METABOLIC FATE OF N-BUTYL-N-(4-HYDROXYBUTYL)NITROSAMINE IN THE RAT*1

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The metabolic fate of N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) was studied in the rat, to investigate the possibility of a relationship between urinary metabolites and organotropic carcinogenicity to the urinary bladder of this N-nitrosamine. The principal urinary metabolite of BBN was identified as N-butyl-N-(3-carboxypropyl)nitrosamine (BCPN). Several minor metabolites characterized were transformation products of BCPN formed by β-oxidation according to the Knoop mechanism, i.e., N-butyl-N-(2-hydroxy-3-carboxypropyl)nitrosamine, N-butyl-N-(carboxymethyl)nitrosamine and N-butyl-N-(2-oxopropyl)nitrosamine; glucuronic acid conjugates of BBN and BCPN were also detected. No BBN was detected in the urine. A possible correlation of the urinary excretion of BCPN with selective induction of bladder tumors by BBN in rats is discussed in relation to the carcinogenic action of BCPN.

Key words: Urinary bladder tumor — Carcinogen metabolite — N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN)

Selective induction of urinary bladder tumors in rats by the oral administration of BBN (Ia)*3 was first reported by Druckrey et al.3) and this finding was confirmed by others in rats6,9) and mice.1,2 It was demonstrated by Ito et al.10) that unilateral ligation of the ureter resulted in a marked increase in the incidence of carcinoma in the renal pelvis and ureter of rats treated with BBN, suggesting that stagnation of urine containing an active carcinogen derived from BBN in the renal pelvis, ureter, and bladder might be responsible for inducing tumors of the urinary system.

In order to investigate the possibility of a relationship between the metabolism and the organotropic effect on the urinary bladder of BBN, the metabolic fate of BBN in the rat was studied. This paper reports the isolation and characterization of urinary metabolites after oral administration of BBN to the rat.

MATERIALS AND METHODS

Instrumentation Melting points were taken on a micro hot-stage apparatus and are uncorrected. Ultraviolet (UV) spectra were measured in 95% ethanol solution with a Hitachi EPS-3T

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*3 The following abbreviations are used: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; BCPN, N-butyl-N-(3-carboxypropyl)nitrosamine; BHCPN, N-butyl-N-(2-hydroxy-3-carboxypropyl)nitrosamine; BCPN, N-butyl-N-(carboxymethyl)nitrosamine; BOPN, N-butyl-N-(2-oxopropyl)nitrosamine; BBN-G, glucuronic acid conjugate of BBN; BCPN-G, glucuronic acid conjugate of BCPN; BCPN-Me, methyl ester of BCPN; BHCPN-Me, methyl ester of BHCPN; BCMN-Me, methyl ester of BCMN.
**METABOLIC FATE OF BBN IN THE RAT**

BBN-G (IVa) and Its Hydrolysis by β-Glucuronidase: A solution of IVa (10 mg) in methanol (2 ml) was treated with 2 N NaOH (0.5 ml) and the mixture was allowed to stand for 5 min at room temperature. After dilution with methanol, the solution was mixed with Amberlite IR-120 (H+) to give pH 4 and filtered. The filtrate was concentrated to dryness in vacuo. The residue was dissolved in acetone and treated with charcoal.

After filtration, the filtrate was concentrated in vacuo to give IVa as a colorless syrup (65 mg) as an oily residue, which was chromatographed on a column of silica gel (400 g) with benzene-ethyl acetate (7:3) to afford the acetate-methyl ester of BBN-G (IVb) (436 mg) as colorless needles after recrystallization from 95% ethanol. mp 107.5–108°. Anal. Calcd. for C$_{21}$H$_{34}$N$_2$O$_{11}$: C, 51.42; H, 6.99; N, 5.71. Found: C, 51.17; H, 6.89; N, 5.51. UV$_{\text{max}}$nm(λ): 234.5 (7700), 352.5 (91), IR$_{\text{KBr}}$$\text{cm}^{-1}$: 1758, 1745, 1438, 1375, 1230.

Partial Purification of Acidic Metabolites: Residues containing acidic metabolites BCPN (IIa), BCMN (VIIa), and BHCPN (Va), were dissolved in methanol and treated with diazomethane in ether in the usual way to give a mixture of methyl esters of acidic metabolites, which was subjected to column chromatography on silica gel for separation and purification. Methyl esters or their mixtures thus obtained were hydrolyzed in methanol with 2 N NaOH for several minutes at room temperature, then the solution was adjusted to pH 4 by adding Amberlite IR-120 (H+) and filtered. The filtrate was concentrated to dryness in vacuo, and the residue was purified by preparative TLC.

Animal Experiments: Male Wistar rats (Fuji Animal Farm, Tokyo) weighing 400–450 g were used. The rats were fasted for one night, then BBN was administered by gastric intubation.
under light ether anesthesia. Three rats received 800 mg (263~270 mg/rat) of BBN and each rat was kept in a metabolic cage. Urine samples were collected under protection from light at 24 hr intervals and stored in a refrigerator; no preservatives were added. In the case of the crystalline acidic compounds, BCPN, BCMN, and BHCPN, a definite amount (ca. 250 mg/rat) was measured accurately and dissolved in 2N NaOH to make a neutral solution, which was given to rats via a stomach tube.

**Extraction Procedures** Urine fractionation by solvent extraction was carried out according to Chart 1.

**Enzymic Hydrolysis with β-Glucuronidase** One volume each of 1M acetate buffer (pH 5) and β-glucuronidase (13000 Fishman units/ml) were added to 10 volumes of urine fraction (adjusted to pH 5), which had been freed from unconjugated metabolites and organic solvents, and the mixture was incubated at 37° for 18 hr. After the incubation, compounds liberated from
glucuronides were extracted with organic solvents as indicated in Chart 1.

**RESULTS**

**Isolation and Identification of Metabolites of BBN**

The pooled 48 hr urine was used in the present work for the isolation and characterization of metabolites, since it was found by quantitative determination using the colorimetric method reported previously that urinary excretion in 48 hr of metabolites retaining the nitrosamino moiety amounted to nearly 80% of the dose, and after this time almost no further excretion into the urine occurred.13)

Treatment of the urine according to Chart 1 gave 6 fractions, Frs. A, B, C, D, E, and F, which were obtained after removal of the solvent under reduced pressure.

Thin-layer chromatographic examination of Fr. A (neutral) with S₁ as a solvent showed the presence of a single metabolite (A) corresponding to BOPN (VI). The oily fraction was subjected to preparative TLC with the same solvent, to give A (7 mg), MS m/e: 158.105 (M⁺, Calcd. for C₇H₁₄N₂O₂, 158.105), 128 (M⁺-NO). This product was identical with an authentic sample of BOPN on direct comparison (TLC, IR and NMR spectra).16,19)

The thin-layer chromatogram of Fr. E (neutral) obtained with solvent S₁ also contained only one metabolite (E) corresponding to BBN. This fraction was purified by preparative TLC with S₁ as a solvent to afford E (16 mg). E was identified as BBN by comparing its spectral (IR, NMR) data with those of an authentic sample.16,20)

On the basis of their thin-layer chromatographic behavior, it was presumed that C₁, D₁, and F are the same compound and that C₂ is identical with D₂. These assumptions were confirmed by thin-layer chromatographic examination of the products obtained by methylation of the three fractions, with S₁ as a solvent. Thus, the methyl esters formed from C₁, D₁, and F were the same and the methyl ester of C₂ was identical with that of D₂. In these thin-layer chromatographic examinations it was found further that C₁ (D₁, F), C₂ (D₂) and D₃ corresponded to BCPN (IIa), BCMN (VIIa) and BHCPN (Va), and their methyl esters to BCPN-Me (IIb), BCMN-Me (VIIb) and BHCPN-Me (Vb), respectively.

For the isolation and identification of C₁ and C₂ from Fr. C, the fraction was methylated and the product was subjected to column chromatography on silica gel with solvent S₁ to give a fraction containing a mixture of methyl esters of C₁ and C₂. Alkaline hydrolysis of this fraction yielded an oily product which was purified by preparative TLC with S₂ as a developing solvent to afford C₁ (353 mg) and C₂ (19 mg), which were identified as BCPN and BCMN, respectively.

C₁ and C₂ were treated with p-bromophenacyl bromide in the usual way to give the corresponding p-bromophenacyl esters, which were identical with the corresponding authentic specimens (IIc, VIIc).17)

The methylated product of Fr. D was similarly treated and purified by column chromatography to give two fractions containing a mixture of methyl esters of D₁ and D₂, and the methyl ester of D₃. The first fraction was hydrolyzed with alkali followed by preparative TLC to give D₁ (20 mg) and D₂ (12 mg), which were identical with BCPN and BCMN, respectively.

The methyl ester of D₃ (34 mg), MS m/e: 218.129 (M⁺, Calcd. for C₉H₁₈N₂O₄, 218.127), 188 (M⁺-NO), obtained from the 2nd fraction was shown...
to be identical with BHCPN-Me (Vb)*4 by direct comparison with an authentic sample.20)

In a similar manner, a single compound (F) recognized in Fr. F by TLC was isolated as its methyl ester (4.5 mg), which was shown to be identical with BCPN-Me (IIb).20)

In order to demonstrate unequivocally the presence of BBN-G (IVa) in the urine, a portion of the 4th aqueous layer from which the ethyl acetate layer giving Fr. D had been separated (Chart 1) was saturated with NaCl and extracted with ethyl acetate several times. The organic layer was evaporated to dryness. Thin-layer chromatographic examination of the residue with synthetic BBN-G (IVa) and S3 as a reference and solvent, respectively, revealed the presence of BBN-G in the aqueous layer. Furthermore, the residue was methylated and the product was subjected to column chromatography on silica gel with solvent S1, followed by chloroform-methanol (9:1). An oily residue obtained after removal of the solvent of the latter fraction was acetylated with acetic anhydride and pyridine. After usual work-up, the product was examined by TLC with ethyl acetate-benzene (3:7) as a solvent to give a spot which was visualized with the reagent for N-nitroso compounds.16) The Rf value was the same as that of the authentic acetate-methyl ester of BBN-G (IVb) described above.

The presence of BBN could not be demonstrated in Fr. A in this experiment. No metabolites with the nitrosamino moiety could be detected in Fr. B by TLC.

Isolation and Identification of Metabolites of BCPN, BCMN, and BHCPN

Groups of 3 rats were orally given BCPN (759 mg), BCMN (765 mg) and BHCPN (660 mg) and 48 hr urines were collected. Fractionation of the urines by solvent extraction, and isolation and characterization of metabolites were carried out as described above for BBN. Metabolites identified in the urine of rats receiving BCPN were: BOPN (2 mg, Fr. A), BCPN (307 mg, Frs. C and D), BCMN (26 mg, Frs. C and D), and BHCPN as BHCPN-Me (18 mg, Fr. D). From the urine of rats given BCMN, more than 60% of the dose was recovered unchanged; 482 mg from Frs. C and D. In the case of BHCPN, about 65% of the dose was excreted as such into the urine: BHCPN as BHCPN-Me (465 mg, Fr. D), BOPN (1.3 mg Fr. A), BCPN (13 mg, Frs. C and D), BCMN (3.2 mg, Fr. D).

DISCUSSION

BBN is unique among a large number of carcinogenic N-nitroso compounds.6 It selectively induces urinary bladder tumors in rats3,6,9 and mice.1,2 It is reasonable to consider that carcinogenic urinary metabolite(s) of BBN might take the leading role in the induction of tumors of the bladder.

After administration of single oral doses of BBN to rats more than 50% of the dose was recovered as metabolites retaining the nitrosamino moiety from the urine in 48 hr. BBN is extensively metabolized in this species, no unchanged BBN being found as such in the urine. In the present work using unlabeled BBN, urinary metabolites, if any, which did not retain the nitrosamino moiety were not pursued.

Based on the urinary metabolites characterized, the metabolic pathway of BBN (Ia) in the rat is shown in Chart 2. The principal urinary metabolite, corresponding to more than 40% of the dose, was BCPN (IIa). Several other N-nitroso compounds were characterized as minor metabolites: these were transformation products formed from BCPN by ω-oxidation according to the Knoop mechanism, i.e., BHCPN (Va), BCMN (VIIa) and BOPN (VI), and gluc-
Separation and purification of acidic metabolites (BCPN, BCMN, and BHCPN) from Frs. C and D were carried out via their methyl esters (IIb, VIIb, and Vb). In particular, the isolation of BCPN and BCMN could be achieved through this process followed by alkaline hydrolysis to generate the original acids, which were purified by preparative TLC.

Urinary excretion of metabolites was also examined after oral administration of BCPN, BHCPN, and BCMN, in order to investigate their further biotransformations. More than 40, 65, and 60% of the doses were recovered unchanged from the urines in 48 hr after administration of BCPN, BHCPN, and BCMN, respectively. It was an unexpected and interesting finding that BCPN was isolated and identified as a metabolite of BHCPN. The mechanism involved in this bioconversion is unknown.

The presence of BBN-G (IVa) as a minor metabolite was unequivocally demonstrated, and that of BCPN-G (III) was inferred on the basis of the finding that a small amount of BCPN was isolated and identified from the urine fraction only after enzymic hydrolysis with β-glucuronidase.

The carcinogenic effects of BBN and its metabolites, BCPN, BCMN, and BOPN, were investigated in rats. Selective induction of bladder tumors in 100% incidence was demonstrated not only by BBN but also by BCPN. The histological type of tumors induced by both compounds was found to be the same. Thus BCPN, the principal urinary metabolite of BBN, is a selective and potent bladder carcinogen in rats, as is its mother compound. Moreover, direct carcinogenic action of BCPN on bladder epithelium was demonstrated by inducing bladder tumors in female rats through intravesicular instillation. Neoplastic transformation of epithelial cells of the rat bladder by BCPN in vitro was also demonstrated. On the other hand, BCMN was estimated not to be carcinogenic, more than 60% of the dose being recovered unchanged from
the urine. Hepatomas were induced by treatment with BOPN,\(^{12}\) while the carcinogenicity of BHCPN, a minor urinary metabolite, has not yet been examined.

Urinary excretion of BCPN after oral administration of BBN to rats amounted to be more than 40% of the dose, and the recovery of BCPN as such from the urine of rats given BCPN orally was also found to be more than 40% of the dose. It seems reasonable therefore to conclude that the induction of bladder cancer by BBN is ascribable to the principal urinary metabolite BCPN. In this connection, it is noteworthy that BCPN has been proved to be mutagenic on *Salmonella typhimurium* strain TA1535 in the absence of a metabolic activation system, while BBN, BHCPN, and BOPN were mutagenic with the S-9 mix and BCMN was nonmutagenic with and without the S-9 mix.\(^{18}\)

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