Bladder Carcinogen N-Butyl-N-(3-carboxypropyl)nitrosamine

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ABSTRACT

4- (N-Butylnitrosamino)-4-hydroxybutyric acid lactone (BBAL) was synthesized as a possible intermediate produced by metabolic activation of a selective bladder carcinogen, N-butyl-N-(3-carboxypropyl)nitrosamine. BBAL was stable in neutral sodium phosphate buffer (ionic strength, 0.2), having a half-life of more than 30 hr at 25°C. The mutagenic effects of BBAL were tested with the use of Salmonella typhimurium TA1535 and Escherichia coli B/rWP2-try"-, WP2-try"his"-, and S4. The gene-damaging effects were assessed by repair tests with Bacillus subtilis H17 (rec+) and M45 (rec-). BBAL showed potent effects in the mutation and repair tests on all the strains tested without activation. A possibility is suggested for the metabolic activation of N-butyl-N-(3-carboxypropyl)nitrosamine to BBAL by α-hydroxylation at the site of the 3-carboxypropyl chain followed by lactonization in target tissues prior to interaction with macromolecules to lead to carcinogenesis.

INTRODUCTION

BCPN3 (Chart 1, II), the principal urinary metabolite of N-butyl-N-(4-hydroxybutyl)nitrosamine, as well as N,N-dibutylnitrosamine, was demonstrated to be the proximate form of these N-nitrosamines as bladder carcinogens in rats (20). The butyl group of N-butyl-N-(4-hydroxybutyl)nitrosamine can be replaced with another alkyl group without affecting the in vivo metabolic pattern and properties of the metabolite as a bladder carcinogen. Thus, besides N-butyl-N-(4-hydroxybutyl)nitrosamine, N-propyl-, N-ethyl-, and N-methyl-N-(4-hydroxybutyl)nitrosamines were carcinogenic to the urinary bladder of rats (19, 22) after p.o. administration and were metabolically converted into the corresponding N-alkyl-N-(3-carboxypropyl)nitrosamines, which were isolated from the urine of rats in 40 to 50% yields (20). BCPN and N-ethyl-N-(3-carboxypropyl)nitrosamine, the principal urinary metabolite of N-ethyl-N-(4-hydroxybutyl)nitrosamine, were demonstrated to be carcinogenic exclusively to the urinary bladder (7, 8). The length of the methylene chain between the amino nitrogen atom and the terminal hydroxyl or carboxyl group markedly affected the carcinogenic properties of N-nitrosamines. N-Butyl- and N-ethyl-N-(3-carboxypropyl)nitrosamines did not have any carcinogenic effects under conditions similar to those used for N-alkyl-N-(4-hydroxybutyl)nitrosamines (19, 22), and were converted metabolically to N-butyl- and N-ethyl-N-(2-carboxyethyl)nitrosamines, which were isolated from urine in 70 and 60% yields, respectively, suggesting that N-alkyl-N-(2-carboxyethyl)nitrosamines are noncarcinogenic, at least to the urinary bladder (20). N-Butyl-N-(2-hydroxyethyl)nitrosamine, on the other hand, induced hepatic tumors in esophagus (19), while its principal urinary metabolite, BCMN, which was isolated in 40% yield from urine, gave a negative result in the carcinogenicity test (19).

BCPN and its N-propyl, N-ethyl, and N-methyl homologs were found to be weakly mutagenic toward Salmonella typhimurium TA1535 without metabolic activation by the S-9 mix (18). However, the direct mutagenic activity of the compounds with a 3-carboxypropyl chain could not explain their exclusive carcinogenic effect on the urinary bladder, since BCEN with a 2-carboxyethyl chain was also found to be weakly mutagenic toward the same strain of microorganism without metabolic activation (18).

It is reasonable to presume that BCPN undergoes further metabolic activation to the ultimate carcinogenic form. It is now generally accepted that α-hydroxylation is probably a critical step in metabolic activation of N-nitrosamines to their ultimate forms (3). BBN was thus synthesized (15) as a masked model compound for the elusive α-hydroxylated active species (Chart 1, III) derivable from BCPN by α-hydroxylation. BCPN has 2 α-carbon atoms susceptible to hydroxylation: one on the butyl group and the other on the 3-carboxypropyl chain. CMPABN (Chart 1, I) with an acetoxy group at the α-carbon atom of the butyl group, in which the carboxyl group was also esterified, was prepared as a model compound for a possible active species (Chart 1, II) derivable from BCPN by α-hydroxylation. Mutagenic and gene-damaging effects of CMPABN were compared with those of the corresponding α-
acetoxy compounds derived from BCEN and BCMN (24). There were no distinct differences in these effects among the 3 model compounds. Hydroxylation at the ω-carbon atom of the ω-carboxyalkyl chains, on the other hand, would give a compound (Chart 1, IV) with BCPN in which the hydroxyl and the carboxyl groups are situated in the proper position to form a stable γ-lactone (Chart 1, V) with BCPN in which the hydroxyl and the carboxyl groups are situated in the proper position to form a stable γ-lactone could be formed, although this is unlikely to occur easily.

Thus, if such an N-nitroso compound with a γ-lactone does exist with some stability and exert any direct biological effect, it could be a key intermediate in the metabolic activation of BCPN. In this communication, synthesis of the γ-lactone BBAL (Chart 1, V) and its biological effects, examined by mutation and repair tests, are described, and the role of BBAL in the carcinogenesis by BCPN is discussed.

MATERIALS AND METHODS

Apparatus. UV spectra were measured with a Hitachi Model EPS-3T spectrometer. IR spectra were run on a Hitachi Model EPI-S2 spectrometer. Nuclear magnetic resonance spectra were determined on a 60-MHz Hitachi Model R-20A spectrometer in CDCl₃ solution and are reported as ppm downfield from tetramethylsilane as internal reference.

Chemicals. BBAL (Chart 1, V) was synthesized according to the procedure reported by Roller et al. (23) with some modifications. To an ice-cooled solution of n-butylamine (1 ml, 10 mmol) in glacial acetic acid (15 ml) and water (1 ml) were added 5 ml of succinic semialdehyde monomer (approximately 15% aqueous solution, 7.4 mmol), which was obtained from Sigma Chemical Co., St. Louis, Mo. Sodium nitrite (1.8 g, 26 mmol) was then added portionwise to the above solution over a period of 3 hr. The mixture was stirred overnight in an ice bath and extracted with methylene chloride. The organic layer was washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent gave a yellow oil which was purified by chromatography on a column of Silica Gel 60 (Merck, Darmstadt, Germany; less than 0.063 mm). BBAL was eluted by washing the column with n-hexane:ether:methylene chloride (4:3:2), concentrated by evaporation of the solvent under reduced pressure, and then distilled in a vacuum to give 263 mg of BBAL (yield, 19%), b.p. 110°C/1 mm Hg 125–126°C. UV spectrum (ethanol), λmax (ε) 233 (6800), 372 (80). IR spectrum (film), 1790, 1465 cm⁻¹. Nuclear magnetic resonance spectrum, 6.62 ppm, 1H, m (NCHO); 3.57 ppm, 2H, t, J = 7 Hz (NCH₂C); 2.82 ppm, 2.77 ppm, 2H, 2H, m, s (OCCH₂CH₂); 1.4 ppm, 0.9 ppm, 4H, 3H, m, m (CH₂CH₂CH₂).

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\text{C}_{2}\text{H}_{10}\text{O}_{3}\text{N}_{2} \\
\text{Calculated: C 51.60, H 7.58, N 15.04} \\
\text{Found: C 51.31, H 7.54, N 15.33}
\]

The elemental analysis was carried out by Central Research Institute, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

Determination of Stability in Aqueous Solution. Stability of the compounds was determined by following the decrease of the UV absorption at 232 nm due to N—NO function at 25°C in sodium phosphate solutions, pH 2 to 12 (prepared from 0.2 n HCl:0.5 M NaH₂PO₄:0.5 M Na₂HPO₄:0.1 M Na₃PO₄:H₂O to give a constant ionic strength of 0.2).

Bacterial Strains. Marburg strains of Bacillus subtilis H17 (rec⁻) and M45 (rec⁻) (12) were gifts from Dr. T. Kado of the National Institute of Genetics, Mishima, Shizuoka-ken, Japan. The culture of S. typhimurium TA1535 was kindly provided by Dr. Bruce N. Ames of the University of California, Berkeley, Calif. (1). E. coli B/rWP2-try^"^ (25), E. coli WP2-try^"^hcr^"^ (10) and E. coli Sd4 (2) were kindly donated by Dr. S. Iwahara, Food and Drug Safety Center, Hadano, Kanagawaken, Japan.

Media. The liquid medium used for growth of all the bacterial strains tested was NB containing 8 g of Difco Bacto-Nutrient Broth and 5 g of NaCl per liter. NB supplemented with 0.004% streptomycin (Kyowa Hakko Kogyo, Tokyo) was used in the case of E. coli Sd4. The minimal media used for mutagenicity assays were as follows: S. typhimurium TA1535, the medium reported by Ames et al. (1); E. coli strains WP2, modified Vogel-Bonner E medium ([NH₄]₂SO₄, 1 g; KH₂PO₄, 10 g; MgSO₄·7H₂O, 0.1 g; trisodium citrate-2H₂O, 0.5 g) supplemented with 20 ml of NB per liter and 0.4% glucose; E. coli Sd4, NB. The agar plate contained 30 ml of the minimal medium containing 1.5% Difco agar.

Repair Tests. B. subtilis strains H17 and M45 were grown in 3 ml of liquid broth [10 g of Difco Bacto-Peptone; 10 g of Difco Bacto-Beef Extracts, and 5 g of NaCl in 1000 ml water (pH 7.0)] for 18 hr at 37°C. One ml of overnight culture of each strain was diluted with 9 ml of NB, and the mixture was grown for 4 hr at 37°C. The culture of H17 was diluted 10 times with NB, and that of M45 was not diluted. The streaks of both cultures and the application of test compounds were carried out according to the method of Kada et al. (12) on NB agar plates.

Mutation Tests. The microorganisms were grown in 5 ml of NB for 16 hr at 37°C. The turbidity of the suspension was checked at 660 nm and adjusted to be constant for each strain, if required. To a tube containing 0.1 ml of appropriately diluted dimethyl sulfoxide solution of a test compound was added 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4), prepared from 0.1 M NaH₂PO₄ and 0.1 M Na₂HPO₄, and then 0.1 ml of a culture of bacterial tester strain. This mixture was mixed with 2 ml of top agar (0.6% Difco agar in 0.6% NaCl solution) at 45°C and poured onto a minimal agar plate. After incubation at 37°C for 2 days with S. typhimurium TA1535 and
Stability of BBAL in Aqueous Solution. The stability of BBAL in aqueous solution at 25° with the pH range of 2 to 12 was compared with that of BABN and CMPABN (Chart 2). BABN and CMPABN showed similar hydrolysis rates and pH profiles; the rate constants at pH 4 to 10 showed a small variation, and acid- or base-catalyzed decomposition was observed at pH below 3 or above 11, respectively. BBAL apparently lacked the constant hydrolysis rate region with change in pH of the solution and showed acid-catalyzed decomposition below pH 4 and base-catalyzed hydrolysis above pH 5. BBAL was most stable at pH 4 to 5. At pH 7, BBAL had a half-life of 34 hr, compared with those of BABN and CMPABN, 16 and 36 min, respectively, thus suggesting stabilization by intramolecular γ-lactone formation. BCNP was stable at the conditions tested, and no decrease in the UV absorption was observed.

Repair Tests with B. subtilis. As shown in Table 1, the gene-damaging effect of BBAL with B. subtilis was demonstrated in the repair test by the increased lethality on recombination-deficient strain M45 (rec−) over the wild strain Hi 7 (rec+/−). N-Methyl-N′-nitro-N-nitrosoguanidine was used as a positive control, and the effect of BBAL was compared with those of BABN (17, 21) and CMPABN (24). The effect of BBAL was found to be the most potent of the 3 compounds tested. No gene-damaging effect was observed with succinic semialdehyde, one of the possible decomposition products of BBAL.

Mutagenic proper ties of BBAL were examined with S. typhimurium TA1535, E. coli B/rWP2-try− and its UV-sensitive mutant strain WP2-try−hcr−, and E. coli Sd4, which is streptomycin dependent, and compared with those of the α-acetoxy derivatives, BABN (17, 21) and CMPABN (24). Succinic semialdehyde showed no effect at concentrations tested. As shown in Chart 3, BBAL was slightly more potent than BABN but considerably less potent than CMPABN with S. typhimurium TA1535, while the strongest mutagenic activities were observed with all the E. coli strains tested. BBAL was peculiar in inducing revertants with a similar order of magnitude in S. typhimurium and E. coli.
strains, while the α-acetoxy compounds, BABB and CMPABN, showed a much more potent activity in S. typhimurium than in E. coli WP2-try-"hcr". The background lawn on the test plates was examined, and no decrease of bacterial growth was observed in the concentration range tested.

**DISCUSSION**

BCPN has 2 α-carbon atoms that can be α-hydroxylated in the metabolic activation, as shown in Chart 1. *N-(1-Hydroxybutyl)-N-(3-carboxypropyl)nitrosamine* (Chart 1, III), which could be produced by hydroxylation at the α-carbon atom of the butyl group, is expected to be formed from CMPABN by hydrolysis. Thus, CMPABN and its homologs with shorter alkyl chains, *N-(2-carboxyalkyl)nitrosamine* and *N-(carboxyalkylnitrosamine)*, were prepared (16), and their biological activities were compared by mutation and repair tests (24). They were all mutagenic without activation, but there were no correlations between their mutagenic activity and the carcinogenicity of their parent compounds; of the 3 N-butyl-N-(α-carboxyalkyl)-nitrosamines, only BCPN was found to be selectively carcinogenic to the urinary bladder.

α-Hydroxylation of BCPN at the site of the 3-carboxypropyl chain would afford N-butyl-N-(1-hydroxy-3-carboxypropyl)nitrosamine (Chart 1, IV), which could form a stable γ-lactone ring to give BBAL (Chart 1, V), while the homologous BCN and BCMN would give N-butyl-N-(1-hydroxy-2-carboxyethyl)- and N-butyl-N(1-hydroxy-2-carboxymethyl)nitrosamines by α-hydroxylation of their ω-carboxylalkyl chains, which could only form unstable β- and α-lactones. Mandolini et al. (6, 13) reported the rate of internal ring closure from ω-bromoalkanoate ions at 50°C as 0.00241, 0.587, 170, and 1.88 sec⁻¹ for α-, β-, γ-, and δ-lactone formations. Thus, the intramolecular γ-lactone formation is 290 and 70,000 times more favorable than are the β- and α-lactone formations, respectively, and 90 times more favorable than is the δ-lactone formation. The distinct difference in the carcinogenicity among the 3 N-butyl-N(ω-carboxyalkyl)nitrosamines may be associated with the possible formation of the lactone ring with the presumed active metabolites produced by α-hydroxylation at the site of ω-carboxyalkyl chains.

BBAL was found to be much more stable in neutral and weakly acidic aqueous solutions than were the α-acetoxy derivatives BABB and CMPABN, and it exhibited potent biological effects on all the microbial systems examined. These results suggest that the lactone BBAL does play some role in activating the selective bladder carcinogen BCPN. Thus, the mechanism of action of the bladder carcinogenic *N,N*-dialkylnitrosamines with a 4-hydroxybutyl chain might be explained as follows. N-Alkyl-4-hydroxybutyl nitrosamines are metabolically transformed in the liver to water-soluble proximate carcinogens, N-alkyl-N(3-carboxypropyl)nitrosamines, which are excreted into the urine in large quantities and which contact and interact with the bladder epithelial cells for a long period of time; the bladder cells incorporate the proximate carcinogens and further activate them through α-hydroxylation; the resulting α-hydroxylated compounds on the 3-carboxypropyl chain are stabilized in the form of γ-lactone to escape instantaneous decomposition before they reach target molecules with enough concentration to alkylate DNA in the nucleus.

**REFERENCES**

Synthesis and Mutagenicity of 4-(N-Butyl Nitrosamino)-4-hydroxybutyric Acid Lactone, a Possible Activated Metabolite of the Proximate Bladder Carcinogen N-Butyl-N-(3-carboxypropyl)nitrosamine
