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A novel pathway for formation of thiol-containing metabolites from cysteine conjugates

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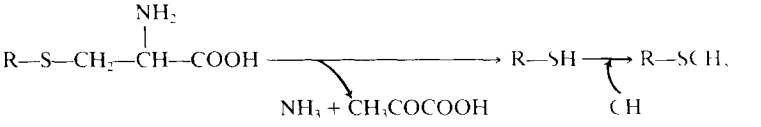
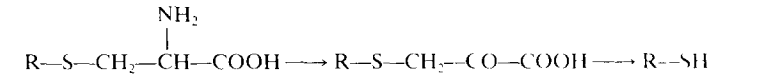
During the past decade the formation of methylthio-containing metabolites has been reported for numerous xenobiotics including drugs, herbicides, and other organic compounds (reviewed by Tateishi [1, 2] and Stillwell [3]) From the extensive studies in our laboratories [4–7] and others [8, 9], various types of methylthio-containing metabolites were shown to be generated from the corresponding cysteine conjugates via thiols (Scheme 1)



Scheme 1

Thus the C—S bond of cysteine conjugates is firstly cleaved by the action of C—S lyases (which are found in mammalian liver, kidney and some kinds of intestinal microorganisms) to give thiols together with equimolar amounts of pyruvic acid and ammonia [5, 7] The thiols thus formed are subsequently methylated by *S*-methyltransferases [5, 10]

In addition to this pathway of formation of thiols we have now found that thiols may be generated from cysteine conjugates via an alternative pathway (Scheme 2) in which the intermediate formation of thiopyruvic acid conjugates is most likely to be involved



Scheme 2

In the present communication we will describe the evidence for this novel pathway

Materials and methods

Materials S-(*p*-Bromophenyl)-L-cysteine and *p*-bromophenyl-3-thiopyruvic acid were synthesized by the method described in our previous report [7] *p*-Bromobenzenethiol was purchased from Aldrich (U S A) All other reagents used in the present study were of analytical special grade and commercially available

The liver was excised from male Sprague–Dawley rats (8 weeks old) and homogenized in 2 vol of ice-chilled 50 mM of potassium phosphate buffer (pH 7 4) The cytosol (105,000 *g* supernatant) and microsomal fractions were obtained by published methods [5] The following *in vitro* experiments were carried out with these enzyme preparations

Deamination of S-(p-*bromophenyl*)-L-*cysteine* The reaction mixture contained in a total volume of 0 5 ml S-(*p*-bromophenyl)-L-cysteine (0 5 μmole) 0 3 ml of potassium phosphate buffer (5 μmole pH 7 4) and 0 2 ml of the enzyme preparation (8 mg protein) The mixture was incubated at 37° for 30 min and the reaction terminated by an addition of 0 5 ml of acetonitrile at 0° After centrifugation a portion of the supernatant was applied to an h p l c column (YMC A-312 ODS 6 × 150 mm Yamamura Chemical, Osaka, Japan) and the column eluted with a solvent system of methanol/water/acetic acid (5 4 1 by vol) at a flow rate of 1 5 ml/min *p*-Bromophenyl-3-thiopyruvic acid which emerged as a single sharp peak at 5 6 min under these conditions was quantitatively analyzed by monitoring the uv absorbance at 254 nm

C—S cleavage of p-*bromophenyl-3-thiopyruvic acid* The reaction mixture contained in a final volume of 0 2 ml *p*-bromophenyl-3-thiopyruvic acid (0 2 μmole) as a substrate dithiothreitol (1 μmole) potassium phosphate buffer (10 μmole pH 7 4) and 0 04 ml of the enzyme preparation The reaction mixture was incubated at 37° for 1 hr under anaerobic conditions The incubation was terminated by an addition of 0 2 ml of acetonitrile containing *p*-fluoroben-

zenethiol as an internal standard (60 μg) at 0°. The amount of *p*-bromobenzenethiol in the solution was determined by h.p.l.c. (μBondapak C_{18}, Waters) with a solvent system of methanol/water/acetic acid (65:35:1, by vol.) by the method described in our previous report [11].

Identification of metabolites. To obtain enough amount of each metabolite for a mass spectral analysis, the incubations were performed on a 10-times larger scale for the two substrates with the cytosol fraction as enzyme source.

The thiopyruvic acid conjugate generated was partially purified by the usual method for a selective extraction of carboxylic acids (i.e. extraction with organic solvent and subsequent back-extraction with saturated aqueous solution of sodium bicarbonate). The extracted conjugate was further purified with the h.p.l.c. by the method described above. After methylation with diazomethane, the derivatized thiopyruvic acid conjugate was analyzed with a GC-mass spectrometer as described below.

The thiophenol formed in the reaction mixture was isolated, methylated to *p*-bromothioanisole with diazomethane, and subjected to GC-mass analysis according to the procedure described by Tomisawa *et al.* [7].

GC-Mass analysis. The mass spectra were obtained with a JEOL JMS DX-300 mass spectrometer coupled to a JEOL GCG-05 gas chromatograph.

For the thiopyruvic acid the gas chromatograph was equipped with a glass column (1 m × 5 mm) packed with 5% OV-17 (Gas-chrom Q, 80/100 mesh). The oven temperature was programmed to rise to 250° at a rate of 30°/min from an initial setting at 160°. The temperatures of the injection port and chamber heater were 200°. Helium was used as carrier gas at a flow rate of 40 ml/min. The ionizing energy was 30 eV. Under these conditions the methylated thiopyruvic acid appeared at 7.2 min.

The conditions for the mass spectral analysis of *p*-bromothioanisole were described in our previous report [7].

Results and discussion

Identification of metabolites. *p*-Bromophenyl-3-thiopyruvic acid was methylated with diazomethane at two positions, i.e. the keto and carboxyl moiety, to yield methyl 2-methoxy-3-S-(*p*-bromophenyl)thiopropenate. Thus, the mass spectrum of this derivative gave a molecular ion at m/z 302 ($C_{11}H_{11}SO_3Br$, relative intensity 36%) with an isotope peak at m/z 304. The major fragment peaks appeared at m/z 188 (Br—C_6H_4—SH, 47%, isotope peak 190), m/z 187 (Br—C_6H_4—S, 19%, isotope peak 189), and m/z 108 (C_6H_4S, base peak). The mass spectrum of the methylated metabolite was similar to that of the authentic compound.

The mass spectrum of the methylated derivative of thiophenol was identical with that of the synthesized sample: molecular ion at m/z 202 (C_6H_5SBr, base peak) with its isotope peak at m/z 204, m/z 187 ($M^+ - CH_3$, relative intensity 20%, isotope peak, 189), m/z 169 ($M^+ - SH$, 7%, isotope peak, 171), m/z 156 ($M^+ - SCH_2$, 7%, isotope peak, m/e 158), m/z 123 ($M^+ - Br$, 10%), and m/z 108 ($M^+ - Br - CH_3$, 8%).

Activity of deamination. The microsomal fraction of the liver virtually lacked deamination activity, while the freshly prepared cytosol fraction showed a high deamination activity towards *p*-bromophenyl-L-cysteine, thus, the amount of *p*-bromophenyl-3-thiopyruvic acid formed from the cysteine conjugate was about 80 pmole/mg protein/min under the conditions described above. The incubation with heat denatured cytosol (95°, 5 min) run simultaneously did not give the thiopyruvic acid conjugate in any detectable amount. The renal cytosol likewise deaminated the cysteine conjugate although the rate was somewhat lower (about 1/3) than that in the liver (data not shown).

In vivo formation of a thiopyruvic acid conjugate or its decarboxylated metabolite (i.e. thioacetic acid conjugate) probably via deamination of corresponding cysteine conjugates has been reported for several xenobiotics [12, 13]. However, there have been no reports concerning the *in vitro* deamination of cysteine conjugates. The present results suggest that by the action of enzyme(s) in the liver and kidney, cysteine conjugates are easily converted to thiopyruvic acid conjugates.

In our preliminary experiment, liver cytosol of guinea pigs was found to possess three times higher deamination activity towards *S*-(*p*-bromophenyl)-L-cysteine than rats (data not shown). The finding is compatible with the observation that cysteine conjugates are not excreted as mercapturic acids but undergo other metabolic pathway(s) in guinea pigs [12, 13].

Activity of C—S *bond cleavage.* Under the present incubation conditions, the cytosol fraction showed enzyme activity to cleave the C—S bond of S-(*p*-bromophenyl)-3-thiopyruvic acid, but the activity was not detected in the microsomal fraction nor the heat denatured cytosol fraction. The formation rate was about 30–50 nmole/mg protein/min of the rat liver. Since the incubation was performed under anaerobic conditions, the C—S bond was unlikely to be cleaved via oxidation of C-3 carbon atom of thiopyruvic acid moiety.

Rat liver β-lyase, which was obtained by our previous method [5], did not cleave the C—S bond of S-(*p*-bromophenyl)-3-thiopyruvic acid under the same incubation conditions described in our previous report [5]. Together with the finding that the simple deamination of the cysteine conjugate was not catalyzed by β-lyase, this result indicates that the activity observed in the present study is most likely to be ascribed to different enzymes.

The present results strongly indicate that thiol-containing metabolites are produced from cysteine conjugates not only by the α, β-elimination reaction (Scheme 1) but also by the two-step reaction shown in Scheme 2. The novel pathway of thiol formation in the present study is of great significance from the viewpoints of the metabolism of cysteine conjugates as well as the production *in vivo* of cytotoxic thiols [14–16].

Purification and characterization of these enzymes are in progress in our laboratory.

In summary, *S*-(*p*-bromophenyl)-L-cysteine was converted to the *p*-bromobenzenethiol via a two-step reaction by the action of enzymes in the rat liver cytosol (105,000 *g* supernatant), thus, the cysteine conjugate was deaminated to give *p*-bromophenyl-3-thiopyruvic acid which subsequently cleaved at the C—S linkage giving the thiol. This is a novel and alternative pathway to generate thiols from corresponding cysteine conjugates.

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