure 2. Effects of antibodies to MC-P-448 and PB-P-450 on the mutagenic activities of DAB (A), MAB (B), 3'-methyl-DAB (C) and 3'-methyl-MAB (D). The experimental conditions were as for Figure 1. o MC-P-448; • PB-P-450.

corresponding antigen by Ouchterlony double diffusion tests and profiles of inhibition of drug oxidation (9).

5) Mutagenesis assay: Ames' *Salmonella typhimurium* strain TA-98 was used. Pretreatment of S-9 fraction with antibodies and mutagenesis assay were carried out as described previously (9), except that 4 mM NADH and 5 mM ATP were added to the system as co-factors and the co-mutagen norharman was added at 200 μg/plate as described by Nagao *et al.* (19).

RESULTS

Since NADPH-cytochrome P-450 reductase catalyzes electron transport from NADPH to all forms of cytochrome P-450, the antibody to this flavoprotein inhibits the total activity of the electron transport system (2). Thus, the antibody technique is very useful for determining whether a certain carcinogen or mutagen is activated by the electron transport system. Fig. 1 shows the inhibition profiles of the mutagenicities of DAB (A) and MAB (B) by this antibody. The mutagenicities of DAB and MAB were inhibited 94 and 92%, respectively, by a concentration of 7.6 mg per nmole of total cytochrome P-450. Similar inhibitions of mutagenicity were observed with their 3'-methyl-derivatives: 84% inhibition with 3'-methyl-DAB and 90% with 3'-methyl-MAB (Fig. 1C, D).

These results indicate that the electron transport system catalyzes the formation of active mutagens from all these four amino azo dyes.

The next question was what form of cytochrome P-450 participates in their mutagenic activation. Fig. 2 shows the inhibition profiles of antibodies against MC-P-448 and PB-P-450 on the mutagenicities of DAB (A), MAB (B), 3'-methyl-DAB (C) and 3'-methyl-MAB (D). The mutagenicities of DAB, MAB, 3'-methyl-DAB and 3'-methyl-MAB were inhibited 94, 89, 97 and 92%, respectively, by antibody to MC-P-448 at a concentration of 4.6 mg Ig per nmole of total cytochrome P-450. On the other hand, under similar conditions the mutagenicities of the dyes were inhibited only 20, 5, 8 and 10%, respectively, by antibody to PB-P-450. Under these conditions, the antibody to PB-P-450 caused more than 95% inhibition of the activity of benzphetamine N-demethylase in microsomes of rats treated with PCB (data not shown). The above results indicate that activation of these amino azo dyes is mainly due to MC-P-448. Since the sum of the percentage inhibitions by anti-MC-P-448 Ig and anti-PB-P-450 Ig almost coincides with that by anti-NADPH-cytochrome P-450 reductase Ig, it is concluded that these two molecular species of cytochrome P-450 mediate mostly in activation of these dyes.

DISCUSSION

In *in vivo* experiments in mice, Tarpley *et al.* showed that the main binding adduct of DAB and MAB to DNA was derived from N-hydroxy-MAB (13). Kadlubar *et al.* reported that N-oxidation of MAB, measured indirectly by formation of N-hydroxy-AB, was catalyzed by a mixed function amine oxidase purified from hog liver microsomes. In rat liver microsomes, 63-84% of the N-oxidation was insensitive to 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine (DPEA), an inhibitor of cytochrome P-450-dependent oxidation (12). These observations suggest that another system than the electron transport system, perhaps mixed function amine oxidase, is most important for covalent binding of DAB or MAB to DNA. It is natural to consider that the enzyme should also catalyze the mutagenic activation of MAB, since the covalent binding of chemical carcinogen to DNA is an obligatory step in chemical mutagenesis.

Our results, however, are inconsistent with the above-mentioned observations. Namely, the inhibition of MAB mutagenesis by anti-NADPH-cytochrome P-450 reductase Ig clearly indicates that the microsomal electron transport system participates in mutagenic activation of MAB (Fig. 1B).

Possible reasons for this discrepancy are as follows: 1) Ziegler and Paulsen reported that the amine oxidase is present in unusually high concentrations in hog and human liver (26). No activation of this enzyme by octylamine was observed in rats or rabbits in contrast to hogs and guinea pigs (27). Kadulbar et al. also reported that the rate of this N-oxidation differed in different animal species (12). Thus, species differences in the activity and characteristics of the amine oxidase should be considered. 2) We used male rats of 7-10 weeks old. These young rats have about 40% of DPEA-sensitive N-oxidation activity, which is regarded as the activity catalyzed by the electron transport system (12). Further evidence suggesting participation of the electron transport system in N-oxidation was obtained by Kimura et al. (28). From these observations and our results, it seems possible that not only amine oxidase but also the electron transport system could be important in oxidation activity in vivo as well as in the in vitro conditions used in the present study. 3) Since chemical carcinogens are metabolized through multiple pathways, several metabolites may have mutagenic activity. In the case of DAB, N-hydroxy-compounds such as N-hydroxy-AB as well as N-hydroxy-MAB have strong mutagenicity (18-21). Kadlubar et al. suggested that both N-demethylation of MAB and N-oxidation of AB are catalyzed by the microsomal electron transport system (12). Thus, our results are consistent with the observations of Kadlubar et al. (12), if the mutagenicity of MAB is mainly due to N-hydroxy-AB or other active metabolites that are formed by the electron transport system.

As shown in Fig. 2, both MC-P-448 and PB-P-450 participate in most of the mutagenic activation of DAB, MAB and their 3'-methyl-derivatives. These results are important in considering activation mechanisms in chemical mutagenesis because even these amino azo dyes, which are thought to be mainly metabolized by other routes than by the microsomal electron transport system, are

mutagenetically activated by both MC-P-448 and PB-P-450, like other chemical carcinogens examined previously (9, 10). Thus, these results strongly support our idea that mutagenic activation of chemical carcinogens is mainly catalyzed by these two P-450s (9).

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