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ACTIVATION OF 6-AMINOCHRYSENE TO GENOTOXIC PRODUCTS BY DIFFERENT FORMS OF RAT LIVER CYTOCHROME P450 IN AN *O*-ACETYLTRANSFERASE-OVEREXPRESSING *SALMONELLA TYPHIMURIUM* STRAIN (NM2009)

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Abstract—Metabolic activation of a potent mutagen, 6-aminochrysene, to genotoxic products in a newly developed tester strain, *Salmonella typhimurium* NM2009, was studied in a rat liver microsomal monooxygenase system containing cytochrome P450 (P450). Since the tester strain was constructed by introducing an *O*-acetyltransferase gene into the original strain *S. typhimurium* TA1535/pSK1002, it is highly sensitive toward the reactive metabolites of carcinogenic arylamines. DNA-damaging activities of 6-aminochrysene were detected at very low concentrations of substrate (between 0.01 and 0.2 μM) and liver microsomes (from 0.2 to 2 μg protein/mL) in the *S. typhimurium* NM2009 strain. Thus, the potency of genotoxic activities induced by 6-aminochrysene was about 10- to 20-times greater than those induced by the well-known mutagens 2-aminoanthracene and 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline. Liver microsomes isolated from rats treated with phenobarbital (PB) and a polychlorinated biphenyl mixture, Kanechlor 500, catalyzed very efficiently the activation of 6-aminochrysene to genotoxic metabolites. Treatment of rats with β-naphthoflavone (BNF) and with dexamethasone also caused moderate induction of the microsomal activation of 6-aminochrysene. Studies employing immunoinhibition of microsomal catalytic activities and reconstitution with purified P450 enzymes suggested that the most important enzymes involved in the activation of 6-aminochrysene were P450 2B1 and 2B2; other enzymes including P450 1A1 and 1A2 participated to some extent. We also found that the microsomal activation of 6-aminochrysene was catalyzed more effectively in an acetyltransferase-overexpressing strain (NM2009) than in the original TA1535/pSK1002 strain and that these activities could be inhibited by an acetyltransferase inhibitor, pentachlorophenol, in liver microsomes from PB-treated rats, but not in those from BNF-treated rats. These results suggest that the P450/acetyltransferase system is one of the most important catalysts for the activation of 6-aminochrysene in liver microsomes of PB-treated rats, and that activation by BNF-induced P450 enzymes occurs by different mechanisms, probably through the ring oxidation pathway.

Various environmental carcinogens and mutagens have been shown to require metabolic activation to ultimate reactive forms by drug-metabolizing enzymes in order to exert their biological activities [1–5]. Cytochrome P450 (P450)† enzymes are a group of hemoproteins that play major roles in the activation of these carcinogenic chemicals, and several lines of evidence support the view that P450 1A1 and 1A2 are the most important in the reactions of numerous carcinogenic chemicals in mammals [6, 7]. Although P450 2B enzymes can also participate in the oxidation of a number of xenobiotic chemicals, there are limited studies to show that these P450 enzymes catalyze metabolic activation of environmental carcinogens and mutagens to ultimate reactive metabolites [6, 8–11].

6-Aminochrysene is one of the most potent mutagens in the environment and has been proposed to be activated by both N-hydroxylation and diolepoxide formation in liver microsomal P450 enzymes in several animal species including rats and humans [12–19]. Employing studies with correlation of catalytic activities using different human liver preparations, immunoinhibition of microsomal activities, and reconstitution of activities with purified P450 enzymes, Shimada *et al.* [12, 20] reported previously that in humans the major P450 enzyme involved in the initial oxidation of 6-aminochrysene is P450 3A4. However, limited information is available to identify the specific rat P450 enzymes involved in the activation of 6-aminochrysene [12]. Recently Lubet *et al.* [15] reported that this chemical is catalyzed by different P450 enzymes in the Ames mutation assay, using rat liver 9000 *g* supernatant fraction as the enzyme source. Since this enzyme system contains various kinds of drug-metabolizing enzymes, it is highly desirable to use a more simplified enzyme system in order to explore the major roles of P450 enzymes in the activation process.

In this study we have examined the roles of rat P450 enzymes in the activation of 6-aminochrysene using a newly developed bacterial tester strain, *Salmonella typhimurium* NM2009. Since this tester

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†Abbreviations: P450, cytochrome P450; BNF, β-naphtho-flavone; PB, phenobarbital; and MeIQ, 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline.

strain has been constructed by introducing an *O*-acetyltransferase gene into the original strain *S. typhimurium* TA1535/pSK1002, it is highly sensitive towards the reactive metabolites of aromatic amines including 6-aminochrysene [21]. The potency of this mutagen was found to be greater than those of the well-known mutagens 2-aminoanthracene and 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ). Using liver microsomes isolated from variously treated rats and the reconstituted monooxygenase system containing purified rat P450 enzymes, we obtained evidence that P450 2B1 and 2B2 enzymes are the major species participating in the metabolic activation of 6-aminochrysene. Other enzymes such as P450 1A1 and 1A2 may also be involved in the activation process, but their activities are rather low compared with those of P450 2B enzymes. We also report that the microsomal activation of 6-aminochrysene was catalyzed more effectively in an acetyltransferase-overexpressing strain (NM2009) than in the original TA1535/pSK1002 strain and that these activities could be inhibited by an acetyltransferase inhibitor, pentachlorophenol, in liver microsomes from PB-treated rats, but not in those from BNF-treated rats. These results suggest that the P450/acetyltransferase system is one of the most important catalysts for the activation of 6-aminochrysene in liver microsomes of PB-treated rats, and that activation by BNF-inducible forms of P450 occurs by different mechanisms, probably through the ring oxidation pathway.

MATERIALS AND METHODS

Chemicals. 6-Aminochrysene, pentoxyresorufin and ethoxyresorufin were purchased from the Sigma Chemical Co., St. Louis, MO. Other chemicals used were from the same sources as described previously [6, 12, 22, 23].

Bacterial tester strains. In the present study we used a newly developed tester strain, *S. typhimurium* NM2009, as well as the original tester strain *S. typhimurium* TA1535/pSK1002 to detect genotoxic activation of 6-aminochrysene by rat liver microsomes; the former strain was donated by Dr. Y. Oda of this institution. Since *S. typhimurium* NM2009 has been constructed by introducing an *O*-acetyltransferase gene into the latter strain, it is highly capable of detecting genotoxic activation of mutagenic aromatic amines by liver microsomal P450 [21]. The acetyltransferase activities in lysates of bacterial tester strains using isoniazid as a substrate were found to be 13 and 175 nmol/min/mg protein for *S. typhimurium* TA1535/pSK1002 and *S. typhimurium* NM2009, respectively, as determined by the method of Hein *et al.* [24].

Enzyme preparations and antibodies. Male Sprague–Dawley rats (weighing about 250 g, Nihon Clea Co., Osaka) were treated i.p. with each of several P450 inducers: PB (80 mg/kg, daily for 3 days), BNF (50 mg/kg, daily for 3 days), dexamethasone (80 mg/kg, daily for 3 days), isoniazid (100 mg/kg, daily for 3 days) or Kanechlor 500 (500 mg/kg, once 3 days before the animals were killed). Rats were starved overnight before being killed. Liver microsomes were prepared as described elsewhere [25] and suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM EDTA and 20% glycerol (v/v).

Rat P450 1A1, 1A2 and 2B1 [4, 26] were purified to electrophoretic homogeneity as described previously. Other rat P450 enzymes including P450 2B2, 2C6, 2C11, 2E1 and 3A2 [27] were gifts from Drs. Y. Funae and S. Imaoka. Rabbit anti-P450 preparations have been characterized elsewhere [6, 12, 22, 23, 28]. IgG fractions were prepared and used to inhibit catalytic activities in the microsomes [26, 29]. Rabbit liver NADPH-P450 reductase and cytochrome b_5 were purified using the general procedure described by Yasukochi and Masters [30] and Taniguchi *et al.* [31], respectively.

Assays. Details of the *umu* gene expression assay for DNA damage have been described elsewhere [6, 32]; in general, rat liver microsomes containing 0.2 to 5 μg of protein were used with 0.01 to 0.5 μM 6-aminochrysene in a volume of 1.0 mL of 50 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system consisting of final concentrations of 5 mM glucose-6-phosphate, 0.5 mM NADP [21], and 1 IU of yeast glucose-6-phosphate dehydrogenase/mL and a suspension of bacterial tester strain *S. typhimurium* NM2009 or *S. typhimurium* TA1535/pSK1002 as described previously [6, 12]. Reconstituted P450 system used to replace microsomes included 2 pmol of purified P450, 4 pmol of cytochrome b_5, 25 pmol of NADPH-P450 reductase, and a phospholipid mixture (2.5 μg/mL) consisting of L-α-dilauroyl-*syn*-glycero-3-phosphocholine, dioleoylphosphatidylcholine and L-α-phosphatidyl-L-serine (1:1:1) and sodium cholate (25 μg/mL) [23]. In some experiments 2-aminoanthracene and MeIQ were also used to compare genotoxic activities in this assay system. The expression of the *umu* gene expression was monitored by measuring β-galactosidase activity (the product of the fused *umuC'' lacZ* gene), and the bioactivation was presented as units of β-galactosidase activity per minute per milligram of protein or nanomoles of P450 [6, 12].

O-Dealkylation of pentoxyresorufin and ethoxyresorufin by liver microsomes was determined by methods described previously [33]. The dealkylations of ethylmorphine and *N*-nitrosodimethylamine were also determined by following procedures described previously [23, 34].

P450 was estimated spectrally from Fe^{2+}·Co vs Fe^{2+} difference spectra as described by Omura and Sato [35]. Protein concentrations were determined using the manufacturer's directions (Pierce Chemical Co., Rockford, IL).

RESULTS AND DISCUSSION

We first attempted to determine the optimal conditions for detecting reactive metabolites of 6-aminochrysene in a newly developed tester strain, *S. typhimurium* NM2009, after metabolism by liver microsomes of PB-treated rats (Figs. 1 and 2). Since the *umuC'' lacZ* fused gene as well as the *O*-acetyltransferase gene were introduced into the tester strain, we were able to detect the genotoxic

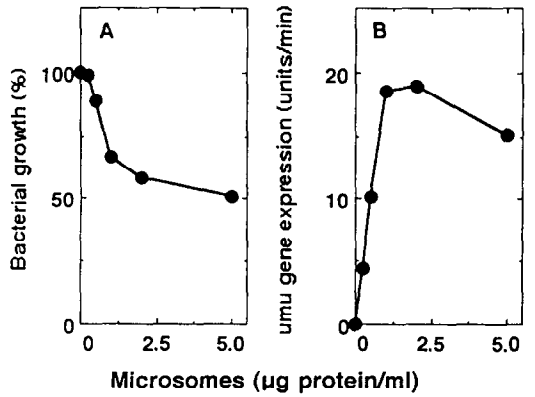

Fig. 1. Effects of liver microsomal concentrations on bacterial cell growth (A) and the metabolic activation of 6-aminochrysene (B) in *S. typhimurium* NM2009. Liver microsomes from PB-treated rats were incubated with 0.1 μM 6-aminochrysene, and the induced DNA damage was detected by measuring β-galactosidase activity in the cells. Bacterial cell growth was determined by measuring the optical density at 600 nm; the 100% value of density was about 0.55 after subtracting the blank value.

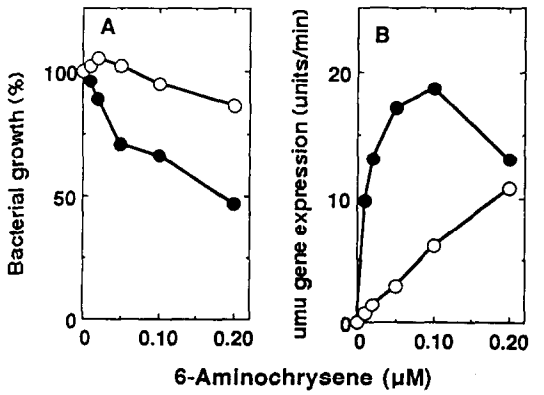

Fig. 2. Effects of the concentration of 6-aminochrysene on bacterial cell growth (A) and the induction of *umu* gene expression in *S. typhimurium* NM2009. Concentrations of liver microsomes from PB-treated rats were 0.5 μg (○) and 2 μg (●) of protein/mL of incubation mixture. Bacterial growth was determined as described in the legend of Fig. 1.

activities of mutagens by measuring β-galactosidase activity as reported previously [36, 37]. Liver microsomal activation of 6-aminochrysene was increased with protein concentrations up to 1 μg protein/mL (Fig. 1B). The reactive metabolites of this mutagen were both genotoxic and cytotoxic (Fig. 1A); the growth rates of the bacteria were retarded when the microsomal protein concentrations increased to over 0.5 μg/mL. The effects of substrate concentration on bacterial cell growth rates and genotoxic activities were determined at liver microsomal protein concentrations of 0.5 and 2 μg/mL (Fig. 2A). At a microsomal protein concentration of 0.5 μg/mL, the genotoxic activities increased linearly with substrate concentrations between 0 and 0.2 μM; with these concentrations the marked cytotoxic effects could not be detected. However, when the protein concentration was increased to 2 μg/mL, the genotoxic activities were saturated at a substrate concentration of about 0.05 μM and the cytotoxic responses became significant. Based on these results the following experiments were done at a protein concentration of 2 μg/mL and a substrate concentration of 0.1 μM.

The effects of treatment of rats with several P450 inducers on liver microsome-catalyzed activation of 6-aminochrysene and on several monooxygenase activities with substrates of pentoxyresorufin, ethoxyresorufin, ethylmorphine, and *N*-nitrosodimethylamine were examined (Table 1). Pentoxyresorufin and ethoxyresorufin are known to be good substrates for P450 2B and 1A1/2, respectively, and N-dealkylations of ethylmorphine and *N*-nitrosodimethylamine have been reported to be catalyzed mainly by P450 3A1/2 and 2E1, respectively, in rat liver microsomes [33, 38–40]. The metabolic activation of 6-aminochrysene by liver microsomes was increased significantly by treatments of rats with PB and a polychlorinated biphenyl mixture, Kanechlor 500. BNF and dexamethasone also caused increases (about 2- to 3-fold) in microsomal activities that catalyze the activation of 6-aminochrysene. The effect of isoniazid was not as great. The activation of 6-aminochrysene by different types of rat liver microsomes correlated well with the activities of pentoxyresorufin *O*-deethylase ($r = 0.94$), but not with those of other reactions with substrates such as ethoxyresorufin, ethylmorphine and *N*-nitrosodimethylamine.

These results suggest that one of the major enzymes involved in the metabolic activation of 6-aminochrysene in rat liver microsomes may be the PB-inducible species of the P450 family, namely P450 2B1 and 2B2; other enzymes which are inducible by BNF and dexamethasone may also contribute to some extent. To obtain more conclusive evidence, we determined the metabolic activation of 6-aminochrysene by a reconstituted monooxygenase system containing various forms of rat P450 enzymes (Table 2). In this experiment we used eight forms of rat P450 enzymes: P450 1A1 and 1A2 are 3-methylcholanthrene- and BNF-inducible forms; P450 2B1, 2B2, and 2C6 are PB-inducible forms; P450 2E1 is an ethanol- and isoniazid-inducible form; and P450 3A2 is a pregnenolone 16α-carbonitrile-inducible form [10, 26, 27, 41]. P450 2C11 is a male specific enzyme that catalyzes xenobiotic chemicals as well as steroids such as testosterone [26, 41, 42]. As expected, P450 2B1 and 2B2 had the highest capacities to catalyze activation of 6-aminochrysene followed by P450 1A1 and 1A2 in the reconstituted monooxygenase system. The other enzymes, P450 2C6, 2C11, 2E1 and 3A2, had lower activities in this reaction.

The effects of specific inhibitors of P450 enzymes and of antibodies raised against rat P450 1A1, 1A2, and 2B1 on the activation of 6-aminochrysene by liver microsomes from PB- and BNF-treated rats were determined to explore the roles of P450

Table 1. Comparison of rates of P450-catalyzed activation of 6-aminochrysene and several monooxygenase activities in liver microsomes of variously treated rats

Treatment of rats	Activation	O-Dealkylation		N-Demethylation	
		Pentoxyresorufin	Ethoxyresorufin	Ethylmorphine	*N*-Nitrosodimethylamine
	6-Aminochrysene (*umu* units/min/mg protein)	(nmol products formed/min/mg protein)			
None	1288 ± 250	0.31 ± 0.03	0.46 ± 0.11	4.29 ± 0.82	2.00 ± 0.19
Phenobarbital	9381 ± 1052*	4.88 ± 0.53*	0.98 ± 0.12*	8.81 ± 0.93*	2.01 ± 0.31
β-Naphthoflavone	3508 ± 319*	0.48 ± 0.04*	12.6 ± 1.21*	2.89 ± 0.32	2.84 ± 0.19*
Dexamethasone	3175 ± 289*	0.77 ± 0.15*	0.89 ± 0.09*	10.8 ± 1.31*	2.42 ± 0.32
Isoniazid	2509 ± 192*	0.41 ± 0.05†	0.53 ± 0.15	3.64 ± 0.42	6.21 ± 0.68*
Kanechlor 500	6441 ± 1021*	1.73 ± 0.23*	16.7 ± 1.78*	7.53 ± 0.86*	4.52 ± 0.66*

Liver microsomal monooxygenase activities were determined as described in Materials and Methods. Values are the means ± SD from three individual rats.

*,† Significantly different from untreated rats (none): $^*P < 0.01$, and $^\dagger P < 0.05$.

Table 2. Metabolic activation of 6-aminochrysene by reconstituted monooxygenase system containing purified P450 isolated from rat liver microsomes

	Activation of 6-aminochrysene (*umu* units/min/nmol P450)
P450 1A1	3838 ± 167
P450 1A2	3228 ± 683
P450 2B1	8117 ± 1116
P450 2B2	7289 ± 1001
P450 2C6	980 ± 77
P450 2E1	891 ± 129
P450 2C11	394 ± 139
P450 3A2	456 ± 83

Each of the purified P450 enzymes (2 nM) was incubated with 0.1 μM 6-aminochrysene in the presence of an NADPH-generating system, and the resulting DNA damage was determined as described in Materials and Methods. Values are averages ± range of duplicate determinations.

enzymes in the reaction (Table 3). Metyrapone caused a decrease in activities of liver microsomes from PB-treated rats at a concentration of 50 μM, but showed weak effects on the activities catalyzed by liver microsomes from BNF-treated rats. α-Napthoflavone showed about 50% inhibition of catalytic activities by liver microsomes of both PB- and BNF-treated rats. Antibodies raised against rat P450 2B1 caused almost complete inhibition of 6-aminochrysene activation catalyzed by liver microsomes of PB-treated rats, but showed weak effects on the reaction catalyzed by liver microsomes from BNF-treated rats. On the contrary, the antibodies raised against rat P450 1A1 and 1A2 inhibited 55 and 73%, respectively, the reaction catalyzed by liver microsomes of BNF-treated rats. These results suggest that both PB- and BNF-inducible forms of P450 are involved in the activation of 6-aminochrysene, and that the roles of P450 2B1 and 2B2 are more important than those of other P450 enzymes in rat liver microsomes.

The potency of reactive metabolites of 6-aminochrysene to induce DNA damage was compared with those of the well-known carcinogens 2-aminoanthracene and MeIQ using liver microsomes from PB- and BNF-treated rats (Fig. 3). 6-Aminochrysene was very effective in inducing DNA damage compared with the other procarcinogens examined; the former chemical caused about 5 to 10 times higher reactivities than 2-aminoanthracene and MeIQ. The latter two carcinogens were activated to a greater extent by liver microsomes from BNF-treated rats than by those from PB-treated rats, similar to previous results in a *S. typhimurium* TA1535/pSK1002 strain [6].

The results presented above support the view that a potential mutagen, 6-aminochrysene, is activated mainly by PB-inducible forms of P450, and is also catalyzed by BNF-inducible P450 1A1 and 1A2 in rat liver microsomes. Lubet *et al.* [15] have reported similar findings; in their reverse mutation assay using the *S. typhimurium* TA98 system, rat P450 2B

Table 3. Effects of chemical inhibitors and antibodies raised against rat P450 enzymes on the activation of 6-aminochrysene by liver microsomes of PB- and BNF-treated rats

Addition	PB-treated rats		BNF-treated rats	
	Activation of 6-aminochrysene			
	units/min/mg protein	(%)	units/min/mg protein	(%)
None	8333 ± 1778	(100)	2115 ± 306	(100)
Metyrapone	2870 ± 439	(34)	1745 ± 16	(83)
α-Naphthoflavone	4113 ± 801	(49)	988 ± 95	(47)
Preimmune	8721 ± 979	(105)	2029 ± 193	(96)
Anti-rat P450 1A1	7645 ± 624	(92)	943 ± 47	(45)
Anti-rat P450 1A2	6504 ± 840	(78)	580 ± 35	(27)
Anti-rat P450 2B1	1068 ± 208	(13)	1554 ± 252	(73)

Liver microsomes from PB- or BNF-treated rats were incubated with 0.1 μM 6-aminochrysene and *S. typhimurium* NM2009 in the absence or presence of 50 μM metyrapone, and 50 μM α-naphthoflavone or 10 mg of preimmune and anti-P-450 IgG/nmol P450. The resulting DNA damage was determined by the method as described in Materials and Methods. Values are averages ± range of duplicate determinations.

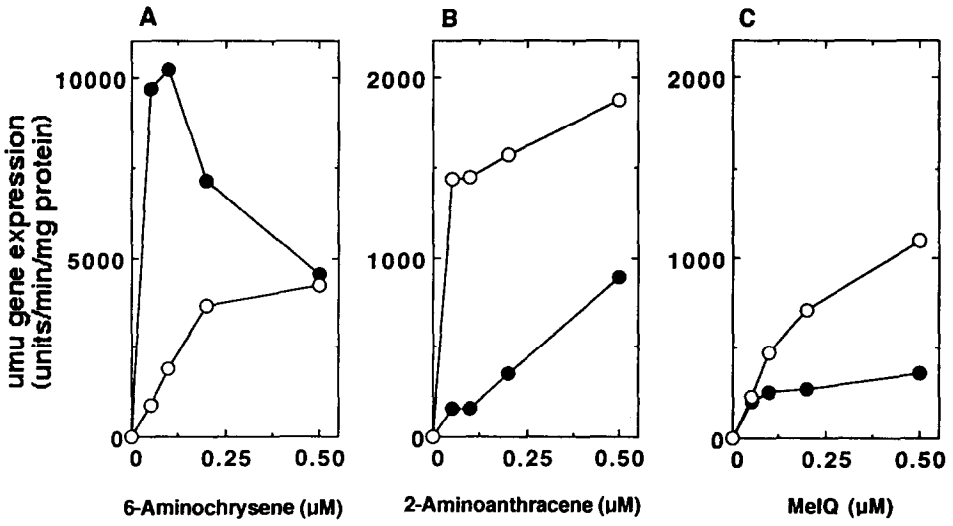

Fig. 3. Comparison of genotoxic activities of 6-aminochrysene (A), 2-aminoanthracene (B), and MeIQ (C) after metabolism by liver microsomes from PB-treated rats (●) and BNF-treated rats (○). The liver microsomal protein concentration used was 2 μg/mL.

enzymes contributed mainly to the mutagenic activation of 6-aminochrysene by hepatocytes or liver 9000 *g* supernatants from control and Aroclor 1254-treated rats. However, their enzyme system was rather crude and we preferred to use a more simplified enzyme system to define the major roles of P450 enzymes in the reaction. In fact, our present results suggested that P450 1A1 and 1A2 enzymes, as well as P450 2B enzymes, also contribute to the activation of 6-aminochrysene in rat liver microsomes. However, the importance of the role of P450 2B enzymes in the activation of 6-aminochrysene is now confirmed; further work involving identification of reactive metabolites of 6-aminochrysene by the P450 system may be necessary to understand the mechanism of carcinogenesis by this chemical.

The findings that this newly developed tester strain with high acetyltransferase activity is very sensitive towards 6-aminochrysene suggest that the activation is through formation of reactive intermediates, possibly N-hydroxylated metabolites, followed by conversion to an ultimate reactive form(s) by acetyltransferase. This activation pathway by the P450/acetyltransferase system is rather common in a variety of aromatic amines as suggested by a number of reports [12–14, 43]. However, the pathway for the activation of 6-aminochrysene has been reported to be more complex [13]. To demonstrate these problems we further examined the genotoxic

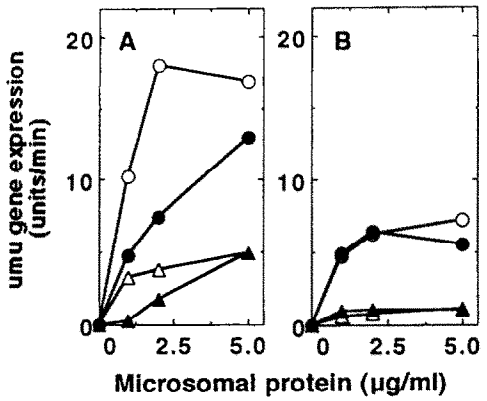

Fig. 4. Effects of pentachlorophenol on the activation of 6-aminochrysene to genotoxic products by liver microsomes from PB-treated rats (A) and BNF-treated rats (B) in tester strains *S. typhimurium* NM2009 (○, ●) and *S. typhimiurium* TA1535/pSK1002 (△, ▲). 6-Aminochrysene (0.1 μM) was incubated with liver microsomes in the presence (●, ▲) or absence (○, △) of 6 μM pentachlorophenol.

activation of 6-aminochrysene by liver microsomes from PB- and BNF-treated rats in both an acetyltransferase-overexpressing *S. typhimurium* NM2009 strain and the original *S. typhimurium* TA1535/pSK1002 strain (Fig. 4). The effects of pentachlorophenol, an inhibitor of acetyltransferase activity [44, 45], were determined in these systems to examine the roles of the P450/acetyltransferase system in the reaction. In liver microsomes from PB-treated rats, the activation of 6-aminochrysene was catalyzed more efficiently in *S. typhimurium* NM2009 than in *S. typhimurium* TA1535/pSK1002 (Fig. 4A). Liver microsomes from BNF-treated rats also catalyzed the activation of 6-aminochrysene effectively in the former strain, but did so very poorly in the *S. typhimurium* TA1535/pSK1002 strain (Fig. 4B). Interestingly, pentachlorophenol, an acetyltransferase inhibitor, was effective in inhibiting activation of 6-aminochrysene by liver microsomes of PB-treated rats at lower concentrations of microsomal proteins. On the contrary, neither of the activities catalyzed by liver microsomes from BNF-treated rats was affected by pentachlorophenol in the two tester strains. These results suggest that the P450/acetyltransferase system may actually be involved in the activation of 6-aminochrysene, particularly in liver microsomes from PB-treated rats. The present results also suggested that PB-inducible forms of P450 may convert 6-aminochrysene mainly to an N-hydroxylated metabolite(s), while BNF-induced P450s activate by different mechanisms, probably through the ring oxidation pathway in rat liver microsomes. Delclos *et al.* [17] reported that *N*-hydroxy-6-aminochrysene is able to interact directly with DNA *in vitro* and the same DNA adducts are detected in isolated hepatocytes *in vivo* after treating with labeled carcinogen. The same group has also shown that 6-aminochrysene-1,2-dihydrodiol can be detected as a proximate carcinogenic metabolite, and the major DNA adduct would be derived from further metabolism of the proximate metabolite of this carcinogen in preweanling mice [16, 18]. To conclude that there are different pathways for the metabolic activation of 6-aminochrysene, the identification of reactive metabolites formed through metabolism by P450 enzymes must be investigated.

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