Some S-Containing Metabolites of Tributyl Phosphate in the Rat

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When rats were given a single intraperitoneal dose of tributyl phosphate, several S-containing metabolites were identified in the urine. The main metabolites were (3-oxobutyl)- and (3-hydroxybutyl)mercapturic acids, and traces of (2-oxobutyl)- and (2-hydroxybutyl)mercarpturic acids were detected. On the other hand, administration of bromobutane gave butyl-, (2-oxobutyl), (3-oxobutyl)-, (2-hyroxybutyl)-, and (3-hydroxybutyl)mercapturic acids, with ratios of 2.1 for 3-oxo/2-oxo and 1.5 for 3-hyroxy/2-hydroxy metabolites. This means that tributyl phosphate is not substantially metabolized via butylmercapturic acid, which is a major metabolite of bromobutane, and that tributyl phosphate undergoes transalkylation of 3-hydroxybutyl or 3-oxobutyl moieties after oxidation of the original butyl moieties. Administration of some probable metabolic intermediates, including dibutyl 3-oxobutyl phosphate, gave almost constant ratios of (3-oxobutyl)mercpaturic acid to (3-hydroxybutyl)mercapturic acid. This indicates that an equilibrium between the 3-oxobutyl compound and 3-hydroxybutyl compound may be set up at some stage after transalkylation.

In the previous paper, we reported that tributyl phosphate (TBP) (I) was mainly metabolized to dibutyl hydrogen phosphate (II) derivatives and finally to butyl dihydrogen phosphate derivatives (Suzuki et al., 1984). However, it has not been clarified whether these metabolites are formed only by drug-metabolizing enzymes (MFO) or by esterase or by glutathione *S*-transferase. It also remains to be determined whether the butyl moieties are removed intact or in an oxidized form.

This paper describes the S-containing metabolites of I and compares the metabolic fate of I with that of bromobutane (III), which was metabolized to (hydroxybutyl)mercapturic acid via *S*-butylglutathione (James et al., 1968).

MATERIALS AND METHODS

Chromatography. Methylated metabolites were analyzed by thin-layer chromatography (TLC) on silica gel HF_{254} (E. Merck, 0.25 mm thick) using a solvent system of 10% MeOH–$CHCl_3$. Detection was carried out with the following chromogenic reagents: (A) KI–platinochloric acid (Toennies and Kolb, 1951); (B) 2,4-dinitrophenylhydrazine reagent, consisting of 0.4 g of 2,4-dinitrophenylhydrazine in 2 mL of concentrated H_2SO_4, 3 mL of H_2O, and 10 mL of EtOH. Column chromatography was carried out using silica gel (100 mesh) for column chromatography (Mallinckrodt).

Gas chromatography (GC) of methylated derivatives was performed in the S mode with a Shimadzu 4BM gas chromatograph equipped with a flame photometric detector (FPD). The GC column was a 1.5 m × 3 mm glass column packed with 1% OV-225 on 80–100-mesh Chromosorb W, AW. Operating conditions were as follows: injection temperature 250 °C, detector temperature 250 °C, nitrogen gas pressure 1.0 kg/cm^2, air flow 80 mL/min, and hydrogen gas flow 180 mL/min. The column temperature is presented in each figure.

Mass Spectrometry (MS). Mass spectra were obtained by coupled GLC–MS on a Shimadzu LKB 9000 mass spectrometer in the electron impact (EI) mode.

Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy. NMR spectra were recorded on a JEOL JNX-FX 200S NMR spectrometer (200 MHz) in $CDCl_3$ by using $(CH_3)_4Si$ as an internal standard (δ value) (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad).

Infrared (IR) Spectroscopy. IR spectra were measured with a Jasco A-102 spectrometer.

Optical Rotation. Optical rotations were determined on a Jasco DIP-180 automatic polarimeter.

Melting Point. All melting points were determined on a Yanagimoto melting point apparatus and are uncorrected.

Determination of Glutathione. Glutathione levels of liver and kidney were in principle determined by the method described by Ellman (1959). A rat under anesthesia was perfused by enforced injection of 20 mL of ice-cold 0.1 M phosphate buffer (5 mM EDTA, pH 7.4) into the heart to avoid contamination with red blood cells, and then the liver and kidneys were removed. To the liver (1 g) and whole kidney (ca. 0.8 g) were added 5 and 2 mL of 10% TCA and then 10 and 5 mL of the buffer mentioned above, respectively. The mixtures were homogenized and then centrifuged at 3000*g*. Then 200 μL of the above supernatant and 0.25 mL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 20 mg/1% sodium citrate) were added to 3 mL of 1 M phosphate buffer (pH 7.4). After 10 min, the absorbance at 412 nm was measured. A similarly processed solution, but without the organ homogenate, was used as a reference.

Treatment. Male Wistar rats (180–220 g) were dosed intraperitoneally with I, III, and other probable intermediates in TBP metabolism.

Determination of Metabolites. Collected urine was adjusted to pH 1 with 10% HCl and then extracted 3 times with EtOAc (each 40 mL). After methylation, the solution was washed with H_2O and then evaporated. The residue was chromatographed on silica gel (15 g) and successively eluted with the following solvent systems. The first eluate ($CHCl_3$, 160 mL) was designated as fraction 1, the second eluate (1% MeOH–$CHCl_3$, 140 mL) fraction 2, and the third eluate (10% MeOH–$CHCl_3$, 100 mL) fraction 3. S-containing metabolites were determined by FPD-GC (S mode) in each fraction.

Acetylation. Two drops of pyridine was added to a solution of a dried sample in acetic anhydride (10 mL), and the solution was allowed to stand at room temperature overnight and then evaporated. The residue was purified on a silica gel column with $CHCl_3$ as the mobile phase.

Methylation. A solution of a sample in a small amount

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of MeOH or EtOAc was treated with an ethereal solution of CH_2N_2.

EXPERIMENTAL PROCEDURE

Synthesis of Authentic Standards. All organic solutions were dried over anhydrous Na_2SO_4 and concentrated in a rotary evaporator under reduced pressure.

Methyl Ester of N-Acetyl-S-butyl-L-cysteine (IV). The methyl ester of *N*-acetyl-*S*-butyl-L-cysteine was synthesized by the method described in the previous paper (Suzuki et al., 1984).

Methyl Ester of N-Acetyl-S-(4-hydroxybutyl)-L-cysteine (V). A trace of benzoyl peroxide was added to a mixture of but-3-ene-1-ol (4.8 g, 0.067 mol), *N*-acetyl-L-cysteine (6.4 g, 0.05 mol), and dry $CHCl_3$ (50 mL), and then the mixture was irradiated with ultraviolet light from a mercury lamp under stirring for 3 h at room temperature. After addition of H_2O (50 mL), the aqueous layer was concentrated to give a syrupy oil, which was methylated. After evaporation of the solvent, the residue was chromatographed on silica gel (80 g) with $CHCl_3$. The eluate from 200 mL to 1.2 L was collected and evaporated to give an oily residue, which was then dissolved in 5% KOH. The solution was allowed to stand at 50 °C for 30 min and then passed through a column of Amberlite CG-400 (H^+ form, 20 mL). The eluates, up to the point of neutral pH, were combined and concentrated at 40–50 °C, and the obtained syrupy oil (1.5 g, 0.0069 mol) was dissolved in a small amount of acetone. This solution was treated with an ethereal solution (10 mL) of dicyclohexylamine (1.2 g, 0.0069 mol). Recrystallization of product from ethanol–acetone afforded the dicyclohexylammonium salt of *N*-acetyl-*S*-(4-hydroxybutyl)-L-cysteine (1.4 g): mp 152–153.5 °C. Anal. Calcd for $C_{21}H_{40}N_2O_4S$: C, 60.54; H, 9.68; N, 6.73; S, 7.70. Found: C, 60.58; H, 9.71; N, 6.72; S, 7.65. This salt (1 g) was dissolved in H_2O (5 mL) and passed through a column of Amberlite GC-400 (H^+ form, 5 mL) and eluted with H_2O until the pH of the eluate reached neutral. After concentration of the eluate, the obtained residue was methylated to give a colorless oil, V (410 mg): IR (film) 1735, 1643 cm^{-1}; MS *m/z* (rel intensity) 60 (38), 71 (100), 88 (89), 101 (28), 113 (34), 117 (25), 113 (28), 140 (93), 157 (23), 172 (28), 190 (70), 191 (7). V-acetate: IR (film) 1732, 1650 cm^{-1}; NMR δ 1.52–1.80 (m, 4 H, CH_2CH_2), 2.06 (2 s, 6 H, CH_3CONH and $OCOCH_3$), 2.55 (t, 2 H, J = 6.8 Hz, CH_2CH_2S), 2.9–3.0 (m, 2 H, SCH_2CH), 3.78 (s, 3 H, OCH_3), 4.07 (t, 2 H, J = 6.4 Hz, OCH_2), 4.78–4.84 (m, 1 H, $CHCOOCH_3$), 6.3–6.4 (br s, 1 H, NH). Anal. Calcd for $C_{12}H_{21}NO_5S$: C, 49.51; H, 7.27; N, 4.81; S, 11.0. Found: C, 49.05; H, 7.25; N, 4.84; S, 10.84.

Methyl Ester of N-Acetyl-S-(2-oxobutyl)-L-cysteine (VI). Chloromethyl ethyl ketone was synthesized by the method described by Terasawa and Okada (1977): bp 130–133 °C. Chloromethyl ethyl ketone was then added dropwise to a solution of *N*-acetyl-L-cysteine (2.0 g, 0.012 mol) in 1 N KOH (24 mL) with stirring under ice cooling. The mixture was stirred for 1 h at room temperature, acidified with 10% HCl, and then extracted with EtOAc. The extract was washed with H_2O, evaporated, and then methylated. Evaporation of the solvent gave an oily residue that was chromatographed on silica gel (80 g) by using $CHCl_3$ as an eluent. The first elution yielded the methyl ester of *N*-acetyl-L-cysteine, followed by the methyl ester of *N*-acetyl-*S*-(2-oxobutyl)-L-cysteine (VI). Recrystallization of VI from ether gave needles (1.6 g): mp 71–72 °C; $[\alpha]^{20}_D$ −37.2° (*c* 1, MeOH); IR (film) 1724, 1659, 1634 cm^{-1}; MS *m/z* (rel intensity) 57 (92), 74 (34), 88 (100), 100 (43), 117 (34), 132 (85), 144 (52), 176 (43), 188 (43), 247 (18); NMR δ 1.09 (t, 3 H, J = 7.3 Hz, CH_3), 2.07 (s, 3 H, CH_3CONH), 2.60 (q, 2 H, J = 7.3 Hz, CH_3CH_2CO), 2.98 (m, 2 H, SCH_2CH), 3.33 (q, 2 H, J = 15.1 or 18.9 Hz, SCH_2CO), 3.77 (s, 3 H, OCH_3), 4.75–4.90 (m, 1 H, $CHNH$), 6.6–6.7 (br s, 1 H, NH). Anal. Calcd for $C_{10}H_{17}NO_4S$: C, 48.57; H, 6.93; N, 5.66; S, 12.97. Found; C, 48.51; H, 6.92; N, 5.68; S, 13.00.

Methyl Ester of N-Acetyl-S-(3-oxobutyl)-L-cysteine (VII). 4-Bromo-2-butane was synthesized by the method described by Marx (1975): bp 74–76 °C/45 mmHg. *N*-Acetyl-L-cysteine (3.0 g, 0.017 mol) and 4-bromo-2-butane (2.4 g, 0.017 mol) were reacted and processed in the same way as described for VI. Purification on silica gel with $CHCl_3$ as the mobile phase and recrystallization from ether gave needles (2.3 g): mp 53.5–54.5 °C; $[\alpha]^{20}_D$ −27.2° (*c* 1, MeOH); IR (film) 1741, 1704, 1635 cm^{-1}; NMR δ 2.05 (s, 3 H, CH_3CONH), 2.17 (s, 3 H, CH_3CO), 2.72 (t, 4 H, J = 3.5 Hz, OCH_2CH_2CO), 3.00 (d, 2 H, J = 5.5 Hz, SCH_2CH), 3.76 (s, 3 H, OCH_3), 4.7–4.9 (m, 1 H, $CHNH$), 6.3–6.6 (br s, 1 H, NH). Anal. Calcd for $C_{10}H_{17}NO_4S$: C, 48.57; H, 6.93; N, 5.66; S, 12.97. Found: C, 48.57; H, 6.92; N, 5.68; S, 13.00.

Methyl Ester of N-Acetyl-S-(3-hydroxybutyl)-L-cysteine (VIII). This compound was synthesized by a slight modification of the method of James et al. (1968). A mixture of *N*-acetyl-L-cystine (1.6 g, 0.012 mol), but-1-ene-3-ol (1.0 g, 0.014 mol) and dry $CHCl_3$ (20 mL) was irradiated and processed in the same manner as in the case of V. Purification of the methyl ester on silica gel (15 g) with $CHCl_3$ as the eluent to give a diastereoisomeric mixture (520 mg). This was hydrolyzed with 5% KOH and passed through a column of Amberlite CG-400 (H^+ form, 20 mL), and the eluate was concentrated to dryness. The residue was converted to the dicyclohexylammonium salt in the same manner as in the case of V to give prisms: mp 181–186 °C. Recrystallization from EtOH–acetone afforded the dicyclohexylammonium salt of *N*-acetyl-*S*-(3-hydroxybutyl)-L-cysteine (220 mg): mp 183–186 °C (lit. mp 187–189 °C). This was dissolved in a small amount of H_2O and passed through a column of Amberlite CG-400 (H^+ form, 5 mL). Concentration of the eluate, followed by methylation and purification of the product on silica gel (5 g) with 1% MeOH–$CHCl_3$ as the eluent, gave a colorless oil, VIII (180 mg): IR (film) 1738, 1650 cm^{-1}; MS *m/z* (rel intensity) 61 (41), 71 (25), 88 (36), 104 (23), 113 (100), 119 (41), 172 (15), 190 (38), 249 (1); NMR δ 1.20 (d, 3 H, J = 6.1 Hz, CH_3), 1.5–1.8 (m, 2 H, OCH_2CH_2), 2.06 (s, 3 H, CH_3CONH), 2.5–2.7 (m, 2 H, SCH_2CH_2), 2.8–3.0 (m, 2 H, $CHCH_2S$), 3.08 (br, s, 1 H, OH), 3.77 (s, 3 H, $COOCH_3$), 3.7–3.9 (m, 1 H, $CHOCOCH_3$), 4.6–4.9 (m, 1 H, $CHNH$), 6.7–7.0 (br s, 1 H, NH). VIII-acetate: IR (film) 1750, 1732, 1653 cm^{-1}; NMR δ 1.23 (d, 3 H, J = 6.4 Hz, CH_3), 1.7–1.9 (m, 2 H, $CH_2CHOCOCH_3$), 1.4–1.6 (m, 2 H, CH_2CH_2S), 2.05 (s, 3 H, CH_3CONH or $OCOCH_3$), 2.06 (s, 3 H, $OCOCH_3$ or CH_3CONH), 2.99 (d, 2 H, J = 5.1 Hz, SCH_2CH), 3.94 (s, 3 H, $COOCH_3$), 4.70–4.83 (m, 1 H, $CHNH$), 4.84–5.00 (br s, 1 H, $CHOCOCH_3$), 6.2–6.4 (br s, 1 H, NH). Anal. Calcd for $C_{12}H_{21}NO_5S$: C, 49.51; H, 7.27; N, 4.81; S, 11.01. Found: C, 49.06; H, 7.30; N, 4.73; S, 11.01.

Methyl Ester of N-Acetyl-S-(2-hydroxybutyl)-L-cysteine (IX). This compound was synthesized by a slight modification of the method of James et al. (1968). A mixture of *N*-acetyl-L-cysteine (3.6 g, 0.015 mol) and 1,2-epoxybutane (1.3 g, 0.018 mol) in 2 N NaOH (40 mL) was stirred at 5 °C for 2 h and then washed with $CHCl_3$. The aqueous layer was acidified to pH 1.0 with 10% HCl and then extracted with EtOAc. The organic layer was washed with H_2O, dried, methylated, and evaporated to dryness. The residue was purified on silica gel with $CHCl_3$ as the eluant.

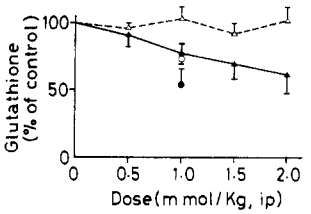


Figure 1. Effects of TBP and Fyrol FR-2 on the levels of glutathione in rat liver and kidney (●) TBP (liver); (○) TBP (kidney); (▲) Fyrol FR-2 (liver); (△) Fyrol FR-2 (kidney).

The oily product (650 mg) was dissolved in 5% KOH (20 mL), and the solution was allowed to stand at 50 °C for 30 min and then applied to a column of Amberlite CG-400 (10 mL), which was washed with H_2O until the pH of the eluate reached neutral. The eluate was concentrated and the residue was dissolved in a small amount of acetone. This solution was treated with dicyclohexylamine (400 mg) in ether (10 mL). Recrystallization of the resulting crude crystals from acetone–ether afforded the dicyclohexylammonium salt of *N*-acetyl-*S*-(2-hydroxybutyl)-L-cysteine (22 mg) as needles: mp 146–148 °C (lit. mp 146–148 °C). Anal. Calcd for $C_{21}H_{40}N_2O_4S$: C, 60.54; H, 9.68; N, 6.72; S, 7.70. Found: C, 60.55; H, 9.66; N, 6.69; S, 7.69. This compound (200 mg) was dissolved in H_2O (5 mL), and the solution was passed throgh a column of Amberlite CG-400 (5 mL). The first eluate (50 mL) was concentrated, and the residue was methylated and purified on silica gel to give an oily material IX (80 mg): $[\alpha]^{20}_D$ −26.4° (*c* 1, MeOH); IR (film) 1740, 1653 cm^{-1}; MS *m/z* (rel intensity) 55 (10), 60 (13), 74 (9), 88 (26), 100 (31), 117 (20), 132 (100), 160 (7), 172 (16), 191 (11), 231 (3); NMR δ 0.97 (t, 3 H, *J* = 7.6 Hz, CH_3), 1.53 (5 lines, 2 H, *J* = 6.8 Hz, CH_3CH_2), 2.06 (s, 3 H, CH_3CONH), 2.4–2.8 [m, 2 H, $SCH_2CH(OH)$], 2.9–3.1(m, 2 H, SCH_2CHNH), 3.4–3.9 [br s, 1 H, $CH(OH)$], 3.78 (s, 3 H, $COOCH_3$), 4.7–4.9 (m, 1 H, $CHNH$), 6.5–6.7 (m, 1 H, NH). Anal. Calcd for $C_{10}H_{19}NO_4S$: C, 48.17; H, 7.69; N, 5.62; S, 12.86. Found: C, 48.48; H, 7.55; N, 5.57; S, 12.86. IX-acetate: NMR δ 0.91 (t, 3 H, *J* = 7.3 Hz, CH_3), 1.5–1.8 (m, 2 H, CH_2), 2.06 (s, 3 H, $OCOCH_3$), 2.09 (s, 3 H, $COCH_3$), 2.67 (d, 2 H, *J* = 6.1 Hz, SCH_2CHO), 2.9–3.2 (m, 2 H, SCH_2CHNH), 3.77 (s, 3 H, $COOCH_3$), 4.6–5.0 (m, 2 H, $CHOCOCH_3$ and $CHNH$), 6.3–6.5 (br s, 1 H, NH). Anal. Calcd for $C_{12}H_{21}NO_5S$: C, 49.50; H, 7.27; N, 4.81; S, 11.01. Found: C, 48.83; H, 7.30; N, 5.20; S, 11.01.

Butyl Bis(3-hydroxybutyl) Phosphate (X), Dibutyl 3-Hydroxybutyl Phosphate (XI), Dibutyl Hydrogen Phosphate (II), and Dibutyl 3-Oxobutyl Phosphate (XII). The syntheses of these compounds were described in the previous report (Suzuki et al., 1984).

RESULTS AND DISCUSSION

Effect of TBP on the Levels of Glutathione of Liver and Kidney. It was reported in the previous paper that I is not metabolized to *S*-butyl-L-cysteine or *N*-acetyl-*S*-butyl-L-cysteine in the rat. However, there is no evidence that other cysteine or mercapturic acid derivatives are not produced. Therefore, the formation of such derivatives were investigated by measuring the changes of endogenous glutathione level (Figure 1). The level of glutathione was determined 2 h after intraperitoneal injection of 1 mmol of TBP. The levels of glutathione decreased to 55% in liver and 75% in kidney, as compared with the initial levels. This suggests that TBP and/or its metabolic intermediates are subjected to transalkylation by glutathione *S*-transferase, producing S-containing metabolities by consuming endogenous glutathione in the liver and kidney. In contrast, the administration of tris(dichloroisopropyl) phosphate (Fyrol FR-2), which had been used in children's sleepwear and is known to be mutagenic, did not cause any fall of glutathione level in the kidney, though a clear dose-dependent decrease in glutathione was observed in liver. This is in accordance with the fact that Fyrol FR-2 is accumulated in the kidney (Nomeir et al., 1981).

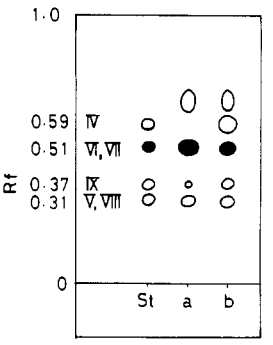


Figure 2. Thin-layer chromatogram of methylated EtOAc extracts of the urine under pH 1.0. S-containing metabolites were detected with a chromogenic reagent (A). Shadowed spots were positive to chromogenic reagents A and B. Standard samples (St) were as follows: IV, methyl ester of *N*-acetyl-*S*-butyl-L-cysteine; V, methyl ester of *N*-acetyl-*S*-(4-hyroxybutyl)-L-cysteine; VI, methyl ester of *N*-acetyl-*S*-(2-oxobutyl)-L-cysteine; VII, methyl ester of *N*-acetyl-*S*-(3-oxobutyl)-L-cysteine; VIII, methyl ester of *N*-acetyl-*S*-(3-hydroxybutyl)-L-cysteine; IX, methyl ester of *N*-acetyl-*S*-(2-hydroxybutyl)-L-cysteine. (a) Extract of urine from rats dosed with TBP (I). (b) Extract of urine from rats dosed with bromobutane (III).

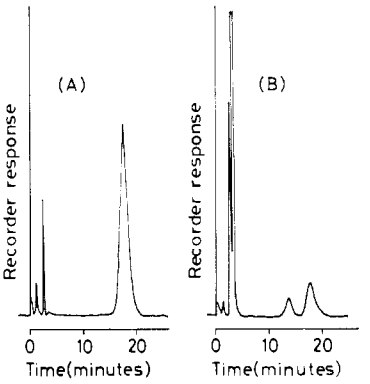


Figure 3. Gas chromatogram of the crude urine extracts (column temperature 180 °C): (A) urine from rats dosed with TBP (I); (B) urine from rats doses with bromobutane (III).

Detection of S-Containing Metabolites of TBP and Bromobutane. TLC and FPD-GC (S mode) results for nuetral and acidic metabolites of I without separation by silica gel column chromatography are shown in Figures 2 and 3, respectively. Chromogenic reagent A revealed four white spots on the TLC plate on a pinkish background. The spot of R_f 0.68 was also observed in the reference urine, so it was judged to be a usual urinary component. The spot of R_f 0.51 was also positive to reagent B.

Identification of Metabolites of TBP. Column chromatography of the crude extract of urine from rats dosed with TBP (250 mg/kg) on silica gel (15 g) was carried out according to the method described under Determination of Metabolites, and the products obtained were designated as M-1–M-5 in order of elution. A gas chromatogram of a mixture of fraction 1 and fraction 2 is shown in Figure 4.

Methyl Ester of M-1. The methyl ester of M-1, corresponding to the metabolite of R_f 0.51 on TLC, was eluted earlier on silica gel column chromatography than the methyl ester of M-2 and could be separated from the

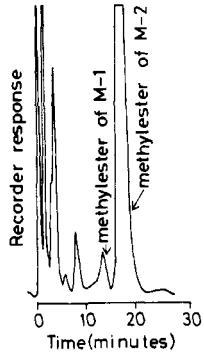


Figure 4. Gas chromatogram of a mixture of fraction 1 and fraction 2 of urine from rats dosed with TBP (column temperature 180 °C).

methyl ester of M-2. In spite of the identity of the retention time with that of an authentic sample of the methyl ester of *N*-acetyl-*S*-(2-oxobutyl)-L-cysteine (VI), the same mass fragment pattern was not obtained because of the small quantity of material available and overlapping with peaks due to other materials, to which FPD is not sensitive. However, several major fragment peaks, m/z 57, 74, 88, 117, 132, 176, 188, and 247, were observed, and the identity of this metabolite was further confirmed by mass chromatography at some of these peaks.

Methyl Ester of M-2. The methyl ester of M-2, corresponding to the metabolite of R_f 0.51 on TLC, showed identical retention time with an authentic sample of the methyl ester of *N*-acetyl-*S*-(3-oxobutyl)-L-cysteine (VII) and with the methyl ester of *N*-acetyl-*S*-(3-hydroxybutyl)-L-cysteine (VIII) on a gas chromatogram and exhibited carbonyl absorptions at 1733, 1703, and 1645 cm^{-1} (film) in the IR spectrum. In the NMR spectrum, the methyl ester of M-2 showed three singlet methyl signals at 2.06, 2.16, and 3.78 ppm due to CH_3CONH, CH_3COCH_2, and $COOCH_3$, respectively, a multiplet (1 H) from 4.5 to 5.0 ppm ascribed to a proton on the carbon atom attached to the carboxyl group, and a multiplet from 6.2 to 6.8 ppm attributed to NH. On the basis of the data described above, this product was assumed to be the methyl ester of *N*-acetyl-*S*-(3-oxobutyl)-L-cysteine, and this was confirmed by comparing its NMR and mass spectra with those of an authentic specimen of VII (Figure 5).

Methyl Ester of M-3. A gas chromatogram of fraction 3 is shown in Figure 6. The methyl ester of M-3 (retention time 5.6 min) cannot be observed in Figure 3A because of its small quantity, but it corresponds to the metabolite of R_f 0.37 on the TLC plate. It was identified as the methyl ester of *N*-acetyl-*S*-(2-hydroxybutyl)-L-cysteine by comparison of its mass spectrum with that of an authentic specimen of IX.

Methyl Ester of M-4. The methyl ester of M-4, which completely overlapped with the methyl ester of M-2 in Figure 3A before separation by silica gel column chromatography, corresponded to the metabolite of R_f 0.31 on the TLC plate. The acetate of M-4 methyl ester showed a doublet methyl signal at 1.23 due to CH_3CH and three singlet methyl signals at 2.00, 2.05, and 3.94 ppm assigned to CH_3CONH, $OCOCH_3$, and $COOCH_3$, respectively, in the NMR spectrum. On the basis of these spectral data, the product was assumed to be the methyl ester of *N*-acetyl-*S*-(3-acetoxybutyl)-L-cysteine, and this was confirmed by comparison of its NMR spectrum with that of an authentic specimen of VIII-acetate. The MS of the methyl ester of M-4 was also completely coincident with that of VIII.

Methyl Ester of M-5. The methyl ester of M-5 (retention time 13.6 min) was identical with an authentic sample of the methyl ester of *N*-acetyl-*S*-(4-hydroxybutyl)-L-cysteine (V) on GC and TLC, but further examination was not carried out because of the small quantity obtained.

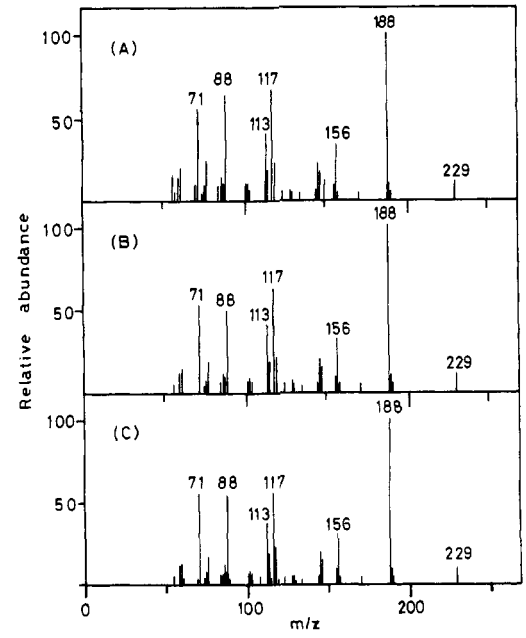


Figure 5. Mass spectra: (A) authentic methyl ester of *N*-acetyl-*S*-(3-oxobutyl)-L-cysteine (VII); (B) methyl ester of M-2; (C) scan no. 51 in Figure 7.

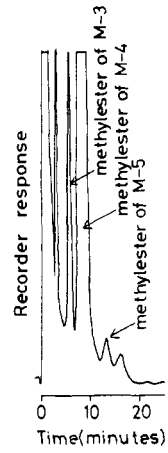


Figure 6. Gas chromatogram of fraction 3 and urine from rats dosed with TBP (column temperature 200 °C).

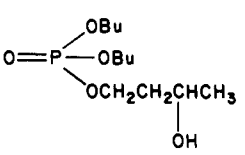
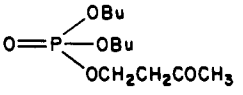
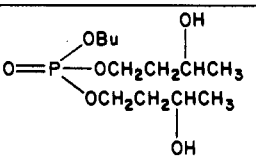
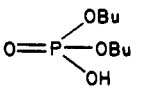
Identification of Metabolites of Bromobutane. TLC and FPD-GC (S mode) of the methylated neutral and acidic extracts of urine from rats given bromobutane (III) (Me-urine-III) are shown in Figures 2 and 3B, respectively. The thin-layer chromatogram of Me-urine-III was clearly different from that of the corresponding fraction from rats given I (Me-urine-I), in that Me-urine-III showed a distinct spot of R_f 0.59 with chromogenic reagent A. Further, an intense peak at a retention time of 3.0 min and two peaks corresponding to the methyl esters of M-1 and M-2, or the methyl esters of M-3 and M-4, were observed (Figure 3B). After separation of Me-urine-III into fractions 1, 2, and 3 by silica gel chromatography in the same way as in the case of I, each fraction was analyzed for S-containing metabolites. The spot of R_f 0.59, which was not found among the metabolites of I was identified as the methyl ester of *N*-acetyl-*S*-butyl-L-cysteine by comparison of its R_f value on TLC, its retention time on GC, and its mass fragment pattern with those of an authentic specimen of IV.

Table I. Amounts of Isolated Metabolites of TBP (I) and BuBr (III)

	% of applied dose[b]							
	S-butylmercapturic acid derivatives						ratio	
compound	*n*-Bu	2-oxo	3-oxo	2-OH	3-OH	4-OH	2-oxo/2-OH	3-oxo/3-OH
TBP[a] (n = 2)	nd	trace	8.9	trace	5.2	trace		1.7
BuBr (n = 3)	5.8 ± 0.7	2.4 ± 0.4	5.1 ± 0.7	1.0 ± 0.2	1.5 ± 0.2		2.4	3.4

[a] The values are expressed as percent of applied dose on the assumption that 1 mol of TBP is equivalent to 1 mol of *S*-butylmercapturic acid derivatives; recoveries are not corrected. [b] TBP, 0.94 mmol/kg; BuBr, 2.2 mmol/kg.

Table II. Amounts of S-Containing Metabolites Excreted in Urine of Rats after Intraperitoneal Administration of Probable Intermediates

	% of applied dose[a]											
												
	XI			XII			X			II		
metabolites[c]	1[b]	2	3	1	2	3	1	2	3	1	2	3
R—$SCH_2CH_2COCH_3$ (A)	10.1	1.7	0.86	7.9	2.1	1.7	7.2	1.11	0.54			
		12.7			11.7			8.8			0.072	
R—$SCH_2CH_2CHCH_3$ (B), OH	5.0	1.1	0.61	4.1	0.89	0.71	3.8	0.96	0.47			
		6.7			5.7			5.2			0.023	
ratio of A/B		1.89			2.03			1.69			3.13	

[a] The values are expressed on the assumption that 1 mol of intermediate is equivalent to 1 mol of *S*-butylmercapturic acid derivatives; recoveries are not corrected. [b] Numbers indicate days after administration. [c] R = $CH_2CH(COOCH_3)NHCOCH_3$.

A mass chromatogram of fraction 2 (Figure 7) and the mass spectrum at scan no. 51 (Figure 5) are shown. The mass fragment pattern in scan no. 38 is coincident with that of VI and that of scan no. 51 is coincident with that of VII (Figure 5). The results of GC–MS of fraction 3 showed that the metabolite of R_f 0.37 was identical with the 2-hydroxy compound IX and that of R_f 0.31 was identical with the 3-hydroxy compound VIII.

Quantitation of Metabolites and Metabolic Fate of the Alkyl Moiety. The results of determination of urinary metabolites of I and III at a dose of 250 mg/kg are shown in Table I. The metabolic fate of III in rat was already investigated by James et al. (1968). They reported that III was transformed to *S*-butylglutathione by alkyl transferase at an early stage of the metabolic process and then to the 2-hydroxy or 3-hydroxy compound by oxidation after its conversion to *N*-acetyl-*S*-butyl-L-cysteine. However, we found that the oxo compounds, which were not detected in their experiment, were the main metabolites. If I were degraded via *S*-butylglutathione, it should be metabolized via the same pathways as III, so that *N*-acetyl-*S*-butyl-L-cysteine (IV) should be detected in the urine of rats dosed with I. However, this was not the case.

In the metabolism of III, the 2-hydroxy compound IX and the 2-oxo compound VI amounted to 67 and 47% of the 3-hydroxy compound VIII and the 3-oxo compound VII, respectively. On the other hand, only traces of IX and VI were detected after the administration of I. This suggest that I is metabolized by a different route from III; that is, the butyl moiety of I is oxidized first by the MFO enzyme system and then subjected to transalkylation by *S*-alkyltransferase.

Determination of S-Containing Metabolites after the Administration of Intermediates of TBP Metabolism. In the previous paper, we determined the phosphorus-containing metabolites after administration of various probable metabolites of I. In this report, S-containing metabolites were measured after the administration

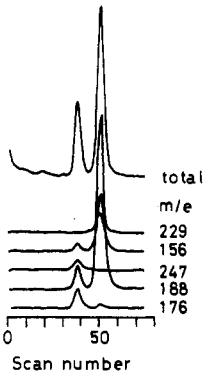


Figure 7. Mass chromatogram of fraction 2 of urine from rats dosed with bromobutane.

of the same compounds. As shown in Table II, the ratios (A/B) of oxomercapturic acid VII to hydroxymercapturic acid VIII in the metabolites of the compounds were between 1.69 and 2.03 except in the case of dibutyl hydrogen phosphate (II) and can be considered to be almost constant.

In the previous paper, it was reported that very little of XII could be metabolically transformed back to XI. The observation that both the oxo compound XII and the hydroxy compound XI were metabolized to give a constant yield ratio of VII and VIII indicates that the metabolic fates of both compounds are identical or that VII and VIII are in equilibrium with each other. If the former is the case, all probable intermediates must be metabolized by the same metabolic pathways and give the same ratios as I, but this is unlikely. If the latter is the case, the ratios of VII to VIII after administration of I and III must be identical.

As shown in Table I, the ratio of VII to VIII after the administration of III was 3.4, somewhat higher than the ratio of 1.7 after the administration of I. This difference may be attributable to the higher dose level of I, since 1

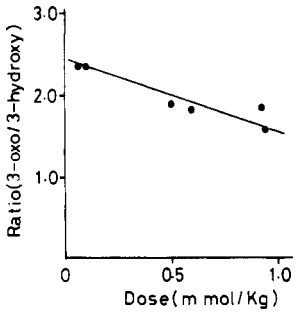


Figure 8. Relationship between TBP dose and ratio of *N*-acetyl-*S*-(3-oxobutyl)-L-cysteine (VII) to *N*-acetyl-*S*-(3-hydroxybutyl)-L-cysteine (VIII).

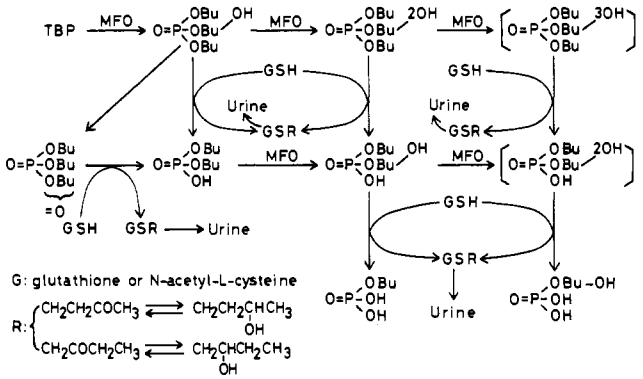


Figure 9. Proposed pathways of metabolic degradation of TBP by glutathione *S*-transferase.

mol of I may be equivalent to 3 mol of III, so the correlation between dose level and the ratio of VII to VIII was investigated (Figure 8). As expected, the lower dose gave a higher ratio, but the values were still rather different from the expected one. Although the reason for this is unclear, the equilibrium between the 3-hydroxy and 3-oxo derivatives might be set up anywhere between the first stage of formation of the glutathione conjugate and another later stage before the formation of the 3-oxo- (M-2) and 3-hydroxymercapturic acid (M-4). It is likely that the compound that has the undesirable "nonbiological" configuration of the hydroxy group will be transformed to the oxo compound and, after transesterification, converted to the hydroxy compound of desirable configuration.

Compounds VII and VIII are also formed from dibutyl hydrogen phosphate (II), indicating that II undergoes transalkylation after hydroxylation at C-3 of the butyl moiety to give butyl dihydrogen derivatives.

On the basis of the above data and discussion, the proposed pathway of metabolic degradation of I is outlined in Figure 9. It is clear that elimination of the alkyl moiety of I can be partially ascribed to the action of glutathione *S*-transferase, but participation of α-hydroxylation by MFO or esterase cannot be excluded. Elimination of butyl moieties of XII undoubtedly occurs as 3-oxobutyl moieties, but there is no evidence that elimination of butyl moieties of XI as 3-hydroxybutyl moieties does not also occur, and further, this does not necessarily imply the involvement of XII as an intermediate in the metabolism of I.

Registry No. I, 126-73-8; II, 107-66-4; III, 26602-89-1; IV, 89197-87-5; V, 86560-44-3; VI, 92079-03-3; VII, 92079-04-4; VIII, 92079-05-5; IX, 92079-06-6; X, 89197-71-7; XI, 89197-69-3; XII, 89197-70-6; glutathione, 70-18-8; Fyrol FR-2, 13674-87-8; but-3-en-1-ol, 627-27-0; *N*-acetyl-L-cysteine, 616-91-1; *N*-acetyl-*S*-(4-hydroxybutyl)-L-cysteine dicyclohexylammonium salt, 92079-02-2; chloromethyl ethyl ketone, 616-27-3; *N*-acetyl-L-cysteine methyl ester, 7652-46-2; 4-bromo-2-butene, 4784-77-4; but-1-en-3-ol, 598-32-3; *N*-acetyl-*S*-(3-hydroxybutyl)-L-cysteine dicyclohexylammonium salt, 22768-33-8; 1,2-epoxybutane, 106-88-7; *N*-acetyl-*S*-(2-hydroxybutyl)-L-cysteine dicyclohexylammonium salt, 22768-32-7; *S*-butylmercapturic acid, 19216-62-7; *S*-(2-oxobutyl)mercapturic acid, 92079-07-7; *S*-(3-oxobutyl)mercapturic acid, 92079-08-8; *S*-(2-hydroxybutyl)mercapturic acid, 19216-63-8; *S*-(3-hydroxybutyl)mercapturic acid, 45159-41-9; *S*-(4-hydroxybutyl)mercapturic acid, 92079-01-1.

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