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REGIOSELECTIVE GLUTATHIONE CONJUGATION OF THE CARCINOGEN, 7,12-DIHYDROXYMETHYLBENZ[a]ANTHRACENE, VIA REACTIVE 7-HYDROXYMETHYL SULFATE ESTER IN RAT LIVER CYTOSOL

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SUMMARY. Potent mutagenicity of 7,12-dihydroxymethylbenz[a]anthracene (DHBA) toward *Salmonella typhimurium* TA 98 in the presence of rat liver cytosol fortified with 3'-phosphoadenosine 5'-phosphosulfate (PAPS) was completely retarded by the addition of glutathione (GSH). The reactive and intrinsically mutagenic metabolite, DHBA 7-sulfate, formed by hepatic cytosolic sulfotransferase disappeared from the incubation mixture by the addition of GSH. Non-mutagenic *S*-(12-hydroxymethylbenz[a]anthracen-7-yl)methylglutathione was isolated from the incubation mixture consisting of the hepatic cytosol, DHBA, PAPS, and GSH and proved to be formed by GSH *S*-transferase directly from DHBA 7-sulfate as an obligatory intermediate.

DHBA, a carcinogenic metabolite of 7,12-dimethylbenz[a]anthracene (DMBA) in rat liver microsomes (1-3), has very recently been demonstrated to be regioselectively conjugated by rat liver cytosolic sulfotransferase in the presence of PAPS to afford the highly reactive and mutagenic hydroxymethyl sulfate ester, DHBA 7-sulfate (4). The sulfate ester has been isolated, identified, and demonstrated to have potently intrinsic mutagenicity toward *Salmonella typhimurium* TA 98, decompose to DHBA with a half life of 8 min in water at pH 7.4 and 37°C and react readily with sodium ethanethiolate in methanol to yield 7-ethylthiomethyl-12-hydroxymethyl-BA (4). Furthermore, the mutagenicity test data have demonstrated that in untreated rat liver, oxidative metabolites play a much less important role than the sulfate ester in metabolic activation of DHBA. During the course of our investigation on the mutagenicity of DHBA toward TA 98 in the presence of rat liver cytosol fortified with PAPS, the authors found GSH retard the mutagenicity markedly. The present communication deals with the enzymatically facile retardation by GSH of the mutagenicity exerted by metabolically formed DHBA 7-sulfate in rat liver cytosol and with the isolation and

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identification of a GSH conjugate formed from the sulfate ester as an obligatory intermediate.

MATERIALS AND METHODS

Materials.

GSH with purity higher than 99.8% and free from GSSG was donated from Yamanouchi Pharmaceutical Co., Ltd., Tokyo. DHBA and DHBA 7-sulfate (Na) were synthesized as previously reported (4). PAPS was biosynthesized as previously reported (5). *Salmonella typhimurium* TA 98 that originated from Dr. B. N. Ames, University of California, was cultured overnight by the method of Ames *et al.* (6) and used for the mutagenicity test without storing.

Isolation and determination of conjugates.

A hepatic cytosolic fraction (35 mg protein/ml), obtained from untreated male Wistar rats, weighing 180-200 g, was dialyzed at 2°C for 12 hr against 0.1 M phosphate buffer, pH 7.4 (2500 vol.), and incubated with DHBA dissolved in dimethyl sulfoxide (DMSO) in the presence of PAPS to isolate its 7-sulfate ester. DHBA 7-sulfate was extracted as an ion-pair complex into ethyl acetate and determined by hplc as previously reported (4).

For isolation of the GSH conjugate, the incubation mixture (10 ml) containing GSH was shaken twice with ether (30 ml each), and the ether dissolved in the separated aqueous phase was removed by bubbling with a nitrogen stream. The GSH conjugate in the aqueous phase was adsorbed on an Amberlite XAD-2 column (1.5 x 10 cm) and eluted with methanol (100 ml) after the column was washed with water (100 ml). The methanolic eluate was condensed at 40°C *in vacuo* and subjected to hplc to isolate and determine the GSH conjugate.

The GSH conjugate isolated by hplc was used for the derivatization to the cysteine conjugate as follows: a porcine kidney γ-glutamyl transpeptidase (γ-GTP) preparation (7 units, Sigma Chemical Co., St. Louis, Mo.) was incubated with the GSH conjugate (2.5 mg) in 0.1 M Tris-HCl buffer, pH 8.5 (10 ml), at 37°C until it was completely hydrolyzed (1 hr), and the mixture was directly poured onto the Amberlite XAD-2 column. The resin column was eluted with methanol (100 ml) after it was washed with water (100 ml). The methanolic eluate was condensed at 40°C *in vacuo* and subjected to hplc to isolate and determine the cysteine conjugate.

Chromatography.

Hplc was carried out on a JASCO Model TWINCLE liquid chromatograph equipped serially with a JASCO Model UVIDEC-100-III uv monitor and a LDC Model 1309 fluoromonitor. Tlc was carried out on Merck F_{254} silica plates (5 x 20 cm) in *n*-butanol-acetic acid-water (4:1:2).

Spectroscopy.

Field desorption mass spectra were recorded on a Hitachi Model M-80 mass spectrometer. Uv-absorption spectra were recorded on a Hitachi Model 557 spectrophotometer.

RESULTS AND DISCUSSION

GSH completely retarded the potent mutagenicity of the carcinogen DHBA which was PAPS-dependently exerted toward *S. typhimurium* TA 98 by rat liver cytosol (Fig. 1A). From the incubation mixture without the bacteria was isola-

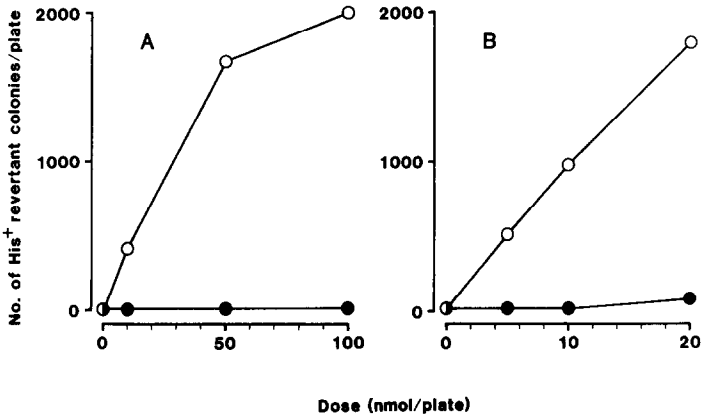

Fig. 1. Effect of GSH on Mutagenicity toward *Salmonella typhimurium* TA 98 of DHBA and Its Active Metabolite DHBA 7-Sulfate in Rat Liver Cytosol. (A) Open circles: each mixture consisting of dialyzed cytosolic fraction from untreated rat liver (50 mg), PAPS (0.6 μmol), and DHBA dissolved in DMSO (0.1 ml) was incubated with an overnight culture (0.1 ml) of TA 98 (10^8 cells) at 37°C for 20 min in a final volume of 1 ml of 0.1 M KH_2PO_4-Na_2HPO_4 buffer, pH 7.4, diluted with soft agar and poured onto a hard agar plate to count His^+ revertant colonies after incubation at 37°C for 48 hr according to the method of Ames *et al.* (6). Closed circles: the cytosolic fraction was mixed with GSH (4 mM) and then with the aforementioned ingredients. The mixture was incubated with TA 98 under the same conditions as mentioned above. (B) Open circles: sodium DHBA 7-sulfate was incubated in the absence of PAPS under the same conditions as mentioned above. Closed circles: the cytosolic fraction containing GSH (4 mM) was used for the incubation of DHBA 7-sulfate.

ted, by adsorption on an Amberlite XAD-2 column followed by hplc, a non-mutagenic polar metabolite with higher polarity than the active metabolite, DHBA 7-sulfate, which was formed in the absence of GSH (Fig. 2). The hplc study also indicated that neither DHBA 7-sulfate nor the more polar metabolite was formed when PAPS was omitted from the incubation mixture, or boiled hepatic cytosol was used.

The more polar metabolite, eluted from the hplc column, showed a ninhydrin-positive spot at Rf 0.52 in the thin-layer chromatogram which was also visualized with a uv lamp (365 nm) as a single bluish violet fluorescence spot characteristic of the 7,12-dialkyl-BA chromophor. This chromophor structure was confirmed by the uv-absorption spectrum of the eluted material; λ_{max}^{EtOH} nm (relative intensity, %): 213 (63), 238 (35), 264 (45), 275 (53), 285 (88), 296 (100), 346 (11), 362 (14), 378 (12), and 398 (5). The metabolite showed a molecular ion peak at *m/z* 577 in a field desorption mass spectrum (Fig. 3),

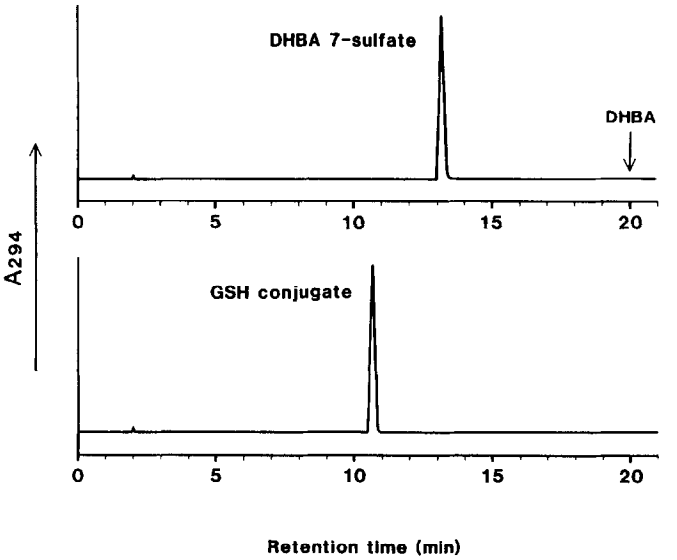

Fig. 2. Hplc of DHBA 7-Sulfate and GSH Conjugate Formed from DHBA in Rat Liver Cytosol Fortified with PAPS Alone and with PAPS and GSH. Hplc was carried out on a Nucleosil $7C_{18}$ octadecylsilicone column (7 μm in particle size, 4 mm x 30 cm) in methanol-water (16:9, 0.6 ml/min) containing 2 mM tetra-*n*-butylammonium bromide (TBA). DHBA 7-sulfate was extracted, after removal of DHBA by extraction with ethyl acetate, as a hydrophobic ion-pair complex with tetra-*n*-butylammonium cation arising from TBA, into ethyl acetate from the incubation mixture without TA 98 and GSH as previously reported (4). The GSH conjugate was isolated from the hepatic cytosolic incubation mixture containing DHBA, PAPS, and GSH as described in the text. TBA was omitted from the developing solvent to isolate the GSH conjugate for subsequent mass spectrometry; under this condition, it was eluted at a retention time (2.1 min) corresponding to the void volume of the column.

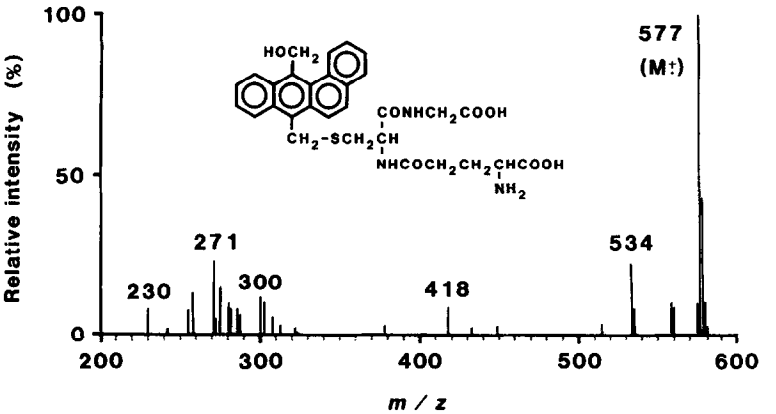

Fig. 3. Field Desorption Mass Spectrum of GSH Conjugate Formed from DHBA in Rat Liver Cytosol Fortified with PAPS and GSH. The hplc column effluent containing the GSH conjugate mentioned in Fig. 2 was used for ms. Ms was recorded at an accelerating voltage of 4 kV and an emitter current of 25-30 mA.

strongly suggesting its structure to be *S*-(hydroxymethyl-BA-yl)methylglutathione. A treatment of the GSH conjugate with a porcine kidney γ-GTP preparation which had also a potent peptidase activity led to its complete hydrolysis into a single less polar material which was also isolated on the XAD-2 column and by hplc. The less polar material was eluted at 13.0 min from the Nucleosil $7C_{18}$ column under the same conditions as described in Fig. 2 and showed a ninhydrin-positive and fluorescent spot at Rf 0.69 on the tl-chromatogram obtained under the same conditions as mentioned above and a field desorption mass spectrum with a molecular ion peak at *m/z* 391 that indicated it to be a cysteine conjugate.

DHBA 7-sulfate, a potently intrinsic mutagen, showed only a little mutagenicity toward TA 98 in the presence of GSH and hepatic cytosol (Fig. 1B). However, no appreciable retardation of the mutagenicity of the sulfate ester was observed when either hepatic cytosol or GSH was omitted from the bacterial assay system or when boiled cytosol was used in the presence of GSH. From the incubation mixture, consisting of DHBA 7-sulfate, hepatic cytosol and GSH, was isolated the polar ninhydrin-positive metabolite that was chromatographically and spectroscopically identical with that from DHBA. The structure of the non-mutagenic GSH conjugate, therefore, was reasonably elucidated to be *S*-(12-hydroxymethyl-BA-7-yl)methylglutathione.

The hplc conditions used for the isolation of the GSH conjugate formed from DHBA in the hepatic cytosolic fraction fortified with PAPS allowed to separate the isomeric conjugates *S*-(12-methyl-BA-7-yl)methyl- (7) and *S*-(7-methyl-BA-12-yl)methyl- (8) glutathiones at retention times of 20.2 and 23.8 min, respectively. Therefore, the 7-GSH conjugate from DHBA as well as from the 7-sulfate would have been homogeneous enough to contain no detectable amount of the 12-GSH conjugate which, if present, would have appeared at a longer retention time than that of the regioisomer in the hpl-chromatogram. This would also support the previously reported fact that the enzymatic sulfate conjugation of DHBA does proceed highly regioselectively to yield the reactive 7-sulfate as the sole product (4), although both carcinogenic 7-hydroxymethyl-12-methyl-BA

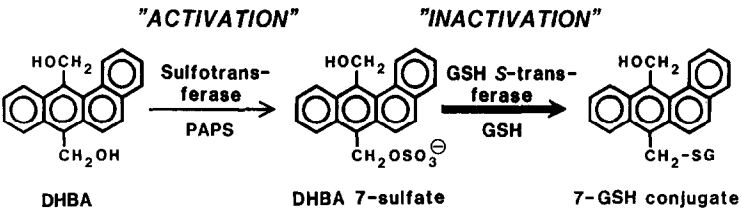

Fig. 4. Metabolic Activation of DHBA by Sulfotransferase and Inactivation of the Active Metabolite by GSH *S*-Transferase in Rat Liver Cytosol.

and 12-hydroxymethyl-7-methyl-BA have been demonstrated to be enzymatically conjugated with sulfate from PAPS and then readily with GSH through their sulfate-bearing methylene carbon with loss of sulfate anion under the same incubation conditions as used in the present investigation (8).

The conjugate formation from DHBA 7-sulfate (0.5 mM) and GSH (4 mM) also occurred non-enzymatically at pH 7.4 and 37°C only to a slight extent: $\lesssim$ 3% of the enzymatic rate (1.3 nmol/mg hepatic cytosolic protein/min). The apparent reaction rate of the enzymatic GSH conjugate formation from DHBA was 0.15 nmol/mg hepatic cytosolic protein/min, approximately equal to the previously estimated rate of the 7-sulfate formation from DHBA (4).

The present investigation indicates that the reactive sulfate ester formed regioselectively from DHBA by sulfotransferase and PAPS is readily scavenged with GSH by GSH *S*-transferase in hepatic cytosol, so that the carcinogen could not show any mutagenicity in the presence of GSH. Thus, the metabolic activation and inactivation of DHBA in hepatic cytosol are summarized as illustrated in Fig. 4.

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THE OUTER MEMBRANE OF Pseudomonas aeruginosa IS A BARRIER AGAINST THE PENETRATION OF DISACCHARIDES

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SUMMARY The outer membrane of Pseudomonas aeruginosa acted as a barrier against the penetration of di- (M_r,342), tri- (M_r,504) and tetrasaccharides (M_r,666), whereas the membrane allowed the penetration of pentose (M_r,150) and methylhexoses (M_r,194) into the periplasm. When the intact cells of P. aeruginosa were treated with 600 mosM saccharides of various sizes and observed under an electron microscope, saccharides of M_r larger than 342 caused the extensive shrinking of the outer membrane. Whereas the cells treated with the saccharides of M_r less than 194 or with sucrose in the presence of EDTA showed plasmolysis. Determination of the extent of saccharide penetration into the periplasm of the cells treated with 600 mosM sodium chloride or with 600 mosM saccharides of various sizes showed that only pentose and hexoses, so far examined, were penetrable but di-, tri- and tetrasaccharides were impenetrable.

A high incidence of the opportunistic infections to Pseudomonas aeruginosa is clinically relevant. A problem in P. aeruginosa infection is its high resistance to a number of antibiotics (1). The recent investigations revealed that the drug resistance of this organism is mainly due to the permeability barrier at the outer membrane (2,3,4). Decad and Nikaido reported that the outer membrane of P. aeruginosa allows the diffusion of polysaccharides of M_r several thousands (5), whereas Caulcott et al reported that the exclusion limit of the membrane is close to the size of disaccharides (6). The former group of the workers confirmed their own results in vitro by the liposome swelling technique (7,8). To explain the intrinsic

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Abbreviations: Na-Mg-PO_4, a buffer containing 25 mM sodium phosphate and 5mM $MgCl_2$, pH7.0; G6PDH, glucose 6-phosphate dehydrogenase.

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