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EFFECT OF GLUTATHIONE DEPLETION ON SULFATE ACTIVATION AND SULFATE ESTER FORMATION IN RATS*

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Abstract—Sulfation of organic compounds requires activation of inorganic sulfate via formation of adenosine 3′-phosphate 5′-phosphosulfate (PAPS). Inorganic sulfate can be formed by sulfoxidation of cysteine, which can be derived from GSH. Thus, a decrease in hepatic GSH may impair formation of inorganic sulfate, the synthesis of PAPS, and the sulfation of chemicals. This hypothesis was tested by investigating the effect of GSH depletion on the levels of inorganic sulfate in serum and of PAPS in liver, and on the capacity to form the sulfate conjugate of harmol in rats. Phorone (2 mmol/kg, i.p.) decreased hepatic GSH (97%), serum inorganic sulfate (63%), and hepatic PAPS (48%). Diethyl maleate and vinylidene chloride (6 mmol/kg, each, i.p.) were less effective than phorone in decreasing GSH in liver and inorganic sulfate in serum, and they did not alter hepatic PAPS levels. Three hours after phorone treatment, the nadir of hepatic PAPS concentration, harmol was injected in order to assess sulfation *in vivo*. After administration of harmol (100 and 300 μmol/kg, i.v.), less harmol sulfate and more harmol glucuronide were found in the serum of phorone-treated rats as compared to control rats. At the higher dosage of harmol, phorone reduced the biliary excretion of harmol sulfate while increasing the biliary excretion of harmol glucuronide. These results indicate that severe GSH depletion decreases PAPS formation and sulfation of chemicals. However, an increase in glucuronidation may compensate for the impaired sulfation.

In addition to glucuronidation, sulfation is a common biotransformation reaction for phenolic compounds [1, 2]. Sulfation is catalyzed by sulfotransferases which utilize adenosine 3′-phosphate 5′-phosphosulfate (PAPS) as the "active sulfate" or cosubstrate. PAPS is synthesized from inorganic sulfate and ATP. Inorganic sulfate may originate from the diet or may be formed via sulfoxidation of cysteine [2, 3]. However, cysteine is also used for synthesis of glutathione (GSH) which, in turn, may serve as a reservoir of cysteine [4, 5]. Therefore, a deficiency in GSH may impair formation of inorganic sulfate and PAPS, and thus may affect sulfation of phenolic compounds. The present work was designed to test this hypothesis. For this purpose, the effects of GSH depletors on the concentration of inorganic sulfate in serum, the concentration of PAPS in liver, and the sulfation of a xenobiotic were determined in rats. Phorone, diethyl maleate (DEM) and vinylidene chloride were used as GSH depletors. Harmol, a metabolite of the hallucinogenic alkaloid harmaline [6], was used as a model compound for testing sulfation capacity *in vivo*. Harmol undergoes both sulfation and glucuronidation and is excreted into bile and urine as harmol-glucuronide and harmol-sulfate [7, 8].

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MATERIALS AND METHODS

Chemicals. DEM, GSH, harmol hydrochloride, PAPS, and phthalic acid potassium salt (potassium hydrogen phthalate) were purchased from the Sigma Chemical Co., St. Louis, MO. Phorone and vinylidine chloride were obtained from the Aldrich Chemical Co., Milwaukee, WI. The sources of chemicals used for analysis of GSH and PAPS have been given elsewhere [9, 10].

Animals and treatments. Male Sprague–Dawley rats, weighing 240–280 g (Sasco, Omaha, NE), were maintained in environmentally controlled rooms at 22–28° with a 12-hr light/dark cycle prior to the experiments. To minimize the supply of dietary inorganic sulfate, rats were deprived of food from 4:00 p.m. The animals were decapitated or used in the biliary excretion studies between 10:00 a.m. and 12:00 noon on the following day. Phorone (2 mmol/kg), DEM and vinylidene chloride (both 6 mmol/kg) were dissolved in corn oil and injected i.p. (2 ml/kg) at various times prior to decapitation or injected of harmol. Control rats received 2 ml/kg corn oil.

Animal experiments. For determination of GSH and PAPS in liver, and of inorganic sulfate in serum, rats were decapitated, and blood and liver were collected. Blood was centrifuged and the serum filtered through a YMT membrane filter by centrifugation (2500 *g* for 25 min) using the Micro Partition System (Amicon Corp., Danvers, MA). The resultant serum ultrafiltrate was used for determination of inorganic sulfate. Two samples were taken from the rapidly removed liver. One was homogenized in 9 vol. of 0.25 M perchloric acid and centrifuged. The resultant supernatant fraction was used for GSH analysis. The other liver sample was

heat denatured, homogenized, and centrifuged followed by a chloroform extraction of the resultant supernatant fraction as described by Hazelton *et al.* [10]. This liver extract was used for determination of hepatic PAPS concentration.

For determination of the biliary excretion of harmol, rats were anesthetized with pentobarbital (60–80 mg/kg, i.p.). The carotid artery and common bile duct were cannulated with PE-50 tubing and with a 25-gauge needle attached to PE-50 tubing, respectively, for blood and bile collection. To eliminate the effect of variations in urine production on biliary excretion and serum concentration of harmol metabolites [8], renal pedicles were ligated prior to injection of harmol. Harmol hydrochloride (100 or 300 μmol/kg) dissolved in saline (10 ml/kg) was injected over a period of 1.5 min into the saphenous vein. Following harmol injection, blood samples (180–220 μl) were taken at 2, 5, 10, 20, 30, 45 and 60 min, and bile was collected in four 15-min periods. Body temperature of the rats was maintained at 37° by means of a heating lamp. Blood and bile samples were protected from light and kept on ice during the experiment. Serum and bile samples were stored at −70° until analysis. Bile flow was determined gravimetrically taking the specific gravity of bile as one. Biliary excretion rates were calculated as the product of biliary concentration and bile flow.

Analytic methods. GSH was determined by HPLC with electrochemical detection as described by Stein *et al.* [9] using an Adsorbosphere HS (C-18, 5 μm) column (Alltech Associates, Deerfield, IL) and a mixture of 0.1 M monochloroacetic acid—3.3 mM 1-heptanesulfonic acid (pH 2.60), methanol, and *N*,*N*-dimethylformamide (96.5:3:0.5, by vol.) as the mobile phase.

The concentration of inorganic sulfate in serum was measured by HPLC using indirect photometric detection [11, 12]. This method involves measuring a decrease in eluate absorbance as sulfate displaces the UV-active component of the mobile phase (phthalate). The chromatographic system consisted of a Waters Assoc. (Milford, MA) model M-6000 pump and a model 450 variable-wavelength detector. Detector polarity was reversed to provide positive peaks for integration. Serum ultrafiltrate was injected using a 50-μl fixed-volume loop Rheodyne 7125 injector (Cotati, CA) onto a Vydac 302IC low-capacity ion-exchange column (250 × 4.6 mm, Hesparia, CA). The analytical column was protected by a direct-connect precolumn (Vydac). Ions were eluted isocratically at a 1.5 ml/min flow rate with 5 mM potassium hydrogen phthalate solution, pH 5.1, at 34°. Effluent absorbance was monitored at 293 nm. Sulfate eluted as a well-isolated peak at 6.5 min. Quantitation of serum sulfate was based on integrated peak areas. The Chromatography Applications Package (Version 1.3, IBM Instruments, Danbury, CT) operating on an IBM 9000 computer performed the integrations. Standard curves were obtained daily and were linear throughout the experimental range of values.

Hepatic PAPS concentration was determined by the method of Hazelton *et al.* [10] which measures the formation of [^{14}C]naphthyl sulfate from [^{14}C]naphthol and PAPS (limiting substrate) via a phenol sulfotransferase-catalyzed reaction.

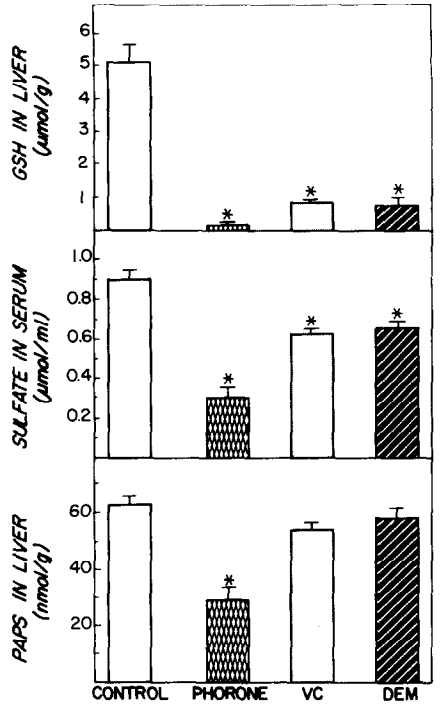

Fig. 1. Effects of GSH depletors on concentration of GSH and PAPS in liver and inorganic sulfate in serum. Rats were injected i.p. with phorone (2 mmol/kg), vinylidene chloride (VC) or DEM (both 6 mmol/kg) 3 hr prior to decapitation. Bars represent means ± SE of six rats. Asterisks indicate values significantly different ($P < 0.05$) from control.

Harmol-sulfate and harmol-glucuronide in bile and serum were separated on TLC plates (Adsorbosil-Plus soft layer prekote, Alltech Associates) which were developed in chloroform:methanol:isopropanol:ammonia (90:10:95:5) as described by Mulder and Hagedorn [7]. The separated compounds were scraped off the plate, extracted with 0.1 M HCl, and quantitated fluorimetrically using an Aminco–Bowman spectrophotofluorimeter at λ_{exc} 335 nm and λ_{em} 415 nm.

Statistics. Data were analyzed by a one-way analysis of variance. Duncan's New Multiple Range test was used to compare the means. $P < 0.05$ was considered significant.

RESULTS

Effects of GSH depletors on GSH, inorganic sulfate and PAPS. The effects of phorone, vinylidene chloride and DEM, 3 hr after administration, on hepatic GSH and PAPS levels as well as serum inorganic sulfate concentration are depicted in Fig. 1. Of the three GSH depletors, phorone was the most effective in decreasing hepatic GSH, serum sulfate and hepatic PAPS concentration. However, phorone diminished the concentration of these substances to different degrees. The largest reduction (97%) was observed in the concentration of GSH in

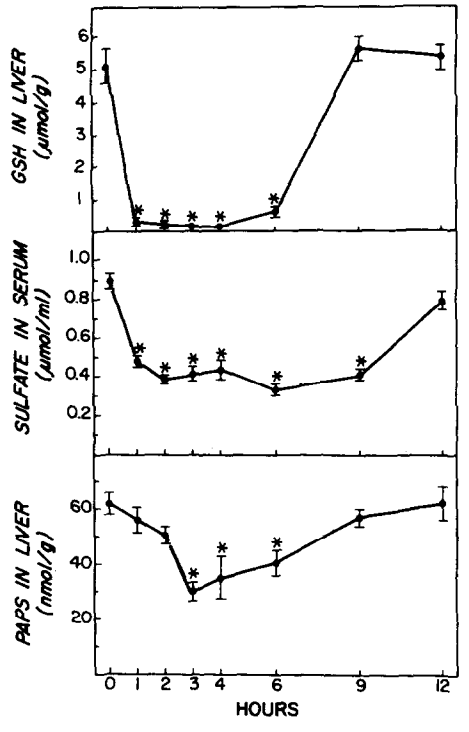

Fig. 2. Time course of effect of phorone on concentration of GSH and PAPS in liver and inorganic sulfate in serum. Rats were injected i.p. with phorone (2 mmol/kg) at 0 time. Symbols represent means ± SE of five to seven rats. Asterisks indicate values significantly different ($P < 0.05$) from values at 0 time.

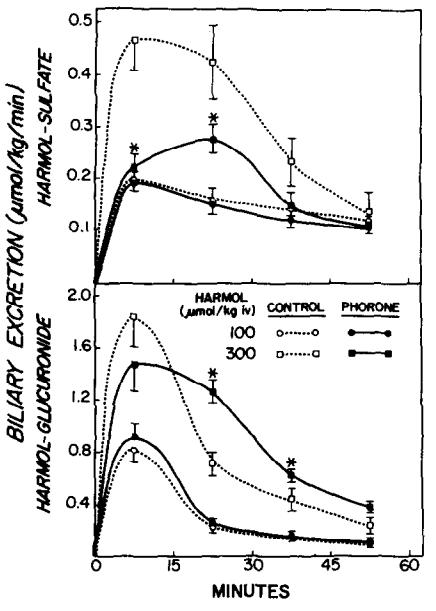

Fig. 3. Effect of phorone on biliary excretion of harmol-sulfate and harmol-glucuronide. Phorone (2 mmol/kg, i.p.) was administered to rats 3 hr prior to administration of harmol. Symbols represent means ± SE of five to six rats. Asterisks indicate values significantly different ($P < 0.05$) from control values.

liver, while serum sulfate was decreased to a lesser extent (65%), and hepatic PAPS levels were reduced the least (54%). Vinylidene chloride and DEM also lowered GSH concentrations in liver (83 and 85% respectively) and serum sulfate levels (30 and 27% respectively) but to significantly lesser degrees ($P < 0.05$) than did phorone. These agents failed to decrease hepatic PAPS concentration.

The time courses of the effect of phorone on hepatic GSH and PAPS levels and inorganic sulfate concentration in serum are shown in Fig. 2. Phorone decreased the concentration of these substances to various degrees. In addition, the time courses for reduction and recovery of GSH, sulfate and PAPS were also different. The concentration of GSH in liver decreased as much as 94% by 1 hr after phorone administration and remained at 3–12% of the pretreatment value between 1 and 6 hr following injection of phorone. Subsequently, hepatic GSH levels rapidly increased and reached control values by 9 hr. The concentration of inorganic sulfate in serum was also diminished significantly by 1 hr following phorone treatment, but to a lesser degree (46%) than was hepatic GSH. However, serum sulfate remained low (37–54% of control) until 9 hr after phorone treatment and also returned to pretreatment values by 12 hr. Although hepatic PAPS concentrations tended to be lower than the control value at 1 and 2 hr following injection of phorone, it was not until 3 hr that the reduction in PAPS concentrations in liver became significant (54%). Decreased hepatic PAPS concentrations (46–65% of control) were observed between 3 and 6 hr after administration of phorone, whereas values at 9 and 12 hr were no longer different from the controls.

Effect of phorone on sulfation and glucuronidation of harmol. At the nadir of hepatic PAPS concentration (i.e. 3 hr after phorone treatment), harmol (100 and 300 μmol/kg, i.v.) was administered, and the biliary excretion of harmol-sulfate and harmol-glucuronide was measured in renal pedicle-ligated rats (Fig. 3). The maximal biliary excretion rate of harmol-glucuronide was approximately four times higher than that of harmol-sulfate in control rats at both harmol dosages. Phorone treatment did not influence the biliary excretion of either harmol conjugate when harmol was injected at the lower dosage. However, when harmol was administered at the higher dosage, the biliary excretion of harmol-sulfate was significantly diminished in the first and second 15-min periods (53 and 35% respectively). In contrast, phorone treatment significantly increased the biliary excretion of harmol-glucuronide in the second and third bile collection period (76 and 45% respectively). The mean biliary excretion of harmol-sulfate and harmol-glucuronide over the 60 min after injection of 300 μmol/kg harmol was 19.0 and 48.5 μmol/kg, respectively, in the controls and 11.3 and 55.6 μmol/kg, respectively, in the phorone-treated rats. The total biliary excretion of harmol conjugates (sulfate plus glucuronide) in control and phorone-treated animals was not significantly different (67.5 and 66.9 μmol/kg respectively).

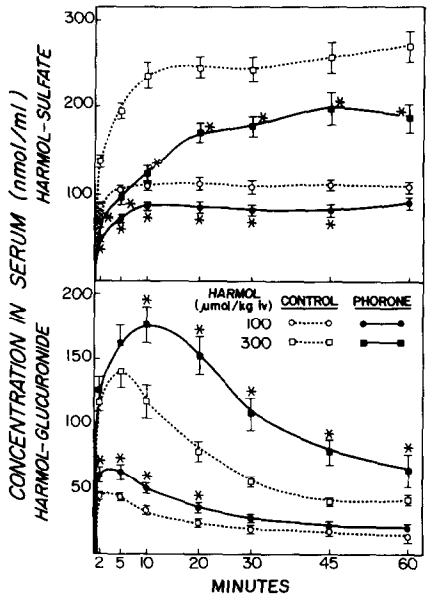

Fig. 4. Effect of phorone on concentration of harmol-sulfate and harmol-glucuronide in serum. Phorone (2 mmol/kg, i.p.) was administered to rats 3 hr prior to administration of harmol. Symbols represent means ± SE of five to six rats. Asterisks indicate values significantly different ($P < 0.05$) from controls.

The effect of phorone treatment on the time course of harmol-glucuronide and harmol-sulfate concentrations in serum after administration of harmol (100 and 300 μmol/kg, i.v.) to bile duct-cannulated, renal pedicle-ligated rats is depicted in Fig. 4. In control rats, at both harmol dosages, the peak concentrations of harmol-sulfate in serum were approximately twice as high as those of harmol-glucuronide. While the concentration of harmol-sulfate in serum was relatively constant between 10 and 60 min after injection of harmol, the concentration of harmol-glucuronide, after reaching a peak at 2–10 min, declined until the end of the observation period. Phorone-induced GSH depletion decreased harmol-sulfate levels and increased the concentration of harmol-glucuronide. Phorone treatment tended to have a greater influence on serum concentrations of harmol conjugates at the higher harmol dosage. For example, at 10 min after administration of harmol, the serum levels of harmol-sulfate were 32 and 44% lower in the rats that received 100 and 300 μmol/kg of harmol, respectively, compared to the controls, while the concentration of harmol-glucuronide was 52 and 94% higher. As a result of these opposite influences, serum concentrations of total harmol conjugates (harmol-sulfate plus harmol-glucuronide) did not appear to change significantly. For example, the mean serum concentration of harmol-sulfate and harmol-glucuronide at 20 min after injection of 300 μmol/kg harmol was 245 and 78.5 nmol/ml, respectively, in the control rats and 171 and 151 nmol/ml in the phorone-treated rats. Thus, the serum levels of total harmol conjugates (sulfate plus glucuronide) in control and phorone-treated animals were 324 and 322 nmol/ml, respectively, which do not differ significantly.

DISCUSSION

GSH depletion by all agents examined resulted in decreased inorganic sulfate concentrations in serum. In theory, there are several mechanisms by which GSH depletion could cause decreased formation of inorganic sulfate from cysteine. GSH is a reservoir of cysteine and an interaction between the liver and kidney regulates its homeostasis [13]. GSH is mainly synthesized in the liver and transported via the systemic circulation to the kidney, the main organ responsible for hydrolysis of GSH. After cysteine is released from the kidney, it is distributed to all tissues. In the liver, cysteine can be resynthesized into GSH or utilized in other synthetic (e.g. protein synthesis) or metabolic (e.g. sulfoxidation) reactions. Because sulfoxidation of cysteine results in the formation of inorganic sulfate, it is very likely that depletion of GSH causes reduced formation of inorganic sulfate due to the limited availability of cysteine from GSH. Additionally, GSH depletion may also impair hepatic uptake of cysteine from blood. It has been hypothesized [14] that uptake of cysteine from cystine, the major form of cysteine in blood plasma, is dependent on the export of GSH from liver to plasma and the subsequent thiol-disulfide exchange that liberates cysteine for uptake into liver. Depletion of GSH may also result in decreased cysteine supply by another mechanism. GSH synthesis is known to be regulated by feedback inhibition of γ-glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis [15]. Therefore, low GSH levels stimulate the synthesis of GSH, which results in increased cysteine consumption by γ-glutamylcysteine synthetase which may reduce the availability of cysteine for sulfoxidation. Our findings do not allow a conclusion as to the relative role of these various mechanisms of decreased inorganic sulfate formation during the entire time course of GSH depletion. However, at 9 hr after administration of phorone, hepatic GSH had returned to control levels whereas serum sulfate concentrations were still low (Fig. 2). This observation makes it reasonable to propose that at this particular time it is the increased utilization of cysteine for GSH synthesis that is responsible for the reduced concentration of sulfate in serum.

Phorone, the most effective GSH depletor, which is remarkably devoid of some unwanted effects such as inhibition of protein synthesis [16] and mixed-function oxidase [17], reduced serum sulfate concentrations more than the less effective GSH depletors DEM and vinylidene chloride. However, the decrease in serum sulfate produced by phorone was less than the decrease in hepatic GSH. For example, while phorone reduced hepatic GSH in liver by 89–97%, inorganic sulfate in serum was decreased by only 46–63%. This observation indicates that, even at very low hepatic GSH concentrations, sulfate levels in serum can be relatively well maintained.

Phorone decreased the hepatic PAPS levels less than it did serum sulfate concentrations (Figs. 1

and 2). Furthermore, DEM and vinylidene chloride, which were less effective than phorone in decreasing either hepatic GSH or serum sulfate concentrations, failed to decrease significantly hepatic PAPS levels (Fig. 1). These findings indicate that, during periods where hepatic GSH is depleted and serum sulfate concentrations are decreased, maintenance of hepatic PAPS concentrations is relatively well preserved.

Phorone-induced depletion of GSH resulted in decreased sulfation of harmol, as evidenced by diminished serum levels and reduced biliary excretion of harmol-sulfate in phorone-treated rats as compared to controls (Figs. 3 and 4). This observation is in accordance with the findings of Galinsky [18] who reported diminished sulfation of acetaminophen in DEM-treated rats. Impairment of harmol sulfation in phorone-treated rats is most likely due to a decreased availability of inorganic sulfate caused by GSH depletion. The importance of serum sulfate for sulfation of harmol [19], as well as acetaminophen [20] and salicylamide [21], has been reported. It has been shown in isolated perfused livers [22] and in intact rats [23] that sulfation of harmol is highly dependent on sulfate concentration in the perfusate and plasma respectively. For example, rats kept on low-protein diets exhibit reduced plasma sulfate concentrations and harmol sulfation [23, 24]. Administration of L- or D-cysteine to these rats brought about increases in serum concentration of inorganic sulfate and sulfation of harmol [24, 25]. In addition, when plasma sulfate levels were varied by infusion of sodium sulfate, it was found that half-maximal and maximal harmol sulfation rates were reached when plasma sulfate concentrations were approximately 0.3 and 1.0 μmol/ml respectively [23]. Thus, serum sulfate levels in the GSH-depleted rats in the present study fell into a range where the sulfation rate of harmol would be expected to be dependent on the concentration of sulfate in serum. Thus, the present results indicate that GSH depletion can affect the sulfation of xenobiotics via an effect on serum sulfate levels.

The inhibitory effect of GSH depletion on the formation of harmol-sulfate was more pronounced when harmol was injected at large dosages, suggesting that the restricted cosubstrate supply for sulfate is more significant when the substrate load is large. This is likely to be due to the greater cosubstrate requirement and thus greater consumption of sulfate at the larger dosage. It has been shown that acetaminophen, which also undergoes sulfation, decreases hepatic PAPS levels at high dosages [26]. These results suggest that the availability of PAPS may be rate-limiting during periods of rapid sulfation.

Impairment of harmol sulfation in phorone-treated rats was accompanied by increased glucuronidation, as indicated by elevated serum levels and enhanced biliary excretion of harmol-glucuronide (Figs. 3 and 4). An increase in the rate of harmol glucuronidation has been observed under various experimental situations when there was reduced sulfation. For example, decreased inorganic sulfate supply in the diet [22], saturation of sulfate conjugation in the isolated perfused liver [27], and treatment with inhibitors of sulfation *in vivo* [28] all reduce the formation rate of harmol-sulfate and increase the glucuronidation of harmol. Therefore, the enhanced glucuronidation of harmol in phorone-treated rats is probably the result of an increased availability of harmol for UDP-glucuronosyltransferase due to the inhibition of harmol sulfation.

In summary, the present results indicate that severe GSH depletion does compromise sulfate activation and formation of sulfate esters. However, glucuronidation may compensate for impaired sulfation.

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