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ENZYMATIC FORMATION OF A CHOLIC ACID DERIVATIVE OF ISETHIONIC ACID

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Abstract—Evidence is presented that preparations of rat liver microsomes are capable of conjugating isethionic acid with cholic acid. Thin-layer chromatography experiments indicate that this newly described conjugated bile acid migrates with the same R_f value as taurocholic acid, a structurally similar analogue. Dilution experiments utilizing both labeled cholic acid (tritium) and isethionic acid (sulfur-35) demonstrate the incorporation of both isotopes into a cholyl-conjugate of isethionic acid. Acid hydrolysis of enzymatically synthesized cholyl[^{35}S]isethionic acid and subsequent thin-layer chromatography of the degradation products also indicate that isethionic acid is conjugated with cholic acid. Formation of the cholyhydrazide derivative of cholyl-isethionic acid confirms an ester linkage between the carboxyl moiety of cholic acid and the hydroxyl moiety of isethionic acid. Rats receiving radioactive isethionic acid by stomach tube synthesized approximately 28 nmoles cholyl-isethionic acid compared with 980 nmoles taurocholic acid when radioactive taurine was administered under similar conditions.

It is widely recognized that liver microsomal preparations are capable of conjugating cholic acid with taurine, hypotaurine or glycine in the presence of ATP and coenzyme A [1–8]. The primary function of these conjugate bile acids (review by Jacobsen and Smith [9]) is to facilitate the intestinal absorption of fat via their detergent action. Recently, other possible roles for taurocholic acid have been postulated and include: (1) stimulation of the transmural electrical potential difference *in vitro* across the rat small intestine [10]; (2) activation of pancreatic lipase [11]; (3) causative agent for back diffusion of hydrogen ions across human gastric mucosa [12]; and (4) reduction of blood supply to the gastric mucosa by initiating mucosal changes which produce and maintain the decreased blood flow [13].

In this paper, it is reported that isethionic acid, a metabolite of taurine in mammalian tissues [14–20], is conjugated with cholic acid, and evidence is presented suggesting that the conjugation of isethionic acid with cholic acid is through an ester linkage.

MATERIALS AND METHODS

Materials. Cellulose thin-layer chromatography sheets (cellulose on Mylar sheets) and the potassium salt of isethionic acid (ISA) were purchased from Eastman Kodak. Silica gel G was purchased from Brinkmann Instruments. Cholic acid and taurocholic acid were obtained from P-L Biochemicals. Hydrazine hydrate (85%) was purchased from Fisher Scientific Co. [^{35}S]Taurine (20–108 mCi/m-mole) and [^{3}H]cholic acid (2·2 Ci/m-mole) were supplied by Amersham/Searle Corp. and New England Nuclear respectively. The anion exchange Dowex resin AG 1-X2 (100–200 mesh) was supplied by Bio-Rad Laboratories. Sprague–Dawley male rats (125–175 g) were purchased from Sprague–Dawley, Madison, Wis.

Preparation of rat liver microsomes. Rat liver microsomes were prepared according to the procedure of Elliott [21] with certain modifications. Male Sprague–Dawley rats were killed by cervical dislocation, and the liver was immediately removed, washed in cold distilled water, weighed and homogenized in 4 vol. of 0·25 M sucrose. The liver homogenate was centrifuged for 15 min at 12,000 *g*, and the supernatant recentrifuged for 30 min at 105,000 *g*. The resulting microsomal pellet was suspended in cold glass-distilled water (1 ml for each 1 g wet wt of liver) and homogenized in a motor-driven Teflon–glass homogenizer. Triton X-100 (app 4% by vol) was added to the homogenate to solubilize the microsomes.

Chemical conversion of [^{35}S]*taurine to* [^{35}S]*isethionic acid.* Sodium nitrite (35 mg in 0·1 ml water) was added to a solution containing 0·1 ml of 1 N HCl and 100 μCi [^{35}S]taurine. The mixture was kept on ice for 15 min while the reaction proceeded. The entire solution (0·2 ml) was applied to Whatman 3 MM filter paper, which was dampened with the same buffer utilized in the electrophoretic procedure [sodium citrate (0·02 M)–potassium phosphate (0·02 M) at pH 6·0] and then subjected to electrophoresis for 20 min at 500 V and for 70 min at 2000 V. The electrophoretic separation of taurine from isethionic acid was checked by applying nonradioactive standards at the edges of the filter paper. Taurine was detected with ninhydrin (0·25% in acetone) and isethionic acid was located with bromophenol blue (5% in alcohol). The radioactive isethionic acid was then eluted from the filter paper with water; 60 μCi [^{35}S]isethionic acid were obtained.

The authenticity of the [^{35}S]isethionic acid was confirmed by thin-layer chromatography with known standards of isethionic acid and taurine (Fig. 1). Further proof that the radioactive isethionic acid was not contaminated with [^{35}S]taurine was demonstrated by passage of an aliquot of the synthesized radioactive isethionic acid (200,000 cpm in 0·3 ml water) through a column containing Dowex anion exchange resin (AG 1-X2, Cl^- form, 2·5 by 20 mm).

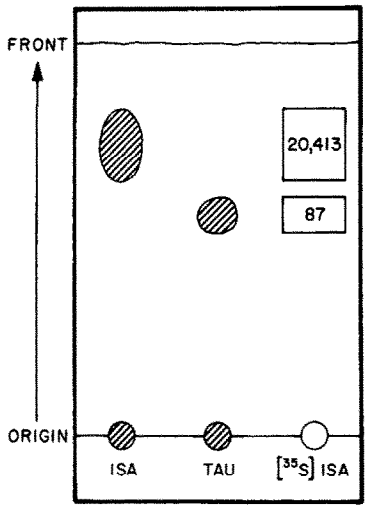

Fig. 1 Cellulose thin-layer chromatogram of isethionic acid (ISA) and taurine (TAU) standards and [^{35}S]isethionic acid ([^{35}S]ISA) prepared from [^{35}S]taurine. The solvent system contained pyridine–*n*-butanol–water (4:2:2, by vol.). The isethionic acid standard was stained with 5% bromophenol blue in alcohol (pH 8·0), while taurine was visualized with 0·25% ninhydrin. Cellulose in areas corresponding to standards was scraped from the Mylar plate into a conical test tube. Water (0·4 ml) was added and the mixture vigorously shaken, and then centrifuged. The water phase was counted in a liquid scintillation counter. The numbers in the boxes are cpm.

The water eluate (2·1 ml) contained only background radioactivity. Since [^{35}S]isethionic acid is a strong acid (sulfonic acid), it is completely removed from solution by the anion exchange resin. However, a [^{35}S]taurine contaminant in the preparation would have been detected in the eluate due to its zwitterion nature.

Enzyme system for conjugation of cholic acid with isethionic acid. Table 1 lists the components (in μmoles contained in a final volume of 0·16 ml) of reaction mixtures A and B for the synthesis of cholyl-isethionic acid. The reaction mixtures differed in that mixture A contained only nonlabeled cholic acid and a constant amount of radioactive isethionic acid, while mixture B contained various quantities of nonlabeled isethionic acid and the radioactive label in the cholic acid. The enzymatic synthesis of cholyl-isethionic acid was performed in conical test tubes in a total volume of 0·16 ml. The reaction was initiated with 200 μg of microsomal protein. Incubations were carried out at 37° for 1 hr. The reaction was terminated after 1 hr by dilution with 2·5 ml water. One ml *n*-butanol was added and the water–*n*-butanol layers were thoroughly mixed on a Vortex Jr. for 30 sec to extract the radioactive cholyl-isethionic acid into the organic phase. Isethionic acid remains in the aqueous phase. An aliquot of the *n*-butanol phase was then evaporated to dryness under a stream of air and the residue dissolved in 10 μl water and chromatographed or processed as indicated.

When [^{3}H]cholic acid and [^{35}S]isethionic acid were utilized to form doubly labeled cholyl-isethionic acid (see Table 2), slightly different conditions were employed. Incubation vessels contained the following reagents (in μmoles): potassium phosphate, 20; magnesium sulfate, 1; ATP, 1; coenzyme A, 0·055; cholic acid, 0·020 (645,000 cpm); nonlabeled isethionic acid, 0 to 0·005; and labeled isethionic acid, 650,000 cpm. The reaction was processed for 30 min at 37° and then stopped by dilution with 2·5 ml water. The unreacted [^{3}H]cholic acid was removed by extraction (five times) with 1 ml dichloromethane [22, 23], and the [^{3}H]cholyl[^{35}S]isethionic acid was extracted with 1 ml *n*-butanol. An aliquot (0·7 ml) of the *n*-butanol layer was counted in 5 ml of Bray's scintillation fluid [24].

Hydrolysis of cholyl[^{35}S]isethionic acid. Cholyl-[^{35}S]isethionic acid, formed by the above enzymatic reaction (reaction mixture A) and extracted into *n*-butanol, was hydrolyzed as follows: 0·5 ml of the *n*-butanol phase (total 1 ml) was placed in a stoppered centrifuge tube and evaporated to dryness in a stream of air. The cholyl-isethionic acid was hydrolyzed by adding 0·5 ml of 6 N HCl and heating on a boiling water bath. An additional 0·5 ml of 6 N HCl was added after 1 hr and hydrolysis continued for a second hr. The centrifuge tube was then removed from the water bath and the liquid evaporated to dryness under a stream of air. The residue was taken up in 20 μl water and chromatographed in the following solvent system: ethyl butyrate–heptane–glacial acetic acid–water (9:1:7:3, by vol).

Formation of methylcholate and cholylhydrazide. Methylcholate and cholylhydrazide were prepared according to the procedures of Cortese [25].

Formation of the hydrazine derivative of [^{3}H]cholyl-isethionic acid. [^{3}H]cholyl-isethionic acid was synthesized by a rat liver microsomal preparation in duplicate incubation vessels (reaction mixture B containing 0·2 μmole isethionic acid). The reaction mixtures were extracted five times with 1 ml dichloromethane to remove [^{3}H]cholic acid [22, 23] and then the [^{3}H]cholyl-isethionic acid was extracted with 1 ml *n*-butanol. An aliquot of the *n*-butanol phase (0·8 ml) was evaporated to dryness in a stream of air. Five mg of methylcholate carrier, 0·2 ml ethanol, and 0·1 ml hydrazine (85%) were added to one test tube containing [^{3}H]cholyl-isethionic acid. To the duplicate tube containing an equal quantity of [^{3}H]cholyl-isethionic acid similar components were added but hydrazine was omitted. Both tubes were refluxed for 8 hr, and the solvent was evaporated by a stream of air. The products were dissolved in 0·1 ml methanol, and an aliquot (0·005 ml) was chromatographed in the following solvent system: *n*-butanol–glacial acetic acid–water (10:1:1, by vol.).

Formation in vivo of taurocholic acid and cholyl-isethionic acid. One hundred μmoles taurine containing 43 μCi sulfur-35 was administered to a male rat (160 g) by stomach tube. The rat was fasted for 24 hr, killed, and the intestines were homogenized in 85 ml of 80% ethanol according to the procedures of Norman and Sjövall [26]. The homogenate was then centrifuged for 20 min at 12,000 *g* and a 10-ml aliquot evaporated in a stream of air to 0·5 ml. The precipitate formed during the concentration procedure was removed by centrifugation. An aliquot (0·1 ml) of the concentrated ethanol supernatant was chromatographed on Silica gel G (500 μm) in the solvent sys-

Table 1. Composition of the enzymatic reaction mixtures used for the synthesis of cholyl-isethionic acid

Component	Reaction mixture A* (μmoles)	Reaction mixture B† (μmoles)
Potassium phosphate buffer, pH 7·4	20	20
$MgSO_4$	1	1
ATP	1	1
Coenzyme A (corrected for 92% purity)	0·055	0·055
Isethionic acid	0·020	0–0·100
Cholic acid	0·1	0·0001

* Reaction mixture A contained 800,000 cpm of [^{35}S]isethionic acid.
† Reaction mixture B contained 500,000 cpm of [^{3}H]cholic acid.

tem: ethyl butyrate–heptane–glacial acetic acid–water (9:1:7:3, by vol). A second rat (158 g) was given 100 μmoles isethionic acid containing 44 μCi sulfur-35 and processed as above.

RESULTS AND DISCUSSION

Enzymatic synthesis of cholyl-isethionic acid using [^{35}S]isethionic acid. Reation mixture A (Table 1) was utilized for the biosynthesis of cholyl[^{35}S]isethionic acid. The cellulose thin-layer chromatogram is presented in Fig. 2.

Cholic acid migrates with the solvent front and contains essentially no radioactivity in either the complete incubation system ('X') or the system in which ATP and coenzyme A were omitted ('Y'). Unfortunately, authentic cholyl-isethionic acid is not available; therefore, commercial taurocholic acid (TC) was utilized as a standard for cholyl-isethionic acid because of its structural resemblance. The area in which taurocholic acid migrates contains significant amounts of radioactivity ('X'). Again, the control incubation vessel ('Y'), minus ATP and coenzyme (A) contained very little radioactivity. Radioactive isethionic acid ([^{35}S]ISA) was chromatographed under the same conditions to eliminate the possibility that it migrated concomitantly with taurocholic acid.

Enzymatic synthesis of cholyl-isethionic acid using tritiated cholic acid. Similar experiments to those presented in Fig. 2 were performed using [^{3}H]cholic acid (reaction mixture B, Table 1). Initial control experiments in which isethionic acid was omitted indicated that the area corresponding to taurocholic acid contained small but significant amounts of radioactivity

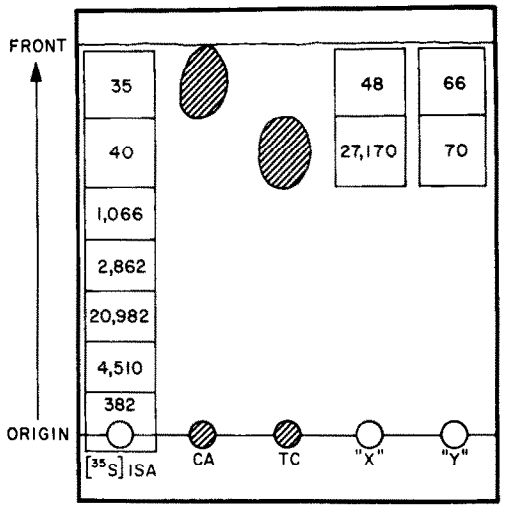

Fig. 2. Cellulose thin-layer chromatogram of the product of the reaction between cholic acid and [^{35}S]isethionic acid. Cholic acid (CA), [^{35}S]isethionic acid ([^{35}S]ISA) and taurocholic acid (TC) were used as standards. The chromatography system contained the following solvents: ethyl butyrate–heptane–glacial acetic acid–water (9:1:7:3, by vol.). Phosphomolybdate (10% in alcohol) was used to stain cholic and taurocholic acids. An aliquot of the *n*-butanol phase (Materials and Methods) designated as 'X' (complete enzyme system) or 'Y' (enzyme system minus ATP and coenzyme A) was chromatographed. Radioactivity was measured after scraping and collecting the cellulose from the areas corresponding to the standards and extracting with water as described in the legend to Fig. 1. The numbers in the boxes are cpm.

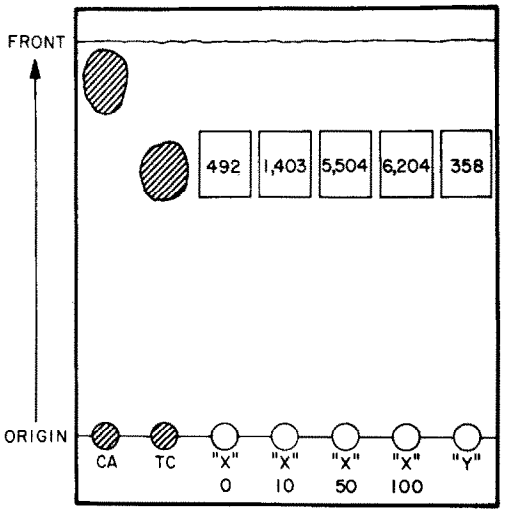

Fig. 3. Cellulose thin-layer chromatogram of [^{3}H]cholyl-isethionic acid. Varying amounts of isethionic acid (0, 10, 50 and 100 nmoles) were added to the incubation vessels containing reaction mixture B (Table 1). After a 1-hr incubation (37°), the reaction was terminated as described in Materials and Methods. Aliquots of the *n*-butanol phase designated as 'X' (complete enzyme system) or 'Y' (enzyme system minus ATP and coenzyme A) were chromatographed. Authentic cholic acid (CA) and taurocholic acid (TC) were used as standards for the chromatography. The solvent system contained the following components: ethyl butyrate–heptane–glacial acetic acid–water (9:1:7:3, by vol.). Both cholic and taurocholic acids were stained with 10% phosphomolybdate in alcohol. The numbers in the boxes refer to the radioactivity in cpm which was measured after scraping the cellulose off the Mylar plates as described in the legend to Fig. 1.

Table 2. Enzymatic formation of cholyl-isethionic acid using tritiated cholic acid and sulfur-35 isethionic acid

Isethionic acid (nmoles)	Tritium channel (cpm)	Sulfur-35 channel (cpm)	Corrected tritium channel* (cpm)	Ratio $^3H/^{35}S$
0	13,951	6,382	13,313	2·09
2·5	16,929	4,380	16,491	3·76
5·0	19,133	3,502	18,783	5·35

* Corrections were made for the spillover of the sulfur-35 radioactivity (10%) into the tritium channel. It is appreciated that the digits shown far exceed the accuracy of the determinations.

(Fig. 3; 492 cpm − 358 cpm = 134 cpm). It was subsequently demonstrated that the microsomal enzyme preparation contained a small quantity of endogenous taurine which was converted to taurocholic acid in the absence of exogenous isethionic acid.

In order to circumvent this source of experimental error, varying quantities of isethionic acid (0–100 nmoles) were added to reaction mixture B. The results in Fig. 3 demonstrate an increase in radioactivity in the area of the chromatogram corresponding to taurocholic acid. This indicates a microsomal synthesis of cholyl-isethionic acid, since isethionic acid was the only variable component in the reaction system.

Enzymatic synthesis of [^3H]cholyl[^{35}S]isethionic acid. [^{3}H]cholic acid and varying amounts of [^{35}S]isethionic acid were employed in the formation of cholyl-isethionic acid, which was then extracted with *n*-butanol and analyzed for radioactivity. By varying the amount of nonlabeled isethionic acid (0–5 nmoles), and increase in the tritium label was demonstrated indicating that a greater quantity of conjugate was formed; conversely, the radioactivity in the sulfur channel decreased due to dilution of the sulfur-35 label by nonradioactive isethionic acid (Table 2). This type of double isotope derivative experiment has been the basis of numerous assays for various biological compounds [23, 27–29].

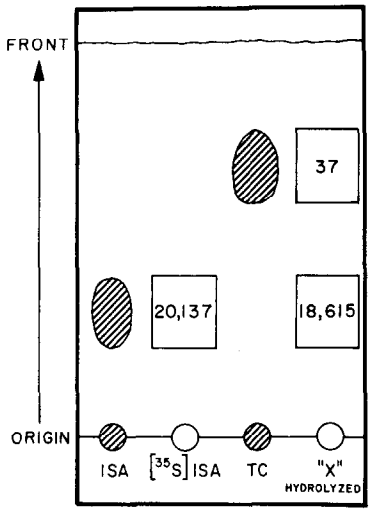

Fig. 4. Cellulose thin-layer chromatogram of the acid hydrolysis products of cholyl[^{35}S]isethionic acid. An aliquot of the *n*-butanol extract containing cholyl[^{35}S]isethionic acid was hydrolyzed in HCl (details described in Materials and Methods). Standard isethionic acid (ISA), [^{35}S]isethionic ([^{35}S]ISA) and taurocholic acid (TC) were used as markers. The chromatography system contained the following solvents: ethyl butyrate–heptane–glacial acetic acid–water (9:1:7:3, by vol). Isethionic acid was stained with 5% bromophenol blue in alcohol; cholic acid was visualized with 10% phosphomolybdate in alcohol. The sample referred to as 'X' was obtained from the complete incubation system (containing ATP and coenzyme A). Radioactivity was measured by scraping and collecting the cellulose from the areas on the chromatogram which corresponded to the standards as described in the legend to Fig. 1. The numbers in the boxes are cpm.

Hydrolysis of cholyl[^{35}S]isethionic acid. To demonstrate that [^{35}S]isethionic acid was a component of the presumptive cholyl-isethionic acid, the conjugated product was hydrolyzed. Chromatography of the hydrolysis products is shown in Fig. 4. The area corresponding to taurocholic acid contained a negligible amount of radioactivity, while the area corresponding to isethionic acid had significant radioactivity.

Conversion of [^3H]cholyl-isethionic acid to [^3H]cholylhydrazide. In order to demonstrate that an ester linkage exists between the carboxyl group of cholic acid and the hydroxyl group of isethionic acid, radioactive [^{3}H]cholyl-isethionic acid was enzymatically synthesized and converted to the hydrazine derivative as described in Materials and Methods. Chromatography of [^{3}H]cholylhydrazide is shown in Fig. 5.

Formation in vivo *of taurocholic acid and cholyl-isethionic acid.* After administration by stomach tube of either [^{3}S]taurine or [^{35}S]isethionic acid to the rats, the cholyl-conjugates were extracted and chromatographed as described in Materials and Methods. The amount of radioactive taurocholic acid formed in a 0·1-ml aliquot of the concentrated ethanol extract of intestine was 23 nmoles (21,800 cpm). The quantity of taurocholic acid formed in the total ethanol extract (85 ml) was calculated to be 980 nmoles.

The quantity of cholyl-isethionic acid which chromatographed with a similar R_f value (0·67) as taurocholic acid was calculated to be approximately 0·65 nmole (680 cpm). The total ethanol extract (85 ml) contained 28 nmoles of cholyl-isethionic acid.

Both taurine (R_f = 0·30) and isethionic acid (R_f = 0·30) migrated in this solvent system [ethyl butyrate–heptane–glacial acetic acid–water (9:1:7:3, by vol.)] with R_f values that were considerably lower than the value for taurocholic acid (R_f = 0·67), and thus contamination from the starting reactants was eliminated as a source of error.

DISCUSSION

It has been demonstrated that a cholyl derivative of isethionic acid can be synthesized by rat liver micro-

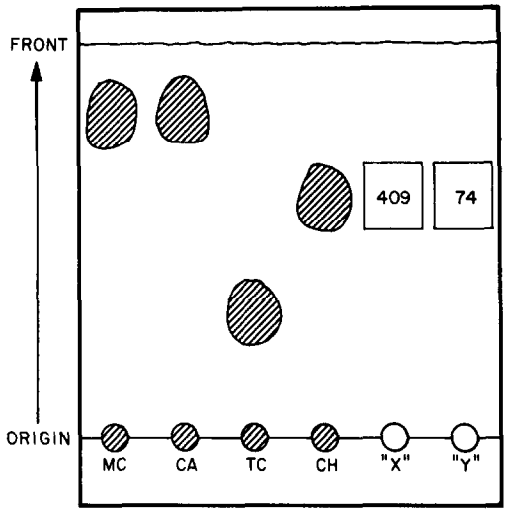

Fig. 5. [^{3}H]cholylhydrazine was synthesized as described in Materials and Methods. Methanol (0·1 ml) was added to the tritiated hydrazide derivative of cholyl-isethionic acid, and an aliquot (0·005 ml) designated as 'X' (refluxed in the presence of hydrazine) or 'Y' (refluxed in the absence of hydrazine) was chromatographed. Authentic methylcholate (MC), cholic acid (CA), taurocholic acid (TC) and cholylhydrazide (CH) were used as standards for the chromatography. The solvent system contained the following components: *n*-butanol–glacial acetic acid–water (10:1:1, by vol.). The chromatogram was stained with 10% phosphomolybdate in alcohol. The numbers in the boxes refer to the radioactivity in cpm which was measured after scraping the cellulose off the Mylar plates as described in the legend to Fig. 1.

somes. This compound is unique in that it appears to contain an ester linkage formed from the alcohol moiety of isethionic acid and the acid moiety of cholic acid.

Evidence for the formation of a conjugated derivative was suggested by demonstrating that isethionic acid was a component of the unknown compound after hydrolysis in HCl (Fig. 4). Further proof that the hydroxyl moiety of isethionic acid was conjugated through an ester linkage with the carboxyl moiety of cholic acid was indicated by formation of the hydrazide derivative. If isethionic acid was linked to cholic acid through one of its hydroxyl groups (3α, 7α or 12α), then the radioactive compound would not have co-chromatographed with synthetic cholylhydrazide (Fig. 5).

In rats given radioactive taurine, a considerable quantity of taurocholic acid was produced within 24 hr. However, when a similar experiment was performed using labeled isethionic acid only a small amount of cholyl-isethionic acid derivative was formed.

Isethionic acid, which has been demonstrated to be a metabolite of taurine in mammalian tissues [14–20], does not have a specific assigned function. Moreover, the significance and function of this new cholic acid derivative of isethionic acid are also unknown.

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