[Chem. Pharm. Bull. 33(7)2877—2885(1985)]

Mechanism of Metabolic Activation of the Analgetic Bucetin to Bacterial Mutagens by Hamster Liver Microsomes

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(Received August 15, 1984)

Bucetin (*N*-(β-hydroxybutyryl)-*p*-phenetidine) was found to be mutagenic to *Salmonella typhimurium* TA100 in the presence of liver 9000 *g* supernatant fractions (S9) prepared from polychlorinated biphenyl (PCB)-treated hamsters and a reduced nicotinamide adenine dinucleotidephosphate (NADPH)-generating system. However, the analgetic was not mutagenic in the presence of NADPH-fortified S9 from PCB-treated rat liver. The mutagenic potency of bucetin was about a quarter of that of the structurally related analgetic, phenacetin. PCB-treated hamster liver microsomes fortified with NADPH activated bucetin to two direct-acting mutagens, *N*-hydroxyphenetidine and *p*-nitrosophenetole, through deacylation followed by *N*-hydroxylation. The nitroso compound arose from *N*-hydroxyphenetidine *via* autoxidation. *N*-(β-Hydroxybutyryl)-*p*-aminophenol, a major metabolite of bucetin under the conditions used, was not mutagenic to TA100 either with or without NADPH-fortified S9 from PCB-treated or untreated rats or hamsters. *N*-Hydroxybucetin, which was about 70 times less mutagenic than *N*-hydroxyphenacetin in the presence of PCB-treated hamster S9, was not detected as a metabolite of bucetin from the NADPH-fortified reaction mixtures. Although no species difference was observed in *p*-phenetidine *N*-hydroxylation, the rate of bucetin deacylation was over 90 times higher in hamsters than in rats. The rate of microsomal deacylation of bucetin was much lower than that of phenacetin or *N*-butyryl-*p*-phenetidine. These results suggest that the species difference in bucetin mutagenicity is due to the difference in deacylating activity between rat and hamster liver microsomes, and also that the β-hydroxyl group in the butyryl side chain makes bucetin poorly hydrolyzable in microsomes, resulting in lower mutagenic activity as compared with phenacetin.

Keywords——mutagenicity; metabolic activation; species difference; deacylation; bucetin; phenacetin; *N*-hydroxybucetin; *N*-hydroxyphenacetin; *N*-hydroxyphenetidine; *p*-nitrosophenetole

Introduction

Bucetin (*N*-(β-hydroxybutyryl)-*p*-phenetidine) has been used instead of phenacetin as a component of analgesic drugs, because it has lower toxicity than phenacetin despite having equivalent analgesic activity when used at appropriate doses.[1] The carcinogenic potential of phenacetin has been extensively studied through epidemiological analysis in abusers of the analgetic as well as through carcinogenicity tests in experimental animals.[2,3] As a result, phenacetin is now classified as a possible human carcinogen, and its use as an analgetic has been severely restricted in several countries.[4]

As compared with phenacetin, little information is available on the carcinogenicity or mutagenicity of bucetin, although it seems to afford a proximate mutagen, *p*-phenetidine, as a result of enzymatic hydrolysis of its acyl side chain. In previous studies, we demonstrated that deacetylation as well as *N*-hydroxylation is an essential step for the metabolic activation of phenacetin to bacterial mutagens.[5,6] The deacetylation and *N*-hydroxylation product, *N*-

hydroxyphenetidine, and its autoxidation product, *p*-nitrosophenetole, are directly mutagenic in *Salmonella typhimurium* TA100, and their mutagenic potency is over 40 times higher than that of phenacetin.[7-9] Since the deacetylating activity is significantly higher in liver microsomes from hamsters than in those from rats,[5,6] the mutagenicity of phenacetin in TA100 is detectable in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-fortified liver 9000 *g* supernatant fractions from polychlorinated biphenyl (PCB)-treated hamsters (PCB-H-S9 mix), but not those from PCB-treated rats (PCB-R-S9 mix).[10,11]

In this study, we investigated the metabolic activation of bucetin to bacterial mutagens by hamster liver microsomes. The purpose of this work was to establish the metabolic activation pathway of bucetin and to compare the metabolic rate and mutagenicity of bucetin with those of phenacetin.

Materials and Methods

Materials——Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Oriental Yeast Co., Ltd. (Tokyo). Paraoxon and sodium fluoride were purchased from Sigma Chemical Co. (Mo.) and Wako Pure Chemicals Co., Ltd. (Osaka), respectively. *N*-Hydroxyphenetidine,[9] *N*-hydroxyphenacetin,[12] *p*-nitrosophenetole,[13] *p*-hydroxyacetoacetanilide,[14] 3-acetoxybutyryl chloride,[15] *N*-butyryl-*p*-phenetidine,[16] *N*-(β-hydroxybutyryl)-*p*-aminophenol,[14] *N*-butyrylaniline[17] and *N*-β-hydroxybutyrylaniline[14] were synthesized according to the previously described methods. Bucetin was supplied by the Ministry of Health and Welfare, Japan. *p*-Phenetidine was purchased from Wako Pure Chemical Co., Ltd. (Osaka) and purified by distillation as previously described.[5] 3-Hydroxybutyric acid was purchased from Aldrich Chemical Co., Inc. (Wis.). Spotfilm silica gel plates were purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo). *Salmonella typhimurium* TA100 was kindly supplied by Dr. B. N. Ames of the University of California.

Synthesis of *N*-Hydroxybucetin——*N*-Hydroxybucetin was obtained by alkaline hydrolysis of *N*-(β-acetoxybutyryl)-*N*-hydroxyphenetidine, which was synthesized by acylation of *N*-hydroxyphenetidine with 3-acetoxybutyryl chloride. The acylation was performed by the application of the same method as reported by Hinson and Mitchell for the synthesis of *N*-hydroxyphenacetin,[12] except that 3-acetoxybutyryl chloride and ethyl acetate were used as an acylating reagent and an extracting solvent, respectively. After hydrolysis of the synthesized *N*-(β-acetoxybutyryl)-*N*-hydroxyphenetidine in 1 N NaOH–methanol (3:7) at room temperature for 1 h, the reaction mixture was neutralized by the addition of solid NaH_2PO_4, and *N*-hydroxybucetin was extracted into ethyl acetate. The ethyl acetate extract was washed with a small volume of water and evaporated under reduced pressure to give crude *N*-hydroxybucetin (700 mg from 2 g of *N*-hydroxyphenetidine). The crude *N*-hydroxybucetin was recrystallized from hot acetone to yield thin-layer chromatographycally (TLC)-pure *N*-hydroxybucetin (250 mg). The *Rf* values of *N*-(β-acetoxybutyryl)-*N*-hydroxyphenetidine and *N*-hydroxybucetin were 0.3 and 0.15, respectively, on a spotfilm silica gel plate in ether as a developing solvent. They gave a purple color when sprayed with a 5% ethanolic solution of $FeCl_3$.[12] Physicochemical data for *N*-hydroxybucetin were as follows: mp 108—109 °C. UV λ_{max}^{EtOH} nm (ε): 205 (16700), 255 (11700). IR ν_{max}^{KBr} cm^{-1}: 3365, 2950, 2850, 1620, 1500, 1405, 1390, 1295, 1244, 1078, 1057, 1040, 835, 825. MS *m/z* (relative intensity, %): 239 (5.5) $M\cdot^+$, 223 (11.6) $M\cdot^+ - O$, 153 (43.4) $M\cdot^+ - COCHCH(OH)CH_3$, 149 (8.2), 137 (64.2) $M\cdot^+ - O - COCHCH(OH)CH_3$, 136 (51.8), 135 (20.3), 124 (22.3), 109 (68.4) $M\cdot^+ - O - COCHCH(OH)CH_3 - C_2H_4$, 108 (100) $M\cdot^+ - O - COCHCH(OH)CH_3 - C_2H_5$.

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.[18] Protein contents were measured by the method of Lowry *et al.*[19]

Mutagenicity Tests——Mutagenicity tests were performed by the method of Ames *et al.*[20] 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, and mixed with 0.5 ml of 1/15 M potassium phosphate buffer, pH 7.4 or S9 mix, and then preincubated for 20 min at 37 °C. In this study, S9 mix (0.5 ml) contained S9 (3 mg protein) prepared from PCB-treated or untreated rats or hamsters, glucose 6-phosphate (2.5 μmol), NADPH (2.0 μmol), NADH (2.0 μmol), $MgCl_2$ (4.0 μmol), KCl (16.5 μmol) and sodium phosphate buffer, pH 7.4 (40 μmol). The mixture was diluted with top agar and poured on minimal agar plates. After 2 d of incubation at 37 °C, the number of His^+ revertants was counted.

Assay of Bucetin Metabolites——The incubation mixture (12 ml) contained liver microsomes (24 mg protein) prepared from PCB-treated hamsters, potassium phosphate buffer pH 7.4 (600 μmol), NADP (10 μmol), $MgCl_2$ (63 μmol), glucose 6-phosphate (124 μmol), glucose 6-phosphate dehydrogenase (8 IU) and bucetin (31 μmol) dissolved in DMSO (50 μl). The mixture was incubated for 20 min at 37 °C with shaking (100 strokes/min), then the

reaction was terminated by rapid cooling on an ice bath followed immediately by shaking with dichloromethane (10 ml). The residual aqueous phase was extracted twice more with the same solvent. The combined 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 high performance liquid chromatography (HPLC) using a reverse-phase column, Toyo Soda TSK ODS-120-A (4 mm i.d. × 300 mm).[5] The column temperature was 5 °C and the flow rate was 1 ml/min. Detection was performed with a ultraviolet (UV) absorbance detector operated at 240 nm. The elution was performed with an acetonitrile and 0.25 mM Na_2HPO_4 solution gradient system: elution times ranged from 0—7 min (15—24% acetonitrile), 7—30 min (24% alone), 30—45 min (24—80%) to after 45 min (80% alone). Under the HPLC conditions used, the retention times of authentic specimens were as follows: *N*-(*β*-hydroxybutyryl)-*p*-aminophenol, 5.7 min; *N*-hydroxyphenetidine, 14.9 min; *N*-hydroxybucetin, 18.3 min; bucetin, 22.3 min; *p*-phenetidine, 24.1 min; and *p*-nitrosophenetole, 46.4 min.

Deacylation of Bucetin, *N*-Butyryl-*p*-phenetidine and Phenacetin——Rates of hepatic microsomal deacylation of bucetin, *N*-butyryl-*p*-phenetidine and phenacetin were measured as follows. Each reaction mixture (3 ml) contained liver microsomes (3 mg protein) prepared from PCB-treated or untreated rats or hamsters, potassium phosphate buffer, pH 7.4 (245 μmol), and the substrate (7.7 μmol) dissolved in DMSO (50 μl). The mixture was aerobically incubated for 20 min at 37 °C, then the reaction was terminated by adding sodium fluoride (0.3 mmol) and the mixture was rapidly cooled on an ice bath. After the addition of *N*-*β*-hydroxybutyrylaniline (0.22 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 of the acetonitrile solution containing metabolites of phenacetin was analyzed by HPLC as described above. The metabolites of *N*-butyryl-*p*-phenetidine or bucetin were analyzed by the same method, except that 40% acetonitrile solution was used as a mobile phase. The amounts of *p*-phenetidine formed were determined from the calibration curve constructed for an authentic sample by using a microcomputer, Chromatopak·CR-1A (Shimadzu Ltd., Kyoto).

***N*-Hydroxylation of *p*-Phenetidine**——Hepatic microsomal *N*-hydroxylation of *p*-phenetidine was measured by the same method as described in Assay of Bucetin Metabolites, except that *p*-phenetidine (8.8 μmol) dissolved in DMSO (50 μl) and *N*-butyrylaniline (0.06 μmol) were used as a substrate and an internal standard, respectively. The amounts of *N*-hydroxyphenetidine and *p*-nitrosophenetole formed were separately determined and summed in order to evaluate the reaction rate.

Results

Mutagenicity of Bucetin and Related Compounds

The mutagenicity of bucetin in *S. typhimurium* TA100 was tested using PCB-treated or untreated rat or hamster S9. The mutagenicity of bucetin was clearly demonstrated in the presence of PCB-H-S9 mix (Fig. 1). No appreciable mutagenic activity was detected when

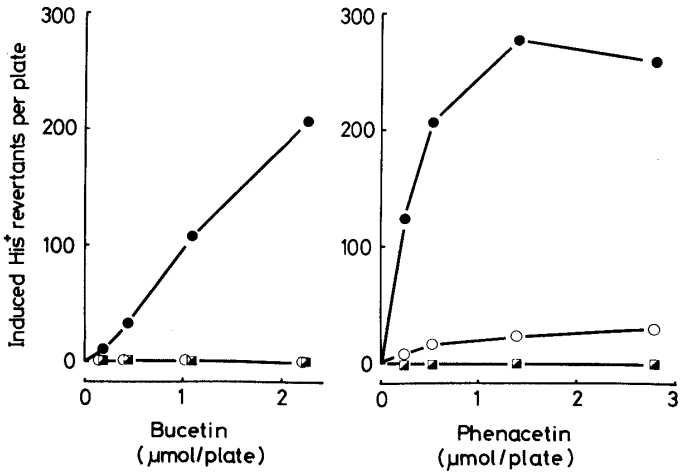
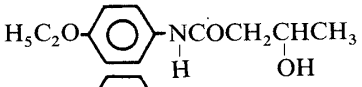
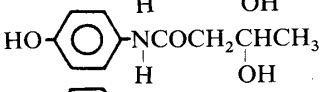
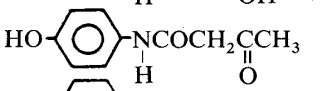
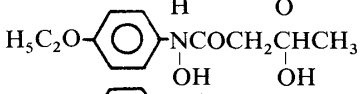
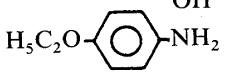
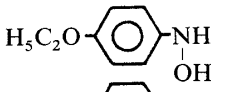
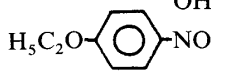
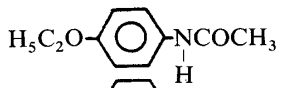
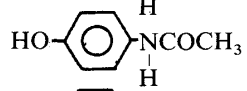
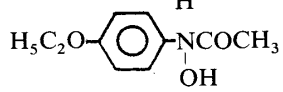


Fig. 1. Mutagenic Activities of Bucetin and Phenacetin in TA100 Mediated by Rat and Hamster Liver Microsomes

The analgetics were incubated with the bacteria, S9 and an NADPH-generating system. Data are expressed as arithmetic mean values of at least three experiments after subtracting the mean number of spontaneous revertant colonies (124/plate). ●, PCB-treated hamster S9; ○, untreated hamster S9; ■, PCB-treated rat S9; □, untreated rat S9.

TABLE I. Mutagenicity of Bucetin, Phenacetin and Related Compounds towards TA100 in the Presence and Absence of PCB-Treated Hamster S9 Mix

Compound	Structure	Mutagenic potency[a] (induced $His^+/\mu mol$)	
		−S9 mix	+S9 mix[b]
Bucetin		0	100
N-(β-Hydroxybutyryl)-*p*-aminophenol		0	0
p-Hydroxyaceto-acetanilide		0	0
N-Hydroxybucetin		0	32[c]
p-Phenetidine		0	608
N-Hydroxy-phenetidine		15400	1740
p-Nitroso-phenetole		22300	1520
Phenacetin		0	366
Acetaminophen		0	0
N-Hydroxyphenacetin		0	2300[c]

a) Data are expressed as arithmetic mean values of at least three experiments after subtracting the mean number of spontaneous revertant colonies (135/plate). *b*) PCB-treated hamster S9 and an NADPH-generating system (S9 mix) were used as an activating system. *c*) PCB-treated hamster S9 without the NADPH-generating system was used.

untreated-H-S9 mix, untreated-R-S9 mix or PCB-R-S9 mix was used. PCB-H-S9 mix was also the most effective for the detection of phenacetin mutagenicity as has previously been demonstrated.[10,11] Therefore, the mutagenic potencies (induced His^+ per μmol) of bucetin, phenacetin and their possible or actual metabolites were compared in the presence of PCB-H-S9 mix, when metabolic activation was required (Table I). The mutagenic potency of bucetin was about a quarter of that of phenacetin. *N*-(β-Hydroxybutyryl)-*p*-aminophenol and acetaminophen, deethylation products of bucetin and phenacetin, respectively, were not mutagenic either with or without metabolic activation. *p*-Hydroxyacetoacetanilide, a known metabolic deethylation and dehydrogenation product of bucetin, was also non-mutagenic even when PCB-H-S9 mix was present. *N*-Hydroxybucetein, a putative metabolic *N*-hydroxylation product, was weakly mutagenic in the presence of PCB-H-S9; its mutagenicity was about one-third and one-70th of those of bucetin and *N*-hydroxyphenacetin, respectively. The mutagenicity tests of the *N*-hydroxy compounds were performed in the presence of PCB-H-S9, but without any fortifying agent, since the mutagenic activity of the compounds was reduced to less than one-half of that without the co-factors (data not shown). *p*-Phenetidine, a deacylation product of bucetin, was about 6 times more mutagenic than bucetin in the presence of PCB-H-S9 mix. *N*-Hydroxyphenetidine and *p*-nitrosophenetole, deacylation and

N-hydroxylation products of bucetin, were strongly mutagenic to TA100 even when PCB-H-S9 mix was absent, and their intrinsic mutagenicities were about 150 times and 220 times higher, respectively, than that of bucetin exerted in the presence of PCB-H-S9 mix.

Metabolism of Bucetin by Hepatic Microsomes

An HPLC study of the dichloromethane extract of an incubation mixture consisting of bucetin, PCB-treated hamster liver microsomes and an NADPH-generating system indicated that *N*-(β-hydroxybutyryl)-*p*-aminophenol, *N*-hydroxyphenetidine, *p*-phenetidine and *p*-nitrosophenetole were formed as metabolites of the analgetic by the microsomes (Fig. 2). The metabolites were separately eluted from the column and shown to be identified with corresponding authentic specimens by mass spectrum (MS) and UV absorption spectroscopy. *p*-Nitrosophenetole could be formed directly from *N*-hydroxyphenetidine by its autoxidation during the incubation and isolation process, since it was also detected in a significant amount from the incubation mixture consisting of boiled microsomes and *N*-hydroxyphenetidine. However, *N*-hydroxybucetin was not detected in the extract of bucetin metabolites under the conditions used. The addition of paraoxon (0.1 mM) or sodium fluoride (0.1 M), inhibitors of microsomal amidase, to the incubation mixture had no effect on the yield of the *N*-hydroxy compound.

A marked species difference was observed in bucetin-deacylating activity between rat and hamster liver microsomes (Table II). Untreated hamsters had about 90 times higher deacylating activity than untreated rats. The activity in hamster liver microsomes was further

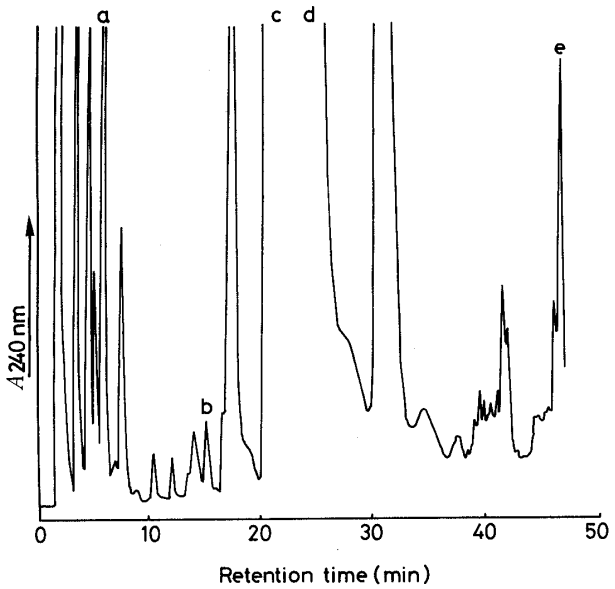


Fig. 2. Chromatogram of Bucetin Metabolites Formed by PCB-Treated Hamster Liver Microsomes and an NADPH-Generating System

The incubation mixture consisted of microsomes (2 mg protein/ml), bucetin (2.6 mM), NADP (0.8 mM), glucose 6-phosphate (10.3 mM), glucose 6-phosphate dehydrogenase (0.67 IU/ml), $MgCl_2$ (5.3 mM), DMSO (0.4%, v/v) and 50 mM potassium phosphate buffer, pH 7.4. After aerobic incubation for 20 min at 37 °C, the mixture was extracted with dichloromethane. The chromatographic conditions were as described in Materials and Methods. When bucetin-deacylating activity was determined, 40% acetonitrile solution was used as a mobile phase, because the peaks of *p*-phenetidine and bucetin were not separated under the above conditions. The small letters indicate the peaks of *N*-(β-hydroxybutyryl)-*p*-aminophenol (a), *N*-hydroxyphenetidine (b), bucetin (c), *p*-phenetidine (d) and *p*-nitrosophenetole (e).

TABLE II. Bucetin-Deacylating and *p*-Phenetidine-*N*-Hydroxylating Activities of PCB-Treated or Untreated Rat and Hamster Liver Microsomes

Animal	Treatment	Bucetin deacylation[a)]	*p*-Phenetidine *N*-hydroxylation[b)]
Rat	None	0.02	0.28
	PCB	0.02	0.84
Hamster	None	1.95	0.47
	PCB	3.55	0.95

a) Activities are expressed as nmol *p*-phenetidine formed/min/mg microsomal protein. *b*) Activities are expressed as total amounts of nmol *N*-hydroxyphenetidine and *p*-nitrosophenetole formed/min/mg microsomal protein.

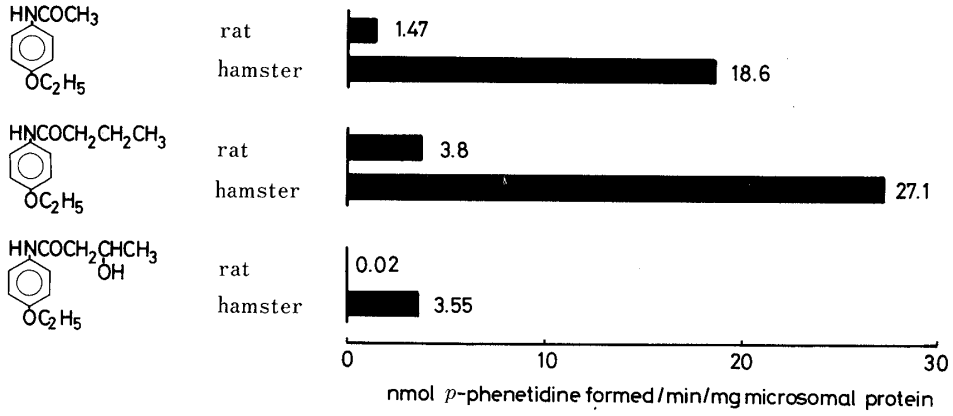


Fig. 3. Deacylation of Phenacetin, *N*-Butyryl-*p*-phenetidine and Bucetin by PCB-Treated Rat and Hamster Liver Microsomes

The incubation mixture consisted of the substrate (2.6 mM), microsomes (1 mg protein/ml), DMSO (1.6%, v/v) and 82 mM potassium phosphate buffer, pH 7.4.

increased two-fold by the PCB treatment, but no detectable increase was observed in rats after the same treatment. Thus, the difference in deacylating activity was about 180 times between PCB-treated rat and hamster liver microsomes. In contrast, *p*-phenetidine-*N*-hydroxylating activity was only 1.5 times higher in untreated hamsters than in untreated rats. The *N*-hydroxylating activity was increased two-fold in hamsters and three-fold in rats by the PCB treatment. Therefore, the activities were almost the same in PCB-treated rat and hamster liver microsomes.

There is a marked difference between the rates of microsomal deacylation of bucetin and phenacetin (Fig. 3). Bucetin was a much poorer substrate than phenacetin. *N*-Butyryl-*p*-phenetidine, a substrate analogous to phenacetin as well as to bucetin, was hydrolyzed at a higher rate than either of them; the relative rate ratios were 5:8:1 and 74:190:1 for phenacetin, *N*-butyryl-*p*-phenetidine and bucetin in PCB-treated hamster and rat liver microsomes, respectively. The order of the rates of deacylation, *N*-butyryl-*p*-phenetidine > phenacetin ≫ bucetin, was the same when untreated rat or hamster liver microsomes were used as an enzyme source. Hepatic soluble supernatant fractions prepared from untreated or PCB-treated rats or hamsters hydrolyzed the three substrates, but the activities were much lower (<5%) than those of the corresponding liver microsomes.

Discussion

Bucetin was found to be mutagenic to *Salmonella typhimurium* TA100 in the presence of PCB-H-S9 mix (Fig. 1). The usually used PCB-R-S9 mix was not effective for detection of the mutagenicity of the analgetic. The mutagenic potency of bucetin was about a quarter of that of the structurally related compound, phenacetin (Table I). Since bucetin, like phenacetin, requires metabolic activation for its mutagenicity, the reason for the species difference in bucetin mutagenicity seems to be the difference of metabolic activation activity between rats and hamsters, and the lower mutagenic activity of bucetin than phenacetin is presumably due to the lower rate of metabolic activation of bucetin. Thus, it is important to know the details of the metabolic pathways of bucetin *in vivo* and *in vitro*. However, little information is available except for some studies on the *in vivo* metabolism of bucetin in rabbits by Ichibagase *et al.*[21] and Shibasaki *et al.*[22] They reported that the main metabolic pathway of bucetin is *O*-deethylation, since a glucuronide conjugate of *N*-(β-hydroxybutyryl)-*p*-aminophenol was detected as the main metabolite from the urine of bucetin-administered rabbits. Glucuronide

conjugates of *p*-hydroxyacetoacetanilide and acetaminophen, both of which may reasonably be assumed to be formed as metabolites of *N*-(β-hydroxybutyryl)-*p*-aminophenol, were also detected from the urine. However, these deethylation products were all non-mutagenic towards TA100 either with or without S9 mix (Table I). Therefore, metabolic pathways other than deethylation could be involved in the metabolic activation of bucetin to bacterial mutagens.

In addition to *N*-(β-hydroxybutyryl)-*p*-aminophenol, other products such as *p*-phenetidine, *N*-hydroxyphenetidine and *p*-nitrosophenetole were detected as metabolites of bucetin formed by PCB-treated hamster liver microsomes fortified with an NADPH-generating system (Fig. 2). Among these metabolites, *p*-phenetidine was about 6 times more mutagenic than bucetin in the presence of PCB-H-S9 mix, and both *N*-hydroxyphenetidine and *p*-nitrosophenetole were strong direct-acting mutagens (Table I). Thus, the metabolic activation of bucetin by PCB-treated hamster liver microsomes is most likely to proceed *via p*-phenetidine to the direct-acting mutagens (Fig. 4). In the case of metabolic activation of phenacetin, not only *p*-phenetidine but also *N*-hydroxyphenacetin plays an important role as a potent mutagenic intermediate, which is further deacetylated to the intrinsic mutagens.[6] *N*-Hydroxybucetin, however, was not detected as an *in vitro* metabolite of bucetin under our assay conditions. The addition of paraoxon or sodium fluoride, both of which have been shown to inhibit microsomal amidase and increase the yield of *N*-hydroxyphenacetin,[12] to the incubation mixture did not lead to the detection of *N*-hydroxybucetin. Moreover, the mutagenic potency of *N*-hydroxybucetin was about one-third of that of bucetin, although the active intermediate, *N*-hydroxyphenacetin, was over 6 times more mutagenic than phenacetin (Table I). The weak mutagenicity of *N*-hydroxybucetin is thought to be due to its resistance to the action of microsomal amidase, since the rate of deacylation of *N*-hydroxybucetin was about 160 times lower than that of *N*-hydroxyphenacetin (unpublished results). Based on these results, we concluded that the metabolic activation of bucetin by hamster liver microsomes proceeds *via p*-phenetidine, but not *via N*-hydroxybucetin, to the intrinsic mutagens.

The marked species difference between rats and hamsters in the mutagenicity of bucetin should exist at the step of either deacylation of bucetin or *N*-hydroxylation of the resulting *p*-phenetidine. Actually, bucetin-deacylating activity was found to be about 180 times higher in liver microsomes from PCB-treated hamsters than in those from PCB-treated rats (Table II). On the other hand, no species difference was observed in the *N*-hydroxylation of *p*-phenetidien. In the previous paper, we reported that *N*-hydroxyphenacetin-deacetylating activity is about 20 times higher in liver microsomes from hamsters than in those from rats.[6] Schut *et al.* also reported that 2-acetylaminofluorene- and *N*-hydroxy-2-acetylaminofluorene-deacetylating activities are about 7 to 10 times higher in liver microsomes from mice than in

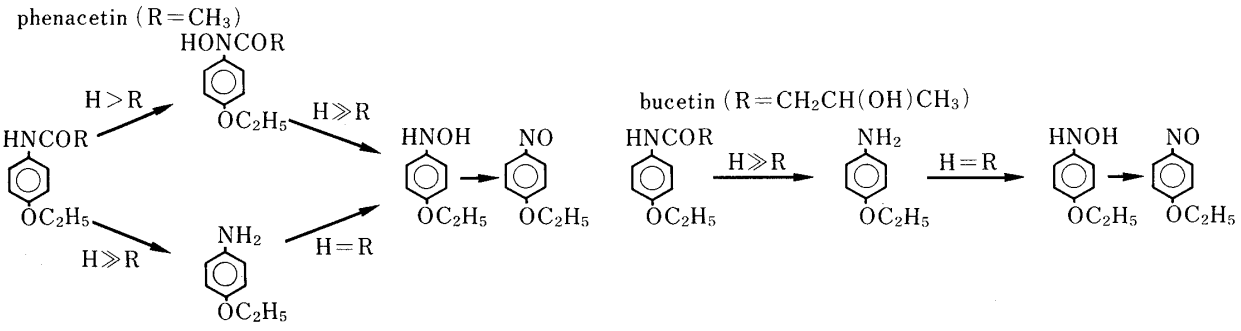


Fig. 4. Proposed Mechanisms for the Metabolic Activation of Phenacetin and Bucetin by PCB-Treated Hamster Liver Microsomes

H and R on the arrows represent hamster and rat, respectively.

those from rats.[23] Thus, it is reasonable to assume that the marked species difference in bucetin mutagenicity (Fig. 1) is due to the difference in bucetin-deacylating activity between rat and hamster liver microsomes. The results of the comparative study on the mechanism of the bacterial mutagenicity of bucetin and phenacetin in relation to the species difference are summarized in Fig. 4. The fact that both bucetin-deacylating and *p*-phenetidine-*N*-hydroxylating activities were doubled in hamsters by the PCB treatment could account for the effectiveness of PCB-H-S9 mix for the detection of bucetin mutagenicity.

The lower mutagenic activity of bucetin than phenacetin can also be ascribed to the difference in the rates of microsomal deacylation between bucetin and phenacetin. Kiese and Renner compared the rates of liver microsomal deacylation of phenacetin and *N*-(α-dimethylamino-β-hydroxybutyryl)-*p*-phenetidine, and reported that the ratio of the rates between the two substrates is 46 : 1 in rabbits, 160 : 1 in dogs and 110 : 1 in guinea pigs.[24] However, it cannot be determined from their results whether the α-dimethylamino group or the β-hydroxyl group makes *N*-(α-dimethylamino-β-hydroxybutyryl)-*p*-phenetidine so poorly hydrolyzable in the microsomes. Thus, in this study, *N*-butyryl-*p*-phenetidine was used as one of the substrates, since the compound has only one point of structural difference from phenacetin and from bucetin. The order of the rates of deacylation among bucetin, *N*-butyryl-*p*-phenetidine and phenacetin by microsomes was *N*-butyryl-*p*-phenetidine > phenacetin ≫ bucetin, regardless of the kind of microsomes used (Fig. 3). The ratio of the rates of deacylation between bucetin and phenacetin was 1 : 5 in PCB-treated and untreated hamster liver microsomes. Therefore, when untreated-H-S9 mix was used, the mutagenicity of bucetin was not detectable, although weak mutagenicity was detected in the case of phenacetin. The deacylation reaction was mediated only by microsomes, since soluble supernatant fractions hydrolyzed the three substrates at extremely low rates. From these results, we concluded that the hydroxyl group at the β-position of the butyryl chain made bucetin poorly hydrolyzable in the microsomes, resulting in lower mutagenic activity of bucetin than phenacetin. It is known that the biological half life of bucetin in rabbits is about 3 times longer than that of phenacetin.[25] The resistant nature of bucetin to microsomal deacylation may be responsible for its longer half life in the experimental animals.

The main metabolic pathway of phenacetin as well as bucetin is *O*-deethylation *in vivo* and *in vitro*, giving rise to acetaminophen from the former.[26] Acetaminophen is known to cause severe liver necrosis at high doses in man and experimental animals.[27,28] The compound also induces chromosome aberrations in Chinese hamster cells *in vitro*.[29] Therefore, it was deduced that the deethylation is the most important step in the conversion of phenacetin to toxic or carcinogenic metabolites. However, the deethylation product, acetaminophen, does not induce mutations in the *Salmonella* tester strains.[7] Neither *N*-hydroxyacetaminophen nor *N*-acetylbenzoquinonimine, both of which are thought to be involved in the induction of liver necrosis of acetaminophen,[30] is mutagenic in the tester strains.[8,31] Moreover, recent studies demonstrated that acetaminophen was not carcinogenic in rats and mice, even when the compound was administered to the animals over 100 weeks.[32] On the other hand, *N*-hydroxyphenetidine and *p*-nitrosophenetole, both of which are metabolic deacylation and *N*-hydroxylation products of phenacetin as well as of bucetin, are potent direct-acting mutagens. *p*-Nitrosophenetole was detected in the blood of *p*-phenetidine-administered dogs.[33] Since *p*-phenetidine is reported to be an *in vivo* metabolite of bucetin as well as of phenacetin,[21] the metabolic activation pathway *via p*-phenetidine to the intrinsic mutagens (Fig. 4) may be applicable not only *in vitro* but also *in vivo*. So far, there is no evidence concerning the carcinogenicity of bucetin in experimental animals and no available epidemiological data on cancer incidence among human bucetin abusers. Nevertheless, there is clearly a potential risk, since potent mutagenic metabolites could be formed from bucetin as well as from phenacetin.

Acknowledgement This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan.

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