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Conjugation of 7-hydroxymethyl-12-methylbenz[*a*]anthracene (7-HMBA) with glutathione via a sulphate ester in hepatic cytosol

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7-Hydroxymethyl-12-methylbenz[*a*]anthracene (7-HMBA), a carcinogenic major metabolite of 7,12-dimethylbenz[*a*]anthracene (DMBA) [1–4], has been demonstrated to be activated to a potent frame shift type of mutagen, 7-HMBA sulphate, by rat liver cytosolic sulphotransferase [5]. The mutagenic activity of 7-HMBA which was exerted by the hepatic 105,000 *g* supernatant fraction (S105) in the presence of a PAPS-generating system (ATP, sulphate and magnesium ions) was much higher than that exerted by the hepatic 9000 *g* supernatant fraction (S9) in the presence of an NADPH-generating system [5]. This strongly suggests that in rat liver sulphate conjugation may play an important role in the metabolic activation of 7-HMBA rather than epoxidation. In addition, the mutagenicity of DMBA was exerted to a greater extent by the addition of the PAPS-generating system to S9 fortified with the NADPH-generating system. The active metabolite, 7-HMBA sulphate, has been isolated as a crystalline sodium salt both from the S9-NADPH-PAPS system containing DMBA and from the S105–PAPS system containing 7-HMBA [5].

During the course of our investigation of the metabolic activation of DMBA and 7-HMBA, we found that the addition of glutathione (GSH) to the S105–PAPS system potentially inhibited the mutagenicity of the carcinogens towards *Salmonella typhimurium* TA 98. The present communication deals with (1) the metabolic inactivation of the intrinsic mutagenicity of 7-HMBA sulphate by GSH *S*-transferase in rat liver S105 and (2) the isolation and identification of a GSH *S*-conjugate formed from 7-HMBA via 7-HMBA sulphate by rat liver S105 fortified with a PAPS-generating system and GSH as well as from 7-HMBA sulphate by S105 in the presence of GSH.

The hepatic soluble supernatant fraction, S105, obtained from untreated male Wistar rats, weighing 180–200 g, was dialysed at 0–2° for 20 hr against 0.1 M phosphate buffer (2500 vol.), pH 7,4, and preincubated at 37° for 20 min with 7-HMBA in the presence of an overnight culture of *S. typhimurium* TA 98 and the PAPS-generating system. His^+ reverse mutation of TA 98 was determined by the method of Ames *et al.* [6] after the mixture was diluted with soft agar, poured onto a hard agar plate, and incubated at 37° for 48 hr. GSH (4 mM) added to the preincubation mixtures containing various amounts of S105 completely inhibited the mutagenicity exerted by 7-HMBA (Fig. 1). In the absence of GSH, 7-HMBA induced 921 His^+ revertant colonies/S105 from 50 mg liver/plate as the maximal activity. Under these conditions using S105 from 50 mg liver, the rate of biological formation of 7-HMBA sulphate determined by the previous method [5] was 53.5 nmole/mg protein/min in the absence of GSH. 7-HMBA, however, neither showed any degree of mutagenicity nor yielded the sulphate when any one of the ingredients of the PAPS-generating system, sodium sulphate, ATP and S105, was omitted from the preincubation medium. The formation of 7-HMBA sulphate was also not observed when GSH was added to the preincubation medium, suggesting that the metabolically formed mutagen was completely scavenged by GSH and GSH *S*-transferase under these conditions.

Synthetic 7-HMBA sulphate (Na) induced 1965 His^+ revertant colonies/0.1 μmole/plate as the maximal activity after 20 min preincubation with TA 98 in the presence and absence of S105 (from 50 mg rat liver). In the presence of S105, the intrinsic mutagenicity of the sulphate ester was potentially inhibited by the addition of GSH (4 mM) to the preincubation medium (Fig. 2).

Isolation of a glutathione conjugate formed from 7-

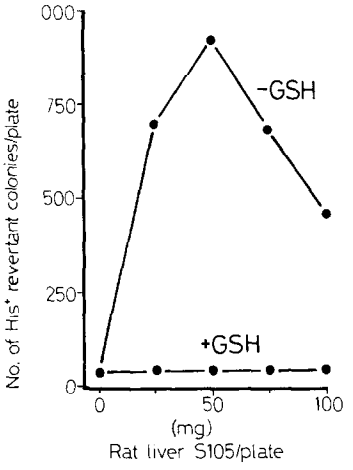

Fig. 1. Hepatic sulphotransferase-induced mutagenicity of 7-HMBA towards *Salmonella typhimurium* TA 98 in the presence and in the absence of GSH. 7-HMBA (1 μmole) dissolved in DMSO (0.1 ml) was preincubated at 37° for 20 min with S105 from various amounts of untreated rat liver and an overnight culture of *S. typhimurium* TA 98 (10^9 cells) in a final volume of 1 ml of 0.1 M phosphate buffer, pH 7.4, containing ATP (5 μmole), Na_2SO_4 (5 μmole), $MgCl_2$ (3 μmole) and EDTA (0.1 μmole). S105 obtained from 100 mg liver contained 0.96 mg protein determined by the method of Lowry *et al.* [7]. GSH (4 μmole) was added to the mixtures before the reaction was started by the addition of the carcinogen.

HMBA as well as from the sulphate ester was carried out as follows: both preincubation mixtures (50 ml each), the S105–PAPS–GSH system for 7-HMBA (1 mM) and the S105–GSH system for 7-HMBA sulphate (0.1 mM), were incubated without TA 98 under the same conditions as mentioned above. After 20 min, the mixture was extracted twice with ether (2 vol.) to remove lipid-soluble materials.

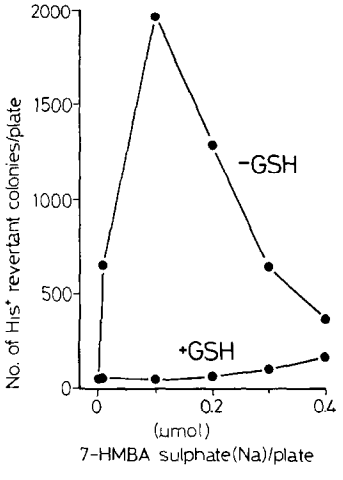

Fig. 2. Intrinsic mutagenicity of 7-HMBA sulphate towards *Salmonella typhimurium* TA 98 in the presence and in the absence of GSH. A preincubation mixture contained TA 98 (10^9 cells), various amounts of 7-HMBA sulphate (Na), S105 (from 50 mg rat liver) and GSH (4 μmole when used) in a final volume of 1 ml of 0.1 M phosphate buffer, pH 7.4. Preincubations were started by the addition of 7-HMBA sulphate (Na) and continued for 20 min at 37°.

The residual aqueous phase was filtered through a diatomaceous bed to remove coagulated protein. The filtrate was poured onto an Amberlite XAD-2 column (3 × 10 cm) after the evacuation of the remaining trace ether *in vacuo*. The column was washed successively with water (5 bed vol.) and 50% (v/v) aqueous methanol (3 bed vol.) and then eluted with methanol (3 bed vol.). The methanolic eluates obtained from both mixtures contained the same single metabolite which showed an inseparable spot when co-chromatographed on cellulose F_{254} plates (Merck, Darmstadt, F.R.G.) in various solvent mixtures, e.g. R_f 0.75, 0.50 and 0.30 in *n*-BuOH–AcOH–H_2O (4:1:2), *n*-PrOH–$CHCl_3$–H_2O (8:1:4) and *n*-BuOH–NH_4OH–H_2O (1:1:2), respectively. The metabolite in the chromatograms was visualized with a ninhydrin reagent as a glutathione-like coloured spot as well as by u.v. ray (365 nm) as an intensely bluish fluorescent spot. Ultraviolet spectra of the ninhydrin-positive metabolites formed from 7-HMBA and 7-HMBA sulphate were recorded in ethanol with the crystalline samples isolated on the XAD-2 column and found to be superimposable on each other: λ_{max}^{EtOH} nm (log ε)—235 (4.33), 265 (4.55), 274 (4.67), 284 (4.90), 294 (4.95), 344 (3.82), 359 (3.99), 375 (3.87). The spectral data strongly suggested that the metabolite has a 7-substituted-methyl-12-methylbenz[*a*]anthracene moiety in its molecule because of the similarity of the data to those for 7-HMBA. The metabolite afforded three ninhydrin-positive materials on heating in concentrated hydrochloric acid at 80° for 1 hr, which were separated on cellulose F_{254} plates: R_f 0.25 (glycine), 0.32 (glutamic acid) and 0.86 (bluish fluorescence by u.v.) in *n*-BuOH–AcOH–H_2O (4:1:2) and R_f 0.17 (glutamic acid), 0.33 (glycine) and 0.92 (bluish fluorescence by u.v.) in *n*-BuOH–NH_4OH–H_2O (1:1:2). A trifluoroacetyl-methyl derivative of the fluorescent hydrolysate was characterized as a methyl ester of *S*-[(12-methylbenz[*a*]anthryl)-7-methyl]-*N*-trifluoroacetyl-cysteine by MS: *m/e* 485 (M^+, base peak at higher *m/e* than 200), 470, 426, 416, 388, 373, 358, 314 and 255. Thus, the ninhydrin-positive metabolites formed from 7-HMBA and its sulphate ester was characterized as *S*-[(12-methylbenz[*a*]anthryl)-7-methyl]glutathione. The GSH conjugate was also chemically synthesized in 62% yield by the reaction of 7-HMBA sulphate (Na) (1 mM) with GSH (4 mM) in 0.1 M carbonate buffer, pH 9, at room temperature for 3 hr. The synthetic specimen was identical with the metabolite in all respects. The cysteine adduct was also synthesized by the reaction of the sulphate with L-cysteine under the same conditions and was chromatographically and spectroscopically identical with that obtained by the acid-treatment of the GSH conjugate.

The GSH conjugate had no intrinsic mutagenicity towards TA 98 even at 5 μmole/plate. A possibility of the direct *S*-conjugation of GSH with 7-HMBA by rat liver S105 was excluded as a result of the incubation of their mixture. The GSH conjugation of 7-HMBA sulphate also occurred nonenzymically at a slow rate at pH 7.4: 12.3 and 2.4% yields from 7-HMBA sulphate (Na) (1 mM) and GSH (4 mM) when incubated at 37° for 20 min in the presence and in the absence of S105 (from 50 mg rat liver), respectively.

The present investigation provides the first evidence for the conjugation of a xenobiotic alcohol with GSH via a metabolically formed sulphate conjugate and also for new metabolic activation and inactivation mechanisms for the promutagen, 7-HMBA or DMBA (Fig. 3). It seems to show that the suspected covalent binding of 7-HMBA sulphate to bacterial DNA takes place via the formation of 7-methylene carbonium ion with loss of sulphate anion since the GHS sulfhydryl group reacts with it to form the 7-methyl sulphide linkage.

The previously demonstrated mutagenic sulphate esters [5] of arylmethanols such as benzyl alcohol, 1-naphthalenemethanol, 2-naphthalenemethanol and 1-pyrenemethanol were all inactivated by incubation with S105 in

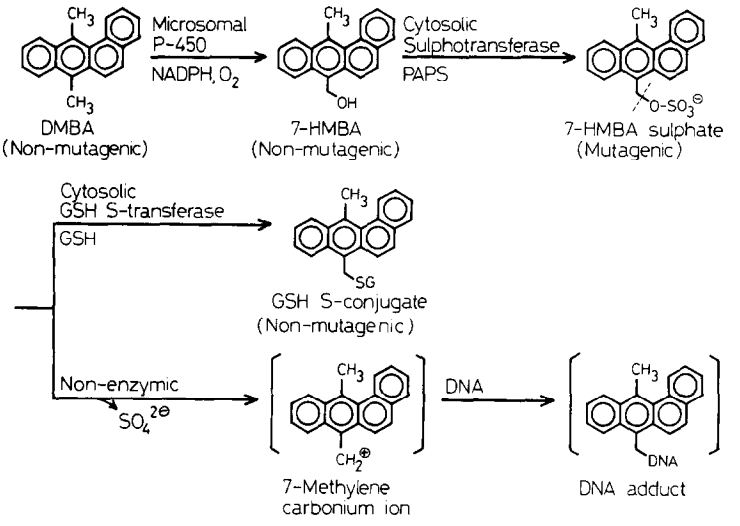

Fig. 3. Metabolic activation and inactivation of DMBA by conjugations of 7-HMBA with sulphuric acid and glutathione. GSH: glutathione.

the presence of GSH under the same conditions as mentioned above. From these incubation mixtures, the corresponding GSH conjugates were isolated and identified. These data will be published in detail elsewhere.

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