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EVIDENCE FOR THE FORMATION OF A STEROID *S*-GLUTATHIONE CONJUGATE FROM AN EPOXYSTEROID PRECURSOR

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SAMMARY—Biotransformation of [4-^{14}C]cholesterol α-epoxide (5α,6α-epoxycholestan-3β-ol) to the *S*-glutathione conjugate, 3β,5α-dihydroxycholestan-6β-yl-*S*-glutathione, by *S*-glutathione transferase of the rat liver soluble supernatant fraction, has been described. After the isolation from the biological incubation mixture by *n*-butanol extraction, followed by the Amberlite XAD-2 column treatment, the conjugate was chromatographically identified with a synthetic specimen which was prepared from cholesterol α-epoxide and glutathione in an ethanolic solution of sodium hydroxide. Further identification of the biologically formed conjugate with the synthetic one, including structural assignment, was established from the result that they yielded the same desulfrization product, 3β,5α-dihydroxycholestane, by the treatment with Raney nickel in an atmosphere of hydrogen.

INTRODUCTION

Steroid *S*-glutathione conjugates are well known as physiologically inactive and excretory forms in estrogens. Estrone and estradiol are converted in the presence of glutathione by rat liver microsomes to 1- and 4-*S*-glutathione conjugates of the corresponding 2,3-catechol estrogens (1-3). The conjugation is interpreted as the sequential reactions involving hydroxylation at the 2-position, further oxidation into a semiquinone radical as a hypothetic intermediate and non-enzymatic coupling with the sulfhydryl group (3-5). In contrast to xenobiotic olefin and arene oxides (6), significance of the hepatic glutathione conjugation of the estrogen is not to require soluble *S*-glutathione transferase.

It is of interest to know whether or not a steroid epoxide can be conjugated with glutathione by hepatic soluble *S*-glutathione transferase. A promising approach to this problem could be to use the naturally occurring stable epoxide, cholesterol α-epoxide (5α,6α-epoxycholestan-3β-ol), which has recently been demonstrated to be formed from cholesterol by the catalytic action of P-450's of liver (7) as well as adrenal cortex microsomes (8, 9). The previously speculated physiological roles of the α-epoxide are of interest, for it has been suggested to be not only a causative material inducing skin carcinoma in mice irradiated by ultraviolet ray (10), but also a material which is closely related to hypercholest-

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erolemic high blood presure in man (11). Many British patients with various type of hypertensive disease have been demonstrated to have extremely high serum concentrations of cholesterol α-epoxide, and especially in the ones with hypercholesterolemia the concentration was approximately 3250 μg/ml serum whereas in the normal it was almost unmeasurable (11).

Although a lot of information have been accumulated on the formation of *S*-glutathione conjugates with a wide variety of epoxides of olefins and arenes as xenobiotic substrates (6, 12), nothing has been known of the peptide conjugation in the endogeneous epoxy substrate. The present communication deals with the first evidence for the enzymatic formation of the glutathione conjugate, 3β,5α-dihydroxycholestan-6β-yl-*S*-glutathione, from cholesterol α-epoxide.

MATERIALS AND METHODS

Materials—[4-^{14}C]5α,6α-Epoxycholestan-3β-ol (58 mCi/mmole), over 99.9% in its radiochemical purity, was synthesized and purified by the previously reported method (9) from [4-^{14}C]cholesterol purchased from Radiochemical Centre, Amersham, England. Unlabeled 5α,6α-epoxycholestan-3β-ol (13) and 3β,5α-dihydroxycholestane (14) were also synthesized by the previously reported methods. Glutathione which was confirmed by tlc on crystalline cellulose powder plates to be practically pure and free from its oxidized form (GSSG), was supplied from Yamanouchi Pharmaceuticals Co., Tokyo.

Synthesis of 3β,5α-dihydroxycholestan-6β-yl-*S*-glutathione—To an admixture of an aqueous solution (10 ml) of glutathione (308 mg, 1 mmole) and a solution of cholesterol α-epoxide (201 mg, 0.5 mmoles) in ethanol (20 ml) was added 5N NaOH (1 ml), and the mixture was refluxed for 3 hr. The reaction mixture was cooled, acidified with glacial acetic acid and brought to dryness *in vacuo*. The residue obtained was dissolved in 1% aqueous acetic acid (10 ml), and extracted three times with two volumes of *n*-butanol presaturated with water. The residue obtained on evaporation of the solvent from the extract was dissolved in water (10 ml), and the solution poured onto an Amberlite XAD-2 column (50 x 3 cm) proccessed successively with ethanol, methanol, methanol-water (1:1), and water (10 bed volumes each). The column was washed with water (5 bed volumes), methanol-water (1:1, 2 bed volumes), and eluted with methanol (5 bed volumes). Evaporation of the solvent from the eluate left an amorphous powder (341 mg) which on silica gel plates (Merck $60F_{254}$) in each solvent system described in Fig. 1 showed only single spots visualized as purple color with a ninhydrin reagent (1% solution in *n*-butanol saturated with citrate buffer, pH 0.5) or as charcoal color with concentrated sulfric acid when heated.

Incubation conditions and isolation of metabolite—A soluble supernatant fraction was obtained by the centrifugation at 105,000 x *g* from a post mitochondrial supernatant fraction of a 3-volume homogenate prepared in 0.25 M sucrose from the liver of male Wistar rats weighing approximately 150 g. The soluble supernatant fraction was dialyzed through the Visking membrane against 250 volumes of water at 2° for 20 hr and adjusted with water so that 3 ml of the solution (53.7 mg protein) was equivalent to 1 g of wet weight of the tissue.

The radioactive substrate solution was made before use as follows: to a solution of [4-^{14}C]cholesterol α-epoxide (9.0 μCi) in benzene (0.25 ml) was added a mixture (5 ml) of 0.1 M Tri-HCl buffer, pH 8.0, and methanol containing 0.1% Tween 80 (4:1), and the mixture was then kept at 50° and agitated vigorously to make a milky suspension. From the suspension was evaporated benzene with a gentle nitrogen stream. The resulting slightly turbid but transparent solution was adjusted with a mixture of 0.1 M Tris-HCl buffer, pH 8.0, and methanol (4:1) to make a final volume of 5 ml.

For the glutathione conjugation reaction, the radioactive substrate solution (1 ml, 1.8 μCi, 3 μM) was incubated at 37° for 30 min with the dialyzed soluble

supernatant fraction (3 ml) in the presence of glutathione (1 mM) and 0.1 M Tris-HCl buffer, pH 8.0, to make a final volume of 10 ml. After the incubation was added unlabeled synthetic 3β,5α-dihydroxycholestan-6β-yl-*S*-glutathione (5 mg), if necessary. The incubation mixture was extracted twice with ethyl acetate (30 ml each) for the elimination of the unchanged radioactive substrate. To the residual aqueous phase was added glacial acetic acid (0.1 ml) to acidify the solution in order to retard the dissociation of the glutathione conjugate, and the mixture was extracted three times with *n*-butanol presaturated with water. The solvent was removed from combined butanol extract *in vacuo* to dryness, and the residue obtained was dissolved in a small volume of water and applied to a silica gel plate (Merck $60F_{254}$). The adsorbent area corresponding to Rf 0.35-0.55 in thin-layer chromatogram obtained in AcOEt-*n*-BuOH-AcOH-H_2O (6:2:3:2) and monitored both by a radiochromatogram scanner and by co-chromatography with the authentic glutathione conjugate was eluted with a mixture of methanol and 28% ammonia (1:1). The clean-up procedure by tlc was made once more using *n*-BuOH-AcOH-H_2O (2:1:1) as the developing solvent. The eluate from the adsorbent area corresponding to Rf 0.5-0.7 in the 2nd chromatogram was used for the characterization or quantification of the conjugate.

Treatment of glutathione conjugate with Raney nickel—A suspension of Raney nickel (50 mg, Wako Pure Chemicals Co., Tokyo) in methanol (2 ml) and 5N NaOH (0.5 ml) was placed in a sealed vessel with a manometer and saturated with hydrogen with vigorous mechanical stirring. Stirring was continued further for 90 min after the introduction of a methanolic solution (1 ml) of the authentic 3β,5α-dihydroxycholestan-6β-yl-*S*-glutathione (5 mg) or the biologically formed radioactive conjugate isolated from the incubation mixture by tlc together with the unlabeled authentic peptide conjugate (5 mg). The reaction mixture was filtrated, and the filtrate was diluted with water (5 ml), saturated with sodium chloride, and extracted twice with ether (20 ml each). The residue obtained on the evaporation of the solvent from the extract dried over sodium sulfate was analyzed by gas-chromatography-mass spectrometry or reverse isotope dilution method when the radioactive specimen was used.

Gas-chromatography-mass spectrometry—The etheral extract obtained after the treatment of the glutathione conjugate with Raney nickel in an atmosphere of hydrogen was trimethylsilylated in anhydrous pyridine in the standard manner by using trimethylsilyl chloride and hexamethyldisilazane and assayed with a Shimadzu LKB gas-chromatograph-mass spectrometer Model 9000; column: 1.5% SE-30 coated on Chromosorb W (60-80 mesh, 3 mm x 1 m), column temperature: 260°, and He as carrier gas: 50 ml/min.

Radioactivity measurements—Tlc-scannograms were recorded on an Aloka radiochromatogram scanner Model TLC-2B. Radioactivities were measured on a Packard TriCarb liquid scintillation counter Model 3300 in a dioxane scintillator, containing 1.3% diphenyloxazole and 0.026% dimethyl-POPOP, for the biologically formed glutathione conjugate and in a toluene scintillator, containing 0.5% diphenyloxazole and 0.03% dimethyl-POPOP, for 3β,5α-dihydroxycholestane derived from the radioactive glutathione conjugate by the treatment with Raney nickel.

RESULTS AND DISCUSSION

Biotransformation of Cholesterol α-Epoxide to 3β,5α-Dihydroxycholestan-6β-yl-*S*-glutathione

[4-^{14}C]Cholesterol α-epoxide (1.8 μCi, 3 μM) was incubated at 37° and pH 8.0 with a dialyzed soluble supernatant fraction (5.3 mg protein/ml) of rat liver homogenate in the presence of glutathione (1 mM), and the incubation mixture was extracted with *n*-butanol following complete removal of the unreacted substrate by the extraction with ethyl acetate. The *n*-butanol extract was co-chromatographed with synthetic 3β,5α-dihydroxycholestan-6β-yl-*S*-glutathione on silica gel plates by

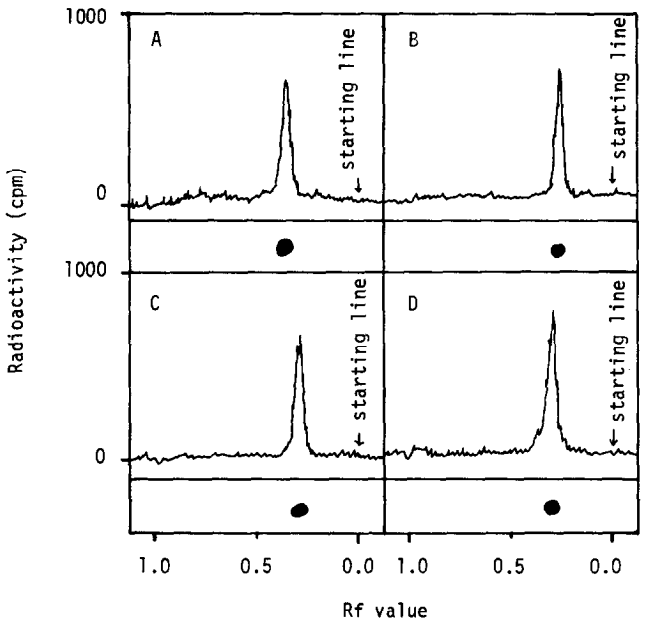

Fig. 1. Tlc scannograms of the biologically formed glutathione conjugate from [4-^{14}C]cholesterol α-epoxide and glutathione by rat liver soluble epoxide *S*-glutathione transferase. Synthetic unlabeled 3β,5α-dihydroxycholestan-6β-yl-*S*-glutathione was added as the carrier to the incubation mixture at the end of incubation, and the cojugate was extracted and isolated as described in the text. Thin-layer chromatograms were obtained on silica gel plates by solvent systems A—ethyl acetate-*n*-butanol-glacial acetic acid- water (6:2:3:2), B—*n*-butanol-glacial acetic acid-water (4:1:5), C—*n*-butanol-formic acid-water (4:1:2), and D—0.1 M phosphate buffer, pH 7.4-*n*-butanol-water-*n*-propanol (1:3:1:2). Chromatograms were recorded on a thin-layer chromatogram scanner and visualized by spraying with a ninhydrin reagent followed by heating.

four different developing solvent systems. Scannograms of the radiochromatograms indicated the butanol extract to contain a single radioactive metabolite which had the same Rf values as the those of the synthetic conjugate visualized with a ninhydrin reagent on the chromatograms (Fig. 1).

The rate of the metabolic conjugation of the α-epoxide with glutathione was determined by the elution of the radioactive spot with methanol and ammonia to be 0.49 pmoles/mg protein/min. Use of the boiled soluble supernatant fraction also yielded a trace amount of the conjugate with the same chromatographic properties as those of the synthetic conjugate. However, it accounted for less than 0.1% of the yield in the above-mentioned enzymatic conjugate formation.

The stereochemical structure of the conjugate was elucidated by the treatment of the biologically formed and chromatographically purified specimen with Raney nickel in an atmosphere of hydrogen. The metal catalyst treatment afforded the desulfrized radioactive steroid, 3β,5α-dihydroxycholestane, in

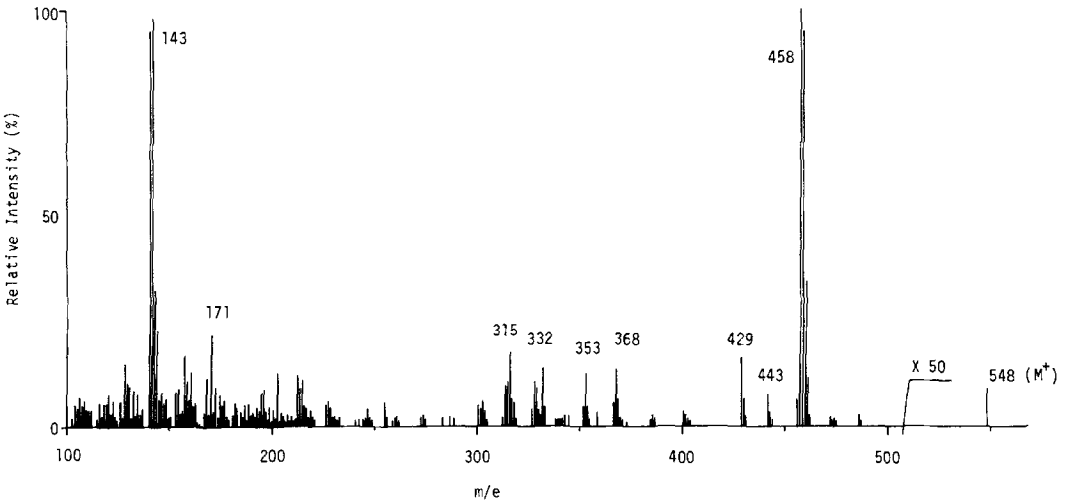

Fig. 2. Glc-mass spectrum of di-trimethylsilyl ether of 3β,5α-dihydroxycholestane yielded from synthetic 3β,5α-dihydroxycholestan-6β-yl-*S*-glutathione by the treatment with Raney nickel in hydrogen. Retention time of the di-trimethylsilyl ether was 11.8 min on a 1.5% SE-30 column at 260°. Other chromatographic data are described in the text.

overall radiochemical yield of 80% which was identified by radiochromatograms scannography using silica gel plates as well as by the reverse isotope dilution method using the authentic unlabeled specimen as the carrier for obtaining constant specific radioactivities by recrystallizations from methanol. The radioscannogram of the thin-layer chromatogram obtained in benzene-ethyl acetate (3:1) indicated the formation of 3β,5α-dihydroxycholestane as the single major radioactive reaction product at Rf 0.31. Treatment of the synthetic conjugate with the Raney nickel catalyst under the same conditions afforded 3β,5α-dihydroxycholestane as the major product which was identified by tlc on silica gel plates as well as by gas-chromatography-mass spectrometry as its di-trimethylsilyl derivative (Fig. 2).

These results reasonably indicate both biologically formed and synthetic conjugates to have the same 5α-hydroxy-6β-*S*-glutathione configuration (Fig. 3).

Stereochemistry of the ring opening of the oxirane caused by the attack of a nucleophile on 5α,6α-epoxysteroids has been known to proceed in concerted manner to yield 6β-substituted 5α-hydroxysteroids stereospecifically with no concomitant formation of 6α-hydroxy isomer (15, 16). The angular C_{10}-methyl group has a remarkable steric hindrance effect on the vicinal 5β-position (axial) so that it may not permit to afford 5β-substituted 6α-hydroxysteroids on the ring opening of 5α,6α-epoxides. The stereospecific introduction of the sulfhydryl anion of

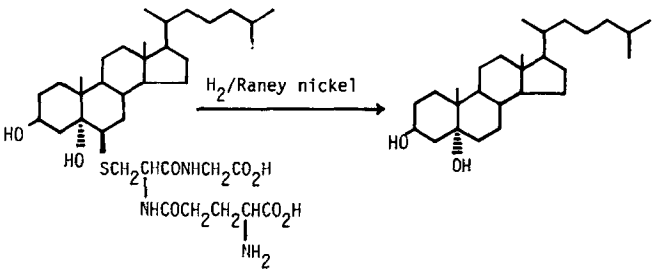

Fig. 3. Formation of 3β,5α-dihydroxycholestane from biologically formed or synthetic glutathione conjugate by the desulfrization with Raney nickel in hydrogen.

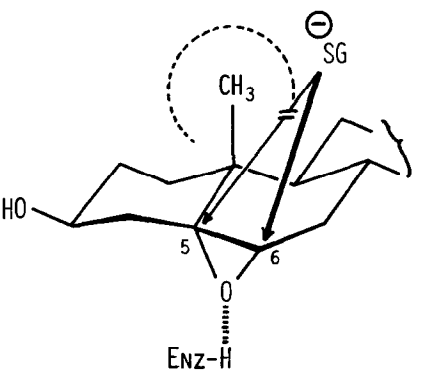

Fig. 4. Stereospecific introduction of sulfhydryl anion of glutathione to 6β-position of cholesterol α-epoxide on the interaction with epoxide *S*-glutathione transferase. Enz-H represents a possible participation of a dissociating hydrogen of the enzyme molecule to form a hydrogen bonding to the oxirane in the interaction with the substrate.

glutathione to the 6β-position of cholesterol α-epoxide, therefore, could be the sole way to form the conjugate both in chemical and enzymatic reactions (Fig. 4).

Further investigation whether or not the metabolic glutathione conjugation of cholesterol α-epoxide occurs *in vivo* is now in progress in our laboratory.

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