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Mechanism of Species Difference in *N*-Hydroxyphenacetin Mutagenicity: The Role of Deacetylation by Rat and Hamster Liver Microsomes

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The mechanism of species difference in *N*-hydroxyphenacetin mutagenicity was investigated by using the *Salmonella*/microsome mutagenicity test and high-performance liquid chromatographic (HPLC) analysis. Mutagenicity of *N*-hydroxyphenacetin in *Salmonella typhimurium* TA100 was about 10 times more efficiently detected with a liver 9000 ***g*** supernatant fraction (S9) from hamsters than with the corresponding fraction from rats in the absence of a reduced nicotinamide adenine dinucleotide phosphate-generating system. Paraoxon and sodium fluoride, both inhibitors of microsomal amidase, not only inhibited the mutagenicity of *N*-hydroxyphenacetin, but also retarded the formation of the deacetylation product, *p*-nitrosophenetole, a strong intrinsic mutagen. The *N*-hydroxyphenacetin-deacetylation activity was about 20 times higher in liver microsomes from the hamster than in those from the rat.

Keywords——mutagenicity; species difference; deacetylation; liver microsome; *N*-hydroxyphenacetin; *N*-hydroxyphenetidine; *p*-nitrosophenetole

The rodent species from which liver 9000 × ***g*** supernatant fractions (S9s) are obtained is an important factor in the efficient detection of carcinogens as mutagens. Liver S9 prepared from polychlorobiphenyl (PCB)-treated rats has widely been used for the metabolic activation of promutagens in the *Salmonella*/microsome mutagenicity test.[1] It has been reported that the mutagenicity of phenacetin, an analgetic drug, in *Salmonella typhimurium* TA100 is detectable in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-fortified liver S9 from hamsters, but not that from rats.[2,3]

N-Hydroxyphenacetin is regarded as one of the proximate forms of phenacetin, since it is not mutagenic *per se* but shows a stronger mutagenic activity in TA100 in the presence of hamster liver S9 than phenacetin does.[4–6] *N*-Hydroxyphenacetin was isolated from an incubation mixture consisting of phenacetin and NADPH-fortified hamster liver microsomes.[7] Thus, it was suggested that *N*-hydroxylation was the rate-limiting step in the species-specific activation of phenacetin by hamster liver microsomes.[8] However, our previous work indicated that there is no significant difference in phenacetin *N*-hydroxylation activity between rat and hamster liver microsomes.[9] In fact, we detected *N*-hydroxyphenacetin in the ratio 1 : 3 in both incubation mixtures (containing rat or hamster liver microsomes). Therefore, we suggested that the aforementioned species difference in phenacetin mutagenicity was due to a metabolic activation step other than *N*-hydroxylation. The possibility that oxidative deethylation is the rate-limiting step of the activation of phenacetin is also ruled out since neither acetaminophen nor *N*-hydroxyacetaminophen is mutagenic in TA100.[5,6]

Deacetylation is most likely to be involved in the further metabolic activation of *N*-hydroxyphenacetin, since we have isolated the *N*-hydroxylation and deacetylation metabolites, *N*-hydroxyphenetidine and *p*-nitrosophenetole, from an incubation mixture contain-

ing phenacetin and NADPH-fortified hamster liver microsomes.[9] Moreover, we showed that both metabolites were strongly mutagenic in TA100 without further metabolic activation. The intrinsic mutagenicity of these compounds was also reported by Wirth *et al.*[10] and Camus *et al.*[6] with synthetic specimens. Unlike *N*-hydroxy-2-acetylaminofluorene, which can be activated by both microsomes and cytosol fraction,[11] the activation of *N*-hydroxyphenacetin is mediated only by microsomes, since this compound shows mutagenicity in TA100 with hamster liver microsomes but not with the cytosol fraction.[5] Thus, the deacetylation of *N*-hydroxyphenacetin by the microsomes seems to be one of the candidates for the rate-limiting step of the species-specific activation of phenacetin.

In this study, we compared the *N*-hydroxyphenacetin-deacetylating activities of rat and hamster liver microsomes. The results of our high-performance liquid chromatography (HPLC) analysis and mutagenicity tests indicated that a marked species difference exists in *N*-hydroxyphenacetin mutagenicity, and this species difference is due to the difference in deacetylating activities between rat and hamster liver microsomes.

Materials and Methods

Materials——*N*-Hydroxyphenetidine,[10] *p*-nitrosophenetole[12] and *N*-hydroxyphenacetin[7] were synthesized according to the methods previously described. Paraoxon (diethyl *p*-nitrophenyl phosphate) and sodium fluoride were purchased from Sigma Chemical Co. (Mo.) and Wako Pure Chemical Industries Ltd. (Osaka), respectively. *S. typhimurium* TA100 was kindly supplied by Dr. B. N. Ames of the University of California.

Preparation of Liver Microsomes——Male Fischer rats (100—120 g) and male Golden Syrian hamsters (80—100 g) were pretreated with a single intraperitoneal injection of PCB (Kanechlor KC-400, 500 mg/kg) dissolved in olive oil (0.2 ml) and sacrificed 5 d later. Control animals received olive oil alone. S9 and microsomes were prepared as described previously.[13]

Mutagenicity Test——Mutagenicity tests were performed according to the method of Ames *et al.*[1] with slight modifications; briefly, the test chemical in 0.1 ml of dimethylsulfoxide (DMSO) and 0.1 ml of overnight culture of *S. typhimurium* TA100 (5×10^8 cells/ml) were placed in a test tube, mixed with 0.5 ml of 1/15 M potassium phosphate buffer, pH 7.4, or S9 solution, and then preincubated for 20 min at 37 °C before being diluted with top agar and placed on minimal agar plates for incubation (2 d). In these experiments, the reaction mixture contained S9 (3 mg of protein as determined by the method of Lowry *et al.*[14]) but no cofactors such as NADPH and reduced nicotinamide adenine dinucleotide (NADH). For inhibition studies, paraoxon dissolved in DMSO (10 μl) or sodium fluoride in distilled water (50 μl) was added to the incubation mixture before the addition of *N*-hydroxyphenacetin.

Deacetylation of *N*-Hydroxyphenacetin——Hepatic microsomal deacetylation of *N*-hydroxyphenacetin was measured as follows. A mixture (3 ml) consisting of microsomes (3 mg protein) from PCB-treated or untreated rats or hamsters, potassium phosphate buffer, pH 7.4 (150 μmol) and *N*-hydroxyphenacetin (7.7 μmol) was aerobically incubated for 20 min at 37 °C, and the reaction was terminated by adding sodium fluoride (0.3 mmol) followed by rapid cooling on an ice bath. For inhibitor experiments, paraoxon (0.3 μmol) dissolved in DMSO or sodium fluoride (0.3 mmol) as a powder was added to the incubation mixture before the addition of the substrate. After the addition of *N-n*-butyrylaniline (0.25 μmol) as an internal standard for subsequent chromatographic analysis, the mixture was extracted three times with dichloromethane (10 ml each) by mechanical shaking. The organic phase (30 ml) was concentrated almost to dryness under a gentle nitrogen stream and the residue was dissolved in acetonitrile (200 μl). An aliquot (20 μl) of the acetonitrile solution was analyzed by HPLC as described previously.[9] The amounts of *p*-nitrosophenetole formed were determined from the calibration curves constructed for the authentic sample by using a microcomputer, Chromatopak C-R1A (Shimadzu Ltd., Kyoto). The mass spectrum of biologically formed *p*-nitrosophenetole was recorded with a gas-chromatograph-mass spectrometer (Hitachi double-focusing mass spectromer) at an ionizing voltage of 70 eV. Gas chromatographic conditions were as follows: 1.5% SE-30 column; column temperature, 110 °C; helium carrier gas, 40 ml/min; retention time, 3.3 min.

Results

S9-Mediated Mutagenicity of *N*-Hydroxyphenacetin

The mutagenicity of *N*-hydroxyphenacetin in *S. typhimurium* TA100 was tested in the presence of PCB-treated or untreated rat or hamster S9 without any fortifying agent. The mutagenicity of *N*-hydroxyphenacetin with hamster S9 was about 10 times higher than that

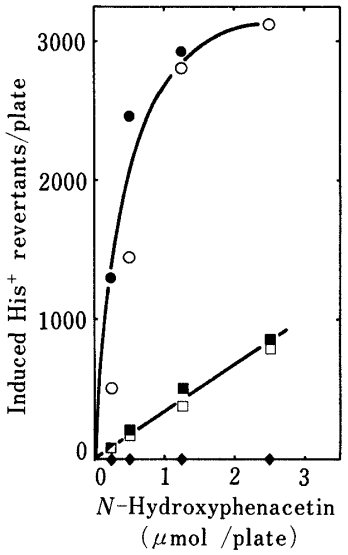

Fig. 1. Mutagenicity of *N*-Hydroxyphenacetin in TA100 Mediated by Rat and Hamster Liver Microsomes

The *N*-hydroxy compound was incubated with the bacteria and S9 without any fortifying agent. Data are expressed as arithmetic mean values of at least six experiments. ●, PCB-treated hamster S9; ○, untreated hamster S9; ■, PCB-treated rat S9; □, untreated rat S9; ◆, without S9.

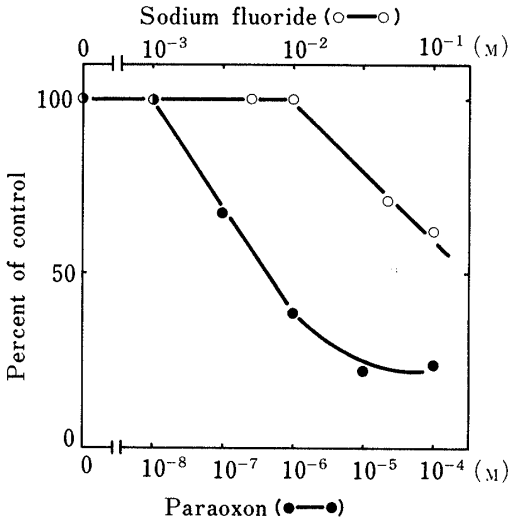

Fig. 2. Effects of Paraoxon and Sodium Fluoride on the Mutagenicity of *N*-Hydroxyphenacetin in TA100 Mediated by Untreated Hamster Liver S9

The mutagenicity test was performed under the same conditions as mentioned in the legend to Fig. 1. *N*-Hydroxyphenacetin was used at a concentration of 0.5 μmol/plate, inducing 2450 His^+ revertant colonies on the control plate (no inhibitor).

with rat S9 (Fig. 1). Treatment of rats or hamsters with PCB had no significant effect on the mutagenicity. When NADPH (or NADH, 4 mM) was added to the preincubation mixture the mutagenic activity of the *N*-hydroxy compound was reduced to less than one-half of that without the co-factors (data not shown).

The amidase inhibitors, paraoxon and sodium fluoride, were added to the preincubation mixture to check the possible involvement of hepatic microsomal amidase in the mutagenicity of *N*-hydroxyphenacetin. Paraoxon, when added at a concentration of 10^{-5} M, inhibited about 80% of the mutagenicity of *N*-hydroxyphenacetin mediated by untreated hamster S9 (Fig. 2). Sodium fluoride was less effective than paraoxon, so that the mutagenicity of *N*-hydroxyphenacetin could be inhibited about 40% at 10^{-1} M. Paraoxon and sodium fluoride, when tested at these concentrations, were neither mutagenic nor toxic to TA100 cells in the presence of hamster S9.

Metabolic Activation of *N*-Hydroxyphenacetin by Hepatic Microsomes

The involvement of deacetylation in the metabolic activation of *N*-hydroxyphenacetin was further supported by isolating *p*-nitrosophenetole from incubation mixtures consisting of *N*-hydroxyphenacetin and either rat or hamster liver microsomes. The metabolite isolated by HPLC after extraction with dichloromethane was identified by mass spectroscopy (Fig. 3). However, *N*-hydroxyphenetidine, the direct deacetylation product, was not detected in the extracts by HPLC. *N*-Hydroxyphenetidine and *p*-nitrosophenetole induced 15400 and 22300 His^+ revertant colonies per μmol, respectively (Fig. 4), which were about 7 to 10 times higher than those induced by *N*-hydroxyphenacetin with hamster S9 (2300 induced His^+ revertant colonies per μmol). *p*-Nitrosophenetole is most likely to arise from *N*-hydroxyphenetidine by autoxidation during incubations, since an authentic specimen of *N*-hydroxyphenetidine, free from *p*-nitrosophenetole, was rapidly converted into the nitroso compound when it was incubated in the buffer alone or in the buffer containing boiled microsomes.

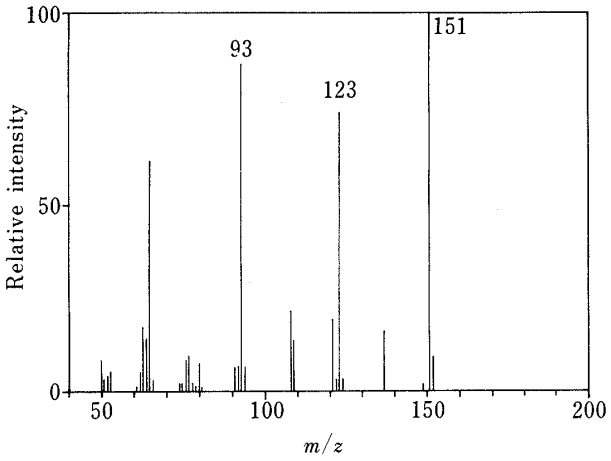

Fig. 3. Mass Spectrum of *p*-Nitrosophenetole Formed from *N*-Hydroxyphenacetin by Hepatic Microsomes

The nitroso compound was extracted and isolated by HPLC from the incubation mixture containing *N*-hydroxyphenacetin and untreated hamster liver microsomes.

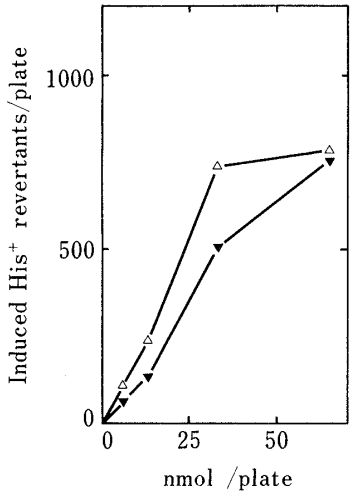

Fig. 4. Mutagenicity of *N*-Hydroxyphenetidine and *p*-Nitrosophenetole in TA100 in the Absence of S9

Data are expressed as arithmetic mean values of at least three experiments. △, *p*-nitrosophenetole; ▼, *N*-hydroxyphenetidine.

TABLE I. Deacetylation of *N*-Hydroxyphenacetin by PCB-Treated or Untreated Animal Liver Microsomes

Animal	Treatment	*N*-Hydroxyphenacetin deacetylation[a]		
		Control	NaF (0.1 M)	Paraoxon (0.1 mM)
Rat	None	0.48	n.d.[b]	n.d.
	PCB	0.52	n.d.	n.d.
Hamster	None	11.2	0.23	0.28
	PCB	11.6	0.29	0.24

a) Activities are expressed as nmol *p*-nitrosophenetole formed/min/mg microsomal protein.
b) n.d. means not detectable.

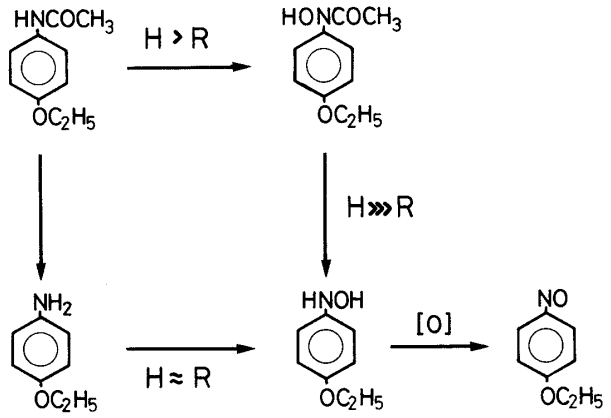

Fig. 5. Species Difference in Metabolic Activation of Phenacetin through Deacetylation and *N*-Hydroxylation by Rat and Hamster Liver Microsomes

H and R represent hamster and rat, respectively.

The *N*-hydroxyphenacetin-deacetylating activity was about 20 times higher in liver microsomes from the hamster than in those from the rat (Table I). PCB treatment of rats and hamsters had no significant effect on the activities. The deacetylating activities were

significantly inhibited when sodium fluoride (0.1 M) or paraoxon (0.1 mM) was added to the incubation mixture.

Discussion

To determine the metabolic activation pathway, the mutagenicity of known and putative phenacetin metabolites has extensively been examined by means of the *Salmonella*/microsome mutagenicity test. The metabolic pathways of phenacetin by the microsomes involve *O*-deethylation,[15,16] ring hydroxylation[17] and *N*-hydroxylation[7] and the main metabolic pathway of phenacetin is *O*-deethylation, giving rise to acetaminophen.[18] However, it has been demonstrated that the deethylation does not lead to mutagenic metabolites.[4-6] Acetaminophen, *N*-hydroxyacetaminophen and *N*-acetylimidoquinone are not mutagenic in TA100 and TA98 either with or without S9 mix. The ring-hydroxylation products, 2-hydroxyphenacetin and 2-acetoxyphenacetin, are also not mutagenic in the tester strains.[6] On the other hand, *N*-hydroxyphenacetin shows stronger mutagenic activity in TA100 than phenacetin does.[4-6] Since the *N*-hydroxy compound requires further metabolic activation for its mutagenesis, it was suggested that some of the metabolites of the *N*-hydroxy compound are direct-acting mutagens. *N*-Hydroxyphenacetin can be converted to its *O*-sulfate or *O*-glucuronide.[19,20] *N*-Acetoxyphenacetin, a model compound for such *O*-conjugates, was tested by Shudo *et al.*, but it was mutagenic only in the presence of S9 mix, as in the case of *N*-hydroxyphenacetin.[4] The only products which are strongly mutagenic *per se* in TA100 are deacetylation metabolites of *N*-hydroxyphenacetin, *i.e.*, *N*-hydroxyphenetidine and *p*-nitrosophenetole.[6,9,10] Therefore, Wirth *et al.* and Camus *et al.* proposed that the activation of phenacetin to the bacterial mutagens proceeds *via* *N*-hydroxyphenacetin to the intrinsic mutagens.[5,6]

Although the *Salmonella*/microsome mutagenicity test is a useful method to investigate the possible metabolic activation pathway, quantitative biochemical analysis is required to reveal the exact mechanism of the aforementioned species difference in phenacetin mutagenicity. Thus, in this study, we used our previously developed HPLC method to demonstrate the formation of the deacetylation product, *p*-nitrosophenetole, from *N*-hydroxyphenacetin and we confirmed the identity of the product by mass spectroscopy (Fig. 3). Moreover, by using this HPLC method, we determined the amounts of *p*-nitrosophenetole formed by rat and hamster liver microsomes, and found that the *N*-hydroxyphenacetin-deacetylating activity was about 20 times higher in liver microsomes from the hamster than in those from the rat (Table I). The inhibitors of microsomal amidase, paraoxon and sodium fluoride, markedly inhibited the formation of *p*-nitrosophenetole (Table I) as well as the mutagenicity of *N*-hydroxyphenacetin with hamster S9 (Fig. 2). It was also reported that paraoxon (0.1 mM) completely inhibits the mutagenicity of *N*-hydroxyphenacetin when hamster liver microsomes instead of S9 are used as an activating enzyme.[5] Both *p*-nitrosophenetole and *N*-hydroxyphenetidine showed strong intrinsic mutagenicity in TA100 and their mutagenic potencies were 10 and 7 times higher than that of *N*-hydroxyphenacetin with hamster S9 (Fig. 4). From these results, we concluded that the marked species difference in *N*-hydroxyphenacetin mutagenicity (Fig. 1) is due to the difference in amidase activities between rat and hamster liver microsomes.

Under our assay conditions, however, *N*-hydroxyphenetidine, the direct deacetylation product, was not detected in the extract from an incubation mixture consisting of *N*-hydroxyphenacetin and rat or hamster liver microsomes. The reason for the failure to detect *N*-hydroxyphenetidine could be its oxidizability in aqueous solutions.[21] Actually, the half-life of the *N*-hydroxy compound in neutral buffer at 37 °C was about 2.5 min (unpublished results). *N*-Hydroxyphenetidine is rapidly and quantitatively converted to *p*-nitrosophenetole

when it is oxidized with ferric chloride or potassium ferricyanide.[21,22] Thus, it was suggested that *N*-hydroxyphenetidine formed from *N*-hydroxyphenacetin was readily converted to the nitroso compound under our aerobic assay conditions. The formation of *N*-hydroxyphenetidine by microsomes was directly demonstrated by the same HPLC method when the NADPH-generating system was included in the incubation mixture.[9] In this case, both *N*-hydroxyphenetidine and *p*-nitrosophenetole were detected as deacetylation products of *N*-hydroxyphenacetin. However, these conditions were not suitable for determining the deacetylating activities of liver microsomes, since NADPH-dependent metabolism such as deethylation of *N*-hydroxyphenacetin by cytochrome P-450 also occurred with concomitant formation of non-mutagenic *N*-hydroxyacetaminophen as the major metabolite.[23] Thus, in this study, we compared the rates of *p*-nitrosophenetole formation by rat and hamster liver microsomes without adding NADPH or the NADPH-generating system to the incubation mixture.

It has been reported that there is a marked species difference in the activity of microsomal amidase, which was shown here to be responsible for the deacetylation of *N*-hydroxyphenacetin. Kiese and Renner measured the phenacetin-deacetylating activities of liver microsomes prepared from dogs, guinea pigs, rabbits and cats, and reported that the ratio of the activities is 17 : 10 : 6 : 1.[24] Schut *et al.* reported that 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene deacetylating activities are 7 to 10 times higher in liver microsomes from mice than in those from rats.[25] However, it is surprising that the *N*-hydroxyphenacetin-deacetylating activity was about 20 times higher in liver microsomes from the hamster than in those from the rat. Since the deacetylation of *N*-hydroxyphenacetin is one of the essential steps for the metabolic activation of phenacetin (Fig. 5),[9,26] we consider that the difference in amidase activities between rat and hamster liver microsomes probably plays an important role in the marked species difference in phenacetin mutagenicity. It should be noted, however, that intrinsic mutagens are also formed through *N*-hydroxylation of phenetidine, a deacetylation product of phenacetin.[9,21] Thus, it is necessary to compare the phenacetin-deacetylating activities of rat and hamster liver microsomes in detail to elucidate the whole mechanism of the species difference in phenacetin mutagenicity. Further studies on this problem are in progress in our laboratory.

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