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Novel Type of Ornithine-Glutathione Double Conjugate Excreted as a Major Metabolite into the Bile of Rats Administered Clebopride

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SUMMARY

Rats orally given radioactive Clebopride [[^{14}C]CP; *N*-(1′-benzyl-4′-piperidyl)-2-[^{14}C]methoxy-4-amino-5-chlorobenzamide], an antiulcer agent, excreted a novel type of ornithine (Orn)-GSH double conjugate in the bile as a major metabolite ([^{14}C]BMCP), corresponding to 18% of the dose. The present study provides the first evidence for Orn conjugation of a xenobiotic in mammals and demonstrates that the structure of the radioactive conjugate differs fundamentally from those known in birds and reptiles. The structure of the biliary metabolite, [^{14}C]BMCP, purified to homogeneity by silica gel thin layer and reverse phase high pressure liquid chromatography, was elucidated as *S*-[2-ornithylamino-4-[^{14}C]methoxy-5-(1′-methyl-4′-piperidylamino)carboxyphenyl]glutathione, based mainly on the following facts: 1) BMCP showed a protonated molecular ion $(M + H)^+$ peak at *m/z* 683 in the secondary ion mass spectrum and 2) [^{14}C]BMCP afforded Orn, glutamic acid, glycine, *S*-(2-amino-4-[^{14}C]methoxy-5-carboxyphenyl)cysteine ([^{14}C]AMCC), and 1-methyl-4-aminopiperidine (MAP) quantitatively, in an equal molar ratio, by complete hydrolysis with peptidase. Thus, BMCP was a metabolite with three enzymatically hydrolyzable amide bonds in addition to the one existing originally in the parent structure of the drug, which produces MAP by peptic digestion. Of the three additional amide bonds of BMCP, one was a novel type of bond formed by condensation of the α-carboxylic acid group of Orn with the primary aromatic amino group of the drug and the other two were in the *S*-glutathionyl residue, substituted for the chlorine atom vicinal to the Orn-conjugating primary amino group in the aromatic ring and affording glutamic acid, glycine, and the *S*-cysteine conjugate AMCC by hydrolysis of BMCP with the peptidase. Substitution of a methyl group for the benzyl group at the piperidine ring nitrogen atom, leading to the formation of MAP by peptic digestion, also occurred during metabolism of CP to BMCP.

CP is an antiulcer agent (1, 2) whose metabolism *in vitro* was first investigated in the rabbit liver 9000 × *g* supernatant fraction (3). Five lipophilic metabolites were isolated and identified in the *in vitro* study. They were an oxidation product bearing a hydroxyl group at the benzylic carbon (metabolite 1), an *N*-debenzylated product (metabolite 2) and its piperidine ring lactam, CP *N*-oxide, and a carboxylic acid formed by hydrolysis of the amide bond of CP (3–6). These metabolites, except metabolite 1, were excreted together with CP N^4-glucuronide into the urine of rats, rabbits, dogs, and humans given CP (7). Major urinary metabolites were *N*-desbenzyl-CP (metabolite 2) in rats (20% of dosed CP) and humans (47%) and the N^4-glucuronide of CP in rabbits (16%).

In rats, 57% of the radioactivity from dosed [^{14}C]CP was excreted into the bile (8). However, no investigation has been made on the biliary metabolites of CP. The present study provides the first evidence for the predominant excretion of a novel type of Orn-GSH double conjugate (BMCP) into the bile of rats given [^{14}C]CP orally. Discussion will be made with respect to a structural difference, in relation to the mode of Orn conjugation, between BMCP and known Orn conjugates of xenobiotics in birds and reptiles.

Experimental Procedures

Materials. [^{14}C]CP (*N*-(1′-benzyl-4′-piperidyl)-2-[^{14}C]methoxy-4-amino-5-chlorobenzamide, 28.5 mCi/mmol), with radiochemical purity higher than 99%, was obtained from Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). Unlabeled CP was supplied by Laboratories Almirall S. A. (Barcelona, Spain). A peptidase preparation (from porcine intestinal mucosa) and the liquid scintillator Atomlight were purchased

ABBREVIATIONS: CP, Clebopride, *N*-(1′-benzyl-4′-piperidyl)-2-methoxy-4-amino-5-chlorobenzamide; Orn, ornithine; BMCP, biliary metabolite of Clebopride; AMCT, 2-amino-4-methoxy-5-carboxythiophenol; N^4-acetyl-CP, *N*-(1′-benzyl-4′-piperidyl)-2-methoxy-4-acetylamino-5-chlorobenzamide; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; AMCC, *S*-(2-amino-4-methoxy-5-carboxyphenyl)cysteine; MAP, 1-methyl-4-aminopiperidine; EI, electron ionization; FD, field desorption; MS, mass spectrometry (spectrum); SI, secondary ion.

from Sigma Chemical Co. (St. Louis, MO) and New England Nuclear (Boston, MA), respectively. Dansyl chloride, *N*-(1′-naphthyl)-ethylenediammonium dichloride, 4-dimethylaminobenzaldehyde, and other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *S*-(*p*-Bromophenyl)-L-cysteine was kindly supplied by Nippon Roche K. K. (Tokyo, Japan). MAP (9) and 2-amino-4-methoxy-5-carboxychlorobenzene (10) were synthesized by previously reported methods.

Synthesis of N^4-acetyl-CP. CP (187 mg) was quantitatively converted into the N^4-acetate in acetic anhydride (10 ml) at room temperature overnight. A residue, obtained on the evaporation of the solvent from the solution *in vacuo*, was recrystallized from methanol/ether to give colorless plates; m.p. 210° (uncorrected); UV: λ_{max} [nm (ϵ) in methanol] 207 (32,200), 235 (10,740), 280 (8,500), and 302 (11,100); ^{1}H NMR: δ (ppm in $CDCl_3$) 1.2–2.8 (m. 9 H, piperidine-H), 2.5 (s, 3 H, N^4-acetyl-CH_3), 3.4 (s, 2 H, α-benzyl-CH_2), 3.8 (s, 3 H, -OCH_3), 6.4 (s, 1 H, C_3-H), 7.2 (s, 5 H, benzyl aromatic-H), and 7.6 (s, 1 H, C_6-H); EI-MS: *m/z* (relative intensity, %) 415 (M^+, 12.0), 324 (12.1), 284 (24.3), 244 (39.2), 226 (68.0), 184 (74.0), 173 (98.2), 91 (96.4), and 82 (100); IR: ν_{max}(cm^{-1} in KBr) 3,350, 2,900, 1,700, 1,590, 1,560, 1,390, and 1,310.

Synthesis of AMCT. To an aqueous sodium nitrite (0.18 g) solution (3 ml), chilled in an ice bath, was added dropwise a cold aqueous solution (20 ml) of 2-amino-4-methoxy-5-carboxyl-chlorbenzene (0.7 g) and hydrofluoroboric acid (0.7 g), with stirring, during 5 min. After 5 min. a suspension of copper powder (1 g) in water (10 ml) containing sodium nitrite (2 g) was added to the above solution, and the mixture was heated at 50° for 5 hr and then extracted with benzene. A yellowish brown residue, obtained on the evaporation of the solvent from the organic extract, was suspended in an aqueous sodium sulfide (4 g) solution (10 ml) and heated under a refluxing condition in nitrogen as a gaseous phase for 5 hr. The reaction mixture was acidified with acetic acid (4 ml), saturated with sodium chloride, and extracted with ethyl acetate. From the organic layer, which was dried over anhydrous sodium sulfate, the solvent was evaporated to dryness *in vacuo*. A residue obtained was recrystalized from acetone/benzene to give colorless plates in 48% yield; m.p. 180–181° (uncorrected); UV: λ_{max}[nm (ϵ) in methanol] 216 (14,500), 238 (10,000), 277 (8,500), and 313 (6,500); ^{1}H NMR: δ (ppm in $CDCl_3$) 3.85 (s, 3 H, -OCH_3), 6.50 (s, 1 H, C_3-H), and 7.70 (s, 1 H, C_6-H); EI-MS: (*m/z* relative intensity, %) 199 (M^+, 6.4), 182 (21.0), 167 (79.0), 152 (35.0), 135 (100), and 107 (97.0); IR: ν_{max} [cm^{-1} in KBr] 3,160 (broad), 2,750, 1,500, 1,430, 1,390, 1,320, 1,245, 1,190, and 1,100.

***In vivo* experiments.** Three groups of three male Wistar rats (280–300 g) were anesthetized by pentobarbital, and their bile ducts and bladders were cannulated with polyethylene tubes. After recovery from anesthesia, the rats were administered [^{14}C]CP (100 mg/50 μCi/ml of 5% gum Arabic solution/kg) or unlabeled CP (100 mg/kg) by gavage and placed in Bollman's cages for collection of their 24-hr bile. The amount of the radioactivity excreted into the pooled 24-hr bile (18–22 ml) of each group of animals was determined by liquid scintillation counting, as described below, after aliquots (10–100 μl) of the bile were diluted with 9 volumes of water, followed by mixing of the diluted bile (50 μl) with a scintillator (5 ml). The remaining bile was frozen and dried *in vacuo* for subsequent analysis and isolation of the radioactive metabolite.

Isolation and purification of BMCP. An equivalent of 6 ml of lyophilized bile from each group of rats administered [^{14}C]CP or unlabeled CP was suspended in methanol (1.5 ml). A methanolic supernatant fraction (1 ml), obtained by centrifugation at 3000 rpm for 10 min, was subjected to HPLC for isolation of [^{14}C]BMCP. HPLC was carried out on a reverse phase column (ODS-120T, 10-μm particle size, 4 × 250 mm; Tosoh Co., Tokyo, Japan) eluted at a flow rate of 1 ml/min, at 37°, with methanol/0.1 M aqueous acetamide (1:1, v/v, solvent A). The radioactivity peak corresponding to [^{14}C]BMCP was collected by repeated injection of the methanolic extract of the dried bile. From the collected radioactive column effluent, the solvent was evaporated to dryness *in vacuo*, and the residue obtained was dissolved in methanol (0.1 ml). The methanolic solution was subjected to preparative TLC, carried out on precoated silica gel plates (Kieselgel $_{60}F_{254}$, 2-mm thickness; E. Merck, Darmstadt, FRG) in chloroform/methanol/28% (w/v) ammonia (10:4:1, v/v, solvent I). The adsorbent area corresponding to [^{14}C]BMCP was scraped and eluted with methanol. The radioactive methanolic eluate was rechromatographed for further purification to homogeneity of [^{14}C]BMCP under the same reverse phase HPLC conditions as described above.

Unlabeled BMCP formed from CP was isolated and purified by the same chromatographic methods as described above. Peak identification of unlabeled BMCP was done by spiking of the methanolic extract of the dried bile with purified [^{14}C]BMCP at an appropriate interval during repeated injections.

Assay of cysteine *S*-conjugate β-lyase in the peptidase preparation. The assay of cysteine *S*-conjugate β-lyase in the porcine intestinal mucosa peptidase preparation was performed by using *S*-(*p*-bromophenyl)cysteine as a substrate in 0.1 M KH_2PO_4/Na_2HPO_4 buffer (pH 7.1), as previously reported (11). Protein was determined by the method of Lowry *et al.* (12).

Digestion of BMCP by the peptidase preparation. [^{14}C]BMCP or unlabeled BMCP (0.5 μmol) was incubated at 37° for 5 hr with the porcine intestinal mucosa preparation (25 mg), containing cysteine *S*-conjugate β-lyase, in 0.1 M KH_2PO_4/Na_2HPO_4 (pH 7.4, 1 ml) in the absence or in the presence of hydroxylamine hydrochloride (0.5, 1, and 2 mM). After the incubations, methanol (2 ml) was added to the mixtures to remove the enzyme preparation as a precipitate. From aqueous methanolic supernatants, obtained by centrifugation at 3000 rpm for 10 min, the solvent was evaporated to dryness *in vacuo*, and the residues were suspended in methanol. Methanolic solutions, obtained after removal of inorganic salts from the suspensions by membrane filtration, were subjected to silica gel TLC for separation and identification of the digestion products.

TLC of digestion products of BMCP. Precoated Kieselgel $_{60}F_{254}$ plates were used for analytical (0.25-mm thickness, 5 or 20 × 20 cm^2) and preparative (2-mm thickness, 20 × 20 cm^2) TLC. Developing solvents used were solvent I, solvent II [ethanol/28% (w/v) ammonia (4:1, v/v)], and solvent III [chloroform/methanol/acetic acid (10:4:1, v/v)].

Visualization of the chromatograms was carried out with an UV lamp (254 nm) for BMCP, AMCC, and AMCT, a ninhydrin reagent [0.5% (w/v) in *n*-butanol] for BMCP, AMCC, and amino acids, and Ehrlich's reagent [1% (w/v) *N*,*N*-dimethyl-4-aminobenzaldehyde in concentrated hydrochloric acid/methanol (1:3, v/v) (13)] for MAP. The primary aromatic amines, CP, AMCC, and AMCT, showed very faint brown coloration when sprayed with the Ehrlich's reagent. However, they showed intense coloration after a diazo-coupling reaction with *N*-(1-naphthyl)ethylene-diammonium dichloride [0.4% (w/v)] in methanol, as a spraying reagent, after their diazotization by spraying with a solution of 1% (w/v) sodium nitrite in concentrated hydrochloric acid, as had been demonstrated with CP (14). Radioactive spots were visualized with a Packard model 7201 readiochromatogram scanner.

Preparative TLC was performed by scraping the adsorbent zones containing the respective products and by eluting them with 50% (v/v) aqueous methanol. Unlabeled peptic digestion products, undetectable with the UV lamp, were visualized by spraying of the corresponding reagents on narrow vertical zones on both sides and in the middle of the plate before the chromatographic zones were marked.

HPLC. A JASCO model BIP-I high pressure liquid chromatograph was used, which was equipped with a model UVIDEC-100V UV spectrophotometer, a model FP-210 spectrofluorometer, and an octadecylsilica column (ODS-120T, 10-μm particle size, 4 × 250 mm; Tosoh Co.). The HPLC column was equipped with a guard column (ODS-120T, 4 × 50 mm) and was maintained at 37° in a JASCO model TU-300 column oven.

Mobile phases used were solvent A [methanol/0.1 M aqueous acetamide (1:1, v/v)], solvent B [methanol/2% (v/v) aqueous acetic acid

(1:2, v/v)], and solvent C [methanol/2% (v/v) aqueous acetic acid (7:5, v/v)].

Fluorescence of dansyl derivatives of amino acids and MAP, prepared by the previously reported method (15), was determined at an excitation wavelength of 340 nm and an emission wavelength of 420 nm.

Spectroscopy. A Hitachi model M-80 mass spectrometer was used for determinations of EI-, FD-, and SI-MS. FD-MS were determined at an emitter current of 14 mA and an accelerating voltage of 3 kV.

UV absorption spectra were determined with a Shimadzu model UV-240 spectrophotometer, IR absorption spectra with a JASCO model A-202 spectrophotometer, and ^{1}H NMR spectra with a JEOL model GX-400 NMR spectrometer, using tetramethylsilane as an internal standard.

Radioactivity determination. A Packard model LS-400 liquid scintillation counter was used for determination of radioactivity of the bile, effluents from the HPLC column, and silica gel scraped from the TLC plates. Atomlight was used as a liquid scintillator. The silica gel was directly suspended in the liquid scintillator for determination of its radioactivity.

Results

Isolation of BMCP from the bile of rats given CP. Male adult rats given [^{14}C]CP orally excreted 46–55% of the radioactivity into the bile within 24 hr. Quantitative extraction of the radioactivity from the lyophilized bile with methanol, followed by radio-HPLC of the methanolic extract, identified at least three polar radioactive metabolites and a small amount of CP (Fig. 1A). The major metabolite, BMCP, was eluted at a retention time of 8.5 min. This peak was collected, subjected to preparative TLC on silica plates, and rechromatographed for further purification to homogeneity [greater than 99% radiochemical purity based on the ratio of radioactivity to absorbance at 305 nm (ϵ = 12,500 in methanol)] under the same HPLC conditions (Fig. 1B). The recovered radioactivity of the purified BMCP corresponded to 17.4–19.5% of the administered dose of [^{14}C]CP.

Purified [^{14}C]BMCP showed a single radioactive spot, at R_F 0.56, 0.78, and 0.63 on silica plates in solvents I, II, and III, respectively, which were also visualized by ninhydrin and with a UV lamp.

Unlabeled BMCP, isolated and purified in the same manner as described above following administration of unlabeled CP, was used for a MS study after identification with purified [^{14}C]BMCP by isotope dilution and preparative HPLC and TLC. BMCP showed a protonated molecular ion peak $(M + H)^+$ at m/z 683 in the SI-MS (Fig. 2) and a UV absorption spectrum with a marked difference in shape from that of CP (Fig. 3A) and a close similarity to that of N^4-acetyl-CP (Fig. 3B); λ_{max} [nm (ϵ) for BMCP in methanol] 208 (14,000), 239 (7,000), 282 (11,000), and 305 (12,500) (Fig. 3C). Unlike the UV absorption spectrum of the primary aromatic amine CP (Fig. 3A), those of N^4-acetyl-CP and BMCP did not show any blue shift after the addition of hydrochloric acid to the methanolic solution (Figs. 3B and 3C). The intensity of the peak maximum at 207–208 nm in the UV absorption spectrum of [^{14}C]BMCP determined in methanol (Fig. 3C) was about half of those for CP (Fig. 3A) and N^4-acetyl-CP (Fig. 3B), suggesting that BMCP no longer retained the N-benzyl chromophore existing in the latter two.

Structural assignment of BMCP. [^{14}C]BMCP was completely digested by the incubation with a commercially available porcine intestinal mucosa peptidase preparation containing a potent cysteine S-conjugate β-lyase activity. The unexpected presence of the β-lyase activity in the peptidase preparation was confirmed by the formation of p-bromophenyl mercaptan from S-(p-bromophenyl)cysteine, with a specific activity of 367 nmol of product formed/mg of protein/min. Silica TLC analysis (in solvent I) of a supernatant of the incubation mixture indicated that the mixture contained at least three products, located at R_F 0.49 and 0.17 and the origin in the chromatogram.

The product at R_F 0.49 was visualized by yellow coloration with Ehrlich's reagent for detection of primary amines. This product, however, did not show any radioactivity, UV absorbance, or reactivity with ninhydrin. It was eluted from the chromatogram and rechromatographed at R_F 0.56 on a silica plate in solvent II for purification. The purified product, eluted from the second chromatogram, showed a molecular ion peak at m/z 114 in the EI-MS and was identified as MAP by comparison with the corresponding authentic specimen (Fig. 4A).

The product at R_F 0.17 in the first TLC had radioactivity and UV absorbance but did not react with ninhydrin. On the silica plate, this product was visualized by reddish purple col-

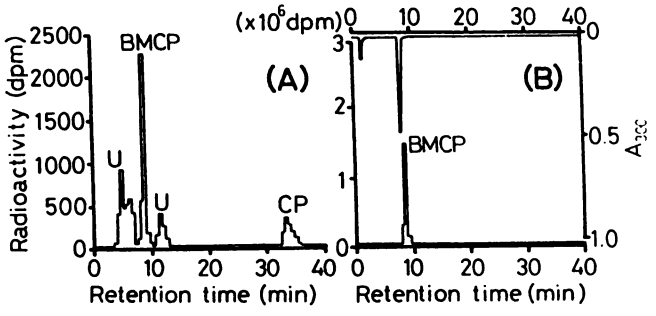

Fig. 1. Radio-HPLC chromatograms of biliary metabolites of [^{14}C]CP in rats (A) and of the major metabolite, [^{14}C]BMCP, isolated and purified thereof by preparative HPLC and TLC (B). HPLC and TLC were carried out on a reverse phase column (ODS-120T) and silica plates, respectively, as described in Experimental Procedures. The effluent from the ODS-120T column, eluted with solvent A [methanol/0.1 M acetamide (1:1, v/v)] at 1 ml/min and 37°, was collected at 30-sec intervals, and radioactivity was determined with a liquid scintillation counter. *U* in chromatogram A, unidentified radioactive metabolites. The purified [^{14}C]BMCP was also monitored by a UV detector (300 nm) in chromatogram B.

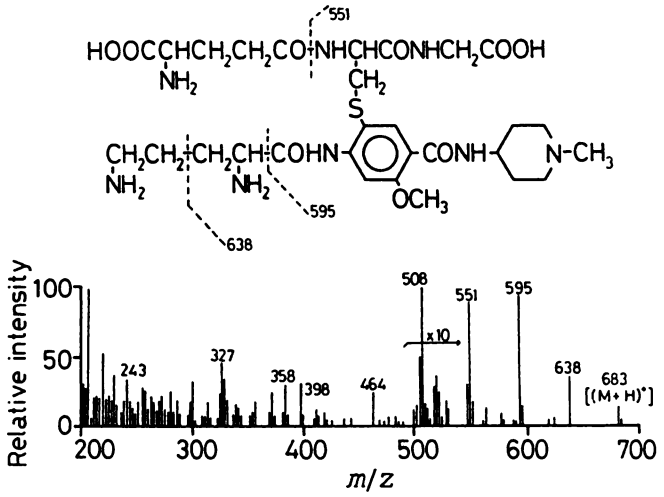

Fig. 2. SI-MS of BMCP isolated and purified from the bile of rats administered CP. The spectrum was recorded by using Xe as a primary ion source, at accelerating voltages of 7 and 3 kV for the primary and secondary ions, respectively.

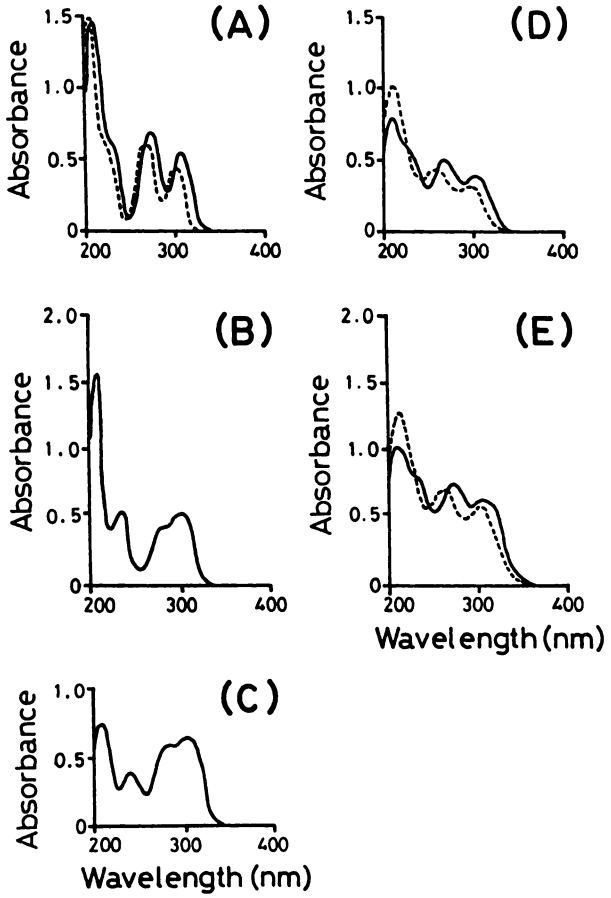

Fig. 3. UV absorption spectra of CP (A), N^4-acetyl-CP (B), BMCP (C), AMCT (D), and AMCC (E) in methanol (—) and methanol/1 N HCl (9:1, v/v) (– – –). The concentration of each compound in both solutions was 50 μM. The absorption spectra of N^4-acetyl-CP (B) and BMCP (C) determined in methanol were superimposable on those in the methanol/1 N HCl.

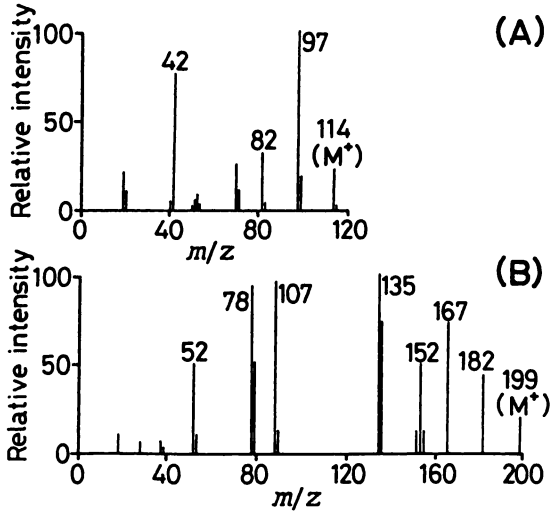

Fig. 4. EI-MS of the digestion products MAP (A) and AMCT (B) formed from BMCP by incubation with the porcine intestinal mucosa peptidase preparation in the absence of the cysteine S-conjugate β-lyase inhibitor hydroxylamine. The spectra were recorded at an ionization chamber temperature of 200°, an ionization voltage of 70 eV, and an accelerating voltage of 3 kV.

oration with a diazo-coupling reagent for detection of primary aromatic amines after diazotization. The same unlabeled peptic digestion product obtained from unlabeled BMCP was purified by preparative TLC on the silica plates in solvents I and II (R_F 0.17 and 0.83) and identified by EI-MS (m/z 199 for M^+) as AMCT by comparison with the corresponding authentic specimen (Fig. 4B). The UV absorption spectrum of AMCT or [^{14}C]AMCT, determined in methanol, showed a marked difference in shape from that of BMCP (Fig. 3C) and showed a blue shift after the addition of hydrochloric acid (Fig. 3D).

The product at the origin in the first TLC was visualized by ninhydrin but did not have any radioactivity or UV absorbance. Development of the chromatogram by the more polar solvent II yielded three ninhydrin-positive products at R_F 0.32, 0.27, and 0.11. These three products were isolated by preparative TLC and identified by SI-MS as glycine [m/z 76, $(M+H)^+$], glutamic acid [m/z 148, $(M+H)^+$], and Orn [m/z 133, $(M+H)^+$], respectively, by comparison with the corresponding authentic specimens (Fig. 5).

In the presence of 2 mM hydroxylamine, a potent cysteine S-conjugate β-lyase inhibitor, [^{14}C]BMCP afforded MAP, glycine, glutamic acid, Orn, and a new radioactive, UV-absorbing, ninhydrin-positive, diazotizable (reddish purple on coupling with 2-naphthol) product located as a single spot at R_F 0.05 and 0.61 on the silica plates developed with solvents I and II, respectively. No detectable amount of [^{14}C]AMCT formation was observed by TLC under the incubation conditions used. The newly formed ninhydrin-positive product, which was also prepared from unlabled BMCP in the same manner as described above, showed an $(M+H)^+$ peak at m/z 287 as the sole ion peak signal in the FD-MS. The UV absorption spectrum of the labeled or unlabeled product was very similar in shape to that of AMCT and demonstrated a blue shift after the addition of

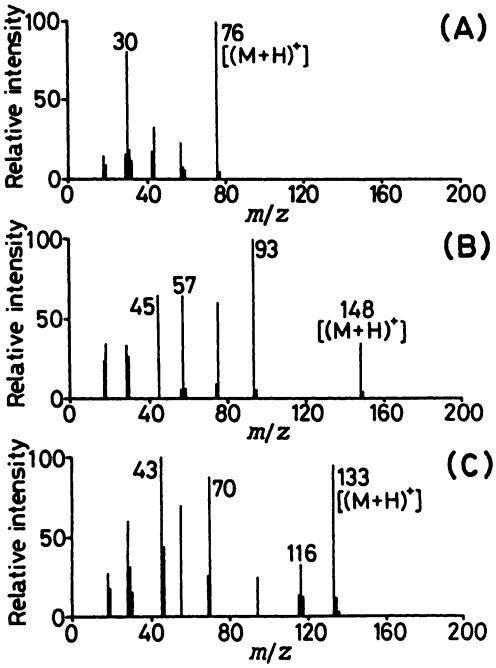

Fig. 5. SI-MS of the digestion products glycine (A), glutamic acid (B), and Orn (C) formed from BMCP by incubation with the porcine peptidase preparation in the presence and in the absence of hydroxylamine. The spectra were recorded under the same conditions as described in the legend to Fig. 2.

hydrochloric acid (Fig. 3E). In the absence of the β-lyase inhibitor, the newly formed radioactive and ninhydrin-positive product, used as a substrate, was quantitatively converted by the incubation with the peptidase preparation into the aminothiophenol [^{14}C]AMCT, which was identified by isotope dilution and preparative HPLC and TLC. Therefore, this product formed from BMCP by the incubation with the peptidase preparation in the presence of hydroxylamine was assigned as AMCC.

When the concentration of the β-lyase inhibitor hydroxylamine was decreased to 0.5 or 1.0 mM, two radioactive products, [^{14}C]AMCT and [^{14}C]AMCC, were simultaneously and stoichiometrically formed from [^{14}C]BMCP by the incubation with the peptidase preparation (Table 1). In the presence of 2 mM hydroxylamine, however, there was no detectable conversion of [^{14}C]AMCC to [^{14}C]AMCT by the peptidase preparation.

Quantitative dansylation of the unlabeled peptic digestion products, glutamic acid, glycine, Orn, and MAP, followed by HPLC of the fluorescent derivatives indicated that they were almost quantitatively formed in an equal molar ratio from [^{14}C]BMCP or BMCP, used as a substrate (Table 2). No detectable amount of these amino acids or MAP was formed when the peptidase preparation was incubated in the absence of the substrate under the same conditions, indicating that the three amino acids did not originate from the enzyme preparation.

TABLE 1

Enzymatic digestion of [^{14}C]BMCP by the peptidase preparation from porcine intestinal mucosa in the absence and in the presence of the cysteine *S*-conjugate β-lyase inhibitor hydroxylamine

[^{14}C]BMCP (1.43 μCi/ml, 50 μM), isolated and purified from the bile of rats given [^{14}C]CP, was incubated with the peptidase preparation (25 mg/ml) from porcine intestinal mucosa at pH 7.1 and 37° for 5 hr, and the radioactive products were isolated and determined as described in Experimental Procedures.

Hydroxylamine	Radioactive products formed	
	AMCC	AMCT
mM	%	
0	ND[a]	99.0
0.5	23.7	75.3
1.0	84.0	14.0
2.0	99.2	ND

[a] ND, not detectable.

TABLE 2

Stoichiometric relationship of all the digestion products formed from [^{14}C]BMCP by the porcine intestinal mucosa peptidase preparation containing cysteine *S*-conjugate β-lyase

Product[a]	HPLC of dansylate[b]	Molar ratios to BMCP	
		Hydroxylamine (2 mM) added	No hydroxylamine
	min		
Gly	18.9	0.96	0.98
Glu	15.7	0.98	0.98
Orn	23.0	0.96	0.96
MAP	90.4	0.96	0.96
[^{14}C]AMCC		0.98	ND[c]
[^{14}C]AMCT		ND	0.96

[a] [^{14}C]BMCP was incubated with the peptidase preparation in the presence and in the absence of 2 mM hydroxylamine, under the same conditions as described in Table 1. The digestion products were separated by ultrafiltration. Aliquots of the filtrate were treated with dansyl chloride in the presence of sodium carbonate to determine the fluorescent derivatives of the three amino acids and MAP, and other aliquots were subjected to HPLC to determine radioactive products, as described in Experimental Procedures.

[b] Monodansylates of glycine, glutamic acid, and MAP were chromatographed on the reverse phase HPLC column in solvent B, and the didansylate of Orn was in solvent C on the same column.

[c] ND, not detectable.

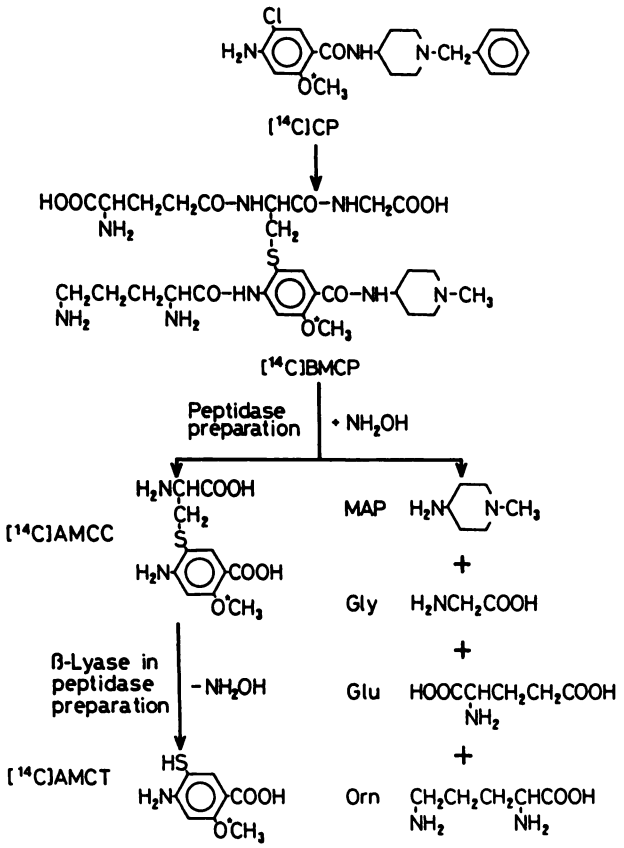

Fig. 6. Structures of [^{14}C]BMCP and its digestion products formed by incubation with the porcine intestinal mucosa peptidase preparation, containing cysteine *S*-conjugate β-lyase, in the absence and in the presence of the β-lyase inhibitor hydroxylamine. ★, ^{14}C. In the absence of hydroxylamine, [^{14}C]BMCP was quantitatively converted into [^{14}C]AMCT, MAP, Gly, Glu, and Orn in an equal molar ratio, without formation of [^{14}C]AMCC. In the presence of 2 mM hydroxylamine, [^{14}C]BMCP was quantitatively converted into [^{14}C]AMCC, MAP, glycine, glutamic acid, and Orn in an equal molar ratio, without formatin of [^{14}C]AMCT. [^{14}C]AMCC used as a substrate was quantitatively converted into [^{14}C]AMCT in the absence of the β-lyase inhibitor but remained unchanged in the presence of 2 mM hydroxylamine. Under the conditions for partial inhibition of the β-lyase in the peptidase preparation by lower concentrations of hydroxylamine (0.5 and 1 mM), both radioactive products, [^{14}C]AMCC and [^{14}C]AMCT, were simultaneously and stoichiometrically formed, together with MAP and the three amino acids, from [^{14}C]BMCP.

Thus, from the aforementioned facts, the structure of the biliary major metabolite, [^{14}C]BMCP, was assigned as *S*-[2-ornithylamino-4-[^{14}C]methoxy-5-(1′-methyl-4′-piperidylamino)carboxyphenyl]glutathione, which had four enzymatically hydrolyzable amide bonds, one of which existed originally in [^{14}C]CP and three of which linked four amino acid residues in the metabolite (Fig. 6).

Discussion

An *S*-glutathionyl residue, binding to the aromatic ring carbon by substitution for the chlorine atom in CP, must be in BMCP. The tripeptide residue can reasonably account for the formation of glutamic acid, glycine, and the *S*-cysteine conjugate AMCC in an equal molar ratio by the peptic digestion of BMCP in the presence of the β-lyase inhibitor. Therefore, Orn must conjugate with the primary aromatic amino group through

its α-carboxylic acid to form an amide bond in BMCP. The existence of the ornithyl-amino group in the aromatic ring was supported by the fact that, unlike the free aromatic amines CP, AMCC, and AMCT, the UV absorption spectrum of BMCP determined in methanol did not demonstrate any blue shift after the addition of hydrochloric acid. The UV absorption spectrum of CP, which produced a blue shift upon addition of hydrochloric acid, did not demonstrate any blue shift upon acid addition after N^4-acetylation; the spectrum of N^4-acetyl-CP shwoed a marked difference in shape from that of CP and closely resembled that of BMCP.

The potent activity of cysteine *S*-conjugate β-lyase, which existed in the commercial peptidase preparation from porcine intestinal mucosa, quantitatively converted both [^{14}C]BMCP and the cysteine *S*-conjugate [^{14}C]AMCC into the thiol [^{14}C] AMCT as the sole product on prolonged incubations. The β-lyase, a pyridoxal phosphate enzyme, has been demonstrated to convert a variety of cysteine *S*-conjugates stoichiometrically into the corresponding thiols and pyruvic acid, with elimination of ammonia (11). The known β-lyase inhibitor hydroxylamine (2 mM), which acts on pyridoxal phosphate with irreversible formation of an oxime (11, 16), completely inhibited the enzymatic transformation of AMCC to AMCT, when used directly as a substrate or when formed as an obligatory intermediate from BMCP. At lower concentrations of the inhibitor (0.5 and 1.0 mM), partial activity of the β-lyase remained, and both radioactive products, [^{14}C]AMCC and [^{14}C]AMCT, were formed from [^{14}C]BMCP. The β-lyase is known to exist at higher concentrations in the liver and kidney than in other tissues of experimental animals (16). However, its existence in the porcine intestinal mucosa has not been demonstrated previously.

[^{14}C]BMCP also quantitatively gave an equal molar ratio of glutamic acid, glycine, Orn, and MAP after acid hydrolysis carried out in 10 N hydrochloric acid at 100° for 24 hr under anaerobic conditions (data not shown). However, the acid hydrolysis failed to yield radioactive AMCC or AMCT, because of rapid decomposition of these aromatic compounds under the acidic conditions used.

CP underwent another structural change in the piperidine ring moiety in the metabolic transformation to BMCP. The *N*-methyl group of BMCP would be formed via oxidation at the benzyl carbon, followed by nonenzymatic elimination of benzaldehyde, probably before the conjugations in the aromatic ring. The oxidation product at the benzyl carbon (metabolite 1) and *N*-desbenzyl-CP (metabolite 2) were actually identified in the *in vitro* study carried out using a rabbit liver 9000 × *g* supernatant fraction fortified with NADPH (3, 6), and the latter was demonstrated to be a nonenzymatic product of metabolite 1 (6). In addition, metabolite 2 was identified as a major urinary metabolite in rats (7). However, *N*-methyl-metabolite 2 has not been reported as a metabolite *in vivo* or *in vitro*. *N*-Methylation is known to occur with a variety of xenobiotic aliphatic amines such as amphetamine (17), normorphine (18), oxprenolol (19), and *N*-desmethyl-nicotine (20) *in vivo*. Oxprenolol, an *N*-isopropyl secondary amine, has been demonstrated to be *N*-deisopropylated and then *N*-methylated in dogs (19). The *N*-methylation of these primary or secondary amines has been demonstrated or suggested to be catalyzed by hepatic *N*-methyltransferases requiring *S*-adenosylmethionine as a cofactor (17–20).

So far as we know, BMCP is the first demonstration of Orn conjugation of xenobiotics in mammals, although the abnormal conjugates ornithocholanic acids, which are amides of cholanic acids with the δ-amino group of Orn, have been reported to be excreted in the bile of humans (21) and guinea pigs (22) when they are infected by *Klebsiella pneumoniae*. Orn conjugation has been known to take place only in birds and reptiles given xenobiotic carboxylic acids (23–26). Furthermore, there exists a fundamental difference in structure between the Orn conjugates in birds and reptiles and BMCP in the rat. In BMCP, Orn is conjugated through its α-carboxylic acid with the primary aromatic amino group to form an amide bond. However, the known Orn conjugates in birds and reptiles are all dicarboxylic acid amides formed by condensation of the α- and δ-amino groups of the amino acid with xenobiotic aromatic carboxylic acids such as benzoic acid (23–25) and phenyl-, 4-chlorophenyl-, 4-nitrophenyl-, and 1-naphthyl-acetic acids (26).

It has been demonstrated that the xenobiotic caboxylic acids are activated by CoA ligase to form CoA-thioesters before enzymatic condensation by acyl CoA-amino acid *N*-acyltransferases with the α- and δ-amino groups of Orn (23, 24), which, in birds and reptiles, has been reported to exist at the highest level among free amino acids in the liver (23). In mammals, however, the CoA-thioesters formed from these xenobiotic carboxylic acids react enzymatically with the most abundant free amino acids, such as glycine, glutamine, and taurine (23). It should be emphasized that no amino acid has been known to conjugate with xenobiotic amines through the α-carboxylic acid group.

The α-carboxylic acid group of amino acids such as serine (27–29) and proline (30, 31) has been demonstrated to form reactive esters with the hepatocarcinogenic aromatic hydroxylamines *N*-hydroxy-4-aminoquinoline *N*-oxide (27), *N*-hydroxy-4-aminoazobenzene (28), and *N*-hydroxy-Trp-P-2 (29, 30). These amino acids are activated by the enzymatic formation of aminoacyl-AMPs and are transferred to the O atom of the hydroxylamines by the catalytic action of tRNA synthases in rat liver. However, nothing is known of whether these aminoacyl-AMPs are transferred to aromatic amines. It is of interest to know what enzymatic system is involved in the activation of Orn, because the existence of a typical active form such as the aminoacyl-AMP has never been reported with Orn.

The following speculations might be possible for the metabolic formation of the double conjugate BMCP: the first conjugation should take place at the primary amino group in the aromatic ring to form the ornithylamino residue, and then the chlorine atom at the vicinal position could be substituted by the sulfhydryl group of GSH, with elimination of hydrogen chloride. GSH *S*-transferase-mediated substitution of GSH for a chlorine atom in the aromatic ring takes place readily with chlorobenzenes bearing the electron-withdrawing nitro group(s) at the *o*- or/and *p*-position, e.g., 1-chloro-2,4-dinitrobenzene (32) and 1,2-dichloro-4-nitrobenzene (33) are the most widely used substrates for the sensitive assay of GSH *S*-transferases.

Considering the results of the extensive studies carried out by Bray *et al.* (34) on the urinary excretion of mercapturic acids in rabbits given the three isomers of mono-chloro-substituted anilines or nitrobenzenes, chloroanilines are not likely to conjugate with GSH *in vivo*. They demonstrated that *o*- and *p*-chloronitrobenzenes were excreted as the corresponding nitro-

phenyl mercapturic acids into the urine. However, they failed to detect the *N*-acetylcysteine conjugates from the urine of animals given *m*-chloronitrobenzene and chloroanilines. Therefore, reduction of the electron-releasing effect of the primary aromatic amino group by amide bond formation with the α-carboxylic acid group of Orn might be needed for the subsequent enzymatic substitution of the sulfhydryl group of GSH for the chlorine atom in the aromatic ring. A study on the double conjugation mechanisms *in vivo* and *in vitro* is now in progress in our laboratories.

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