Biochemical Pharmacology, Vol. 56, pp. 1391–1402, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S006-2952(98)000190–7

Enhancement of Selenium Excretion in Bile by Sulfobromophthalein: Elucidation of the Mechanism

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ABSTRACT. This work was intended to explore the mechanism whereby sulfobromophthalein (BSP), an electrophilic and cholephilic organic acid, increases the biliary excretion of selenium in rats injected with sodium [^{75}Se]selenite. In such animals, neither BSP-glutathione conjugate nor dibromosulfophthalein, nonelectrophilic congeners of BSP, enhanced the hepatobiliary transport of selenium, suggesting that reaction of nucleophilic selenite metabolites formed *in vivo* with the injected BSP may be involved. Indeed, HPLC analysis of bile from rats receiving [^{75}Se]selenite and BSP revealed two peaks (X and Y) that were simultaneously detected both by absorbance as BSP metabolites and by radioactivity as [^{75}Se] metabolites, indicating that these represent selenium-containing BSP metabolites. Pretreatment of rats with inhibitors of selenium methylation, such as periodate-oxidized adenosine (PAD) and ethionine, drastically diminished the size of peak X, while increasing (PAD) or not influencing (ethionine) the size of peak Y. This finding indicates that production of metabolite X, but not Y, is dependent on formation of methylated selenium metabolites. A compound chromatographically indistinguishable from that in peak X was formed *in vitro* during incubation of BSP with methylselenol, suggesting that biliary metabolite X is identical to the reaction product of BSP and selenite-derived methylselenol. Incubation of BSP with selenite in the presence of a thiol, namely glutathione, cysteine or *N*-acetylcysteine (which convert selenite into nucleophilic products, i.e. the respective selenopersulfides and hydrogen selenide) resulted in product(s) chromatographically identical to the biliary selenium-containing BSP metabolite(s) of peak Y, irrespective of the nature of the thiol used. Thus, biliary metabolite(s) Y may be reaction products of BSP and hydrogen selenide. Finally, BSP significantly diminished exhalation of dimethyl selenide in selenite-injected rats, purportedly because it reacted with precursors of dimethyl selenide, that include hydrogen selenide and methylselenol. In summary, BSP increases biliary excretion of selenium in rats receiving selenite because it forms selenium-containing BSP metabolites that are readily transported into bile. It is suggested that the *in vivo* reaction of nucleophilic selenite metabolites with electrophilic compounds may influence the fate of selenium and may contribute to some of the effects of this essential and anticarcinogenic metalloid. BIOCHEM PHARMACOL **56**;10:1391–1402, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. selenium; sulfobromophthalein; biliary excretion; methylation; glutathione

Selenium is a biologically important element for at least three reasons. First, it is essential for life because in the form of selenocysteine, selenium is a critical component of some crucial proteins, such as glutathione peroxidase, iodothyronine 5′-deiodinase, and thioredoxin reductase [1]. Selenium deficiency causes severe degenerative diseases, such as the Keshan cardiomyopathy, and has been implicated in increased incidence of human cancer and some chronic ailments as well as ageing [2–4]. Second, administration of selenium compounds, such as sodium selenite, protects animals against chemical carcinogens [5], heavy metals [6], arsenic [7], and other toxicants [8], and it markedly reduces the incidence of various neoplasms in humans [9, 10]. Third, selenium has a significant toxic potential and its excessive intake has caused toxic manifestations in farm animals (e.g. alkali disease and blind staggers) and humans [11–13]. As discussed in more detail later in this paper, selenite is not an inert oxyanion of selenium but is biotransformed extensively to glutathione-containing metabolites (e.g. glutathionyl selenol), hydrogen selenide, and methylated metabolites (e.g. methylselenol and dimethyl selenide). Biotransformation of selenite is important for its physiological functions, as well as its anticarcinogenic and toxic effects [13, 14].

On studying the biliary excretion of selenium in rats following injection of sodium selenite, we observed that administration of BSP§ (Fig. 1), an electrophilic organic acid that is subject to conjugation with glutathione and hepatobiliary transport in both unconjugated and conju-

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Received 9 September 1997; accepted 9 April 1998.

§ *Abbreviations*. BSP, sulfobromophthalein; GS-Se-H, glutathionyl selenol; H-Se-H, hydrogen selenide; PAD, periodate-oxidised adenosine; and SAHC, *S*-adenosylhomocysteine.

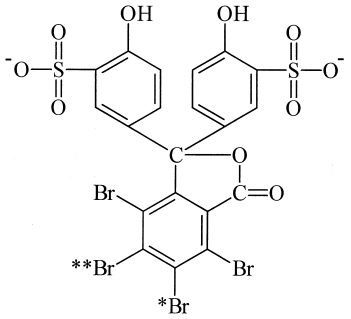

FIG. 1. The structural formula of BSP. In BSP-SG, the Br atom marked with one asterisk is thought to be substituted by glutathione, whereas in DBSP the two Br atoms marked with asterisks are substituted by H atoms.

gated forms [15, 16], increases the biliary excretion of selenium several-fold [17]. Because of the potential significance, we characterised this unexpected interaction with respect to dose- and time-dependence [17] and also designed experiments to decipher the underlying mechanism of this phenomenon. For this purpose, we examined: 1) whether nonelectrophilic derivatives of BSP, namely DBSP and BSP-SG, also enhance the biliary excretion of selenium; 2) whether BSP alters the metabolic profile of selenium in bile; 3) whether BSP reacts chemically with selenite metabolites; 4) whether prevention of the formation of methylated selenite metabolites influences the effect of BSP to increase the excretion of selenium in bile; and 5) whether BSP interferes with the formation of the major methylated selenite metabolite, i.e. the volatile dimethyl selenide. These studies indicate that direct chemical reactions take place *in vivo* between the injected BSP and both methylated and non-methylated metabolites of the injected selenite, and this reaction is proposed to account for the dramatic BSP-induced increase in selenium excretion into bile.

MATERIALS AND METHODS

Chemicals

Sodium selenite ($Na_2SeO_3 \cdot 5H_2O$), D,L-ethionine, glutathione, adenosine, acetonitrile, sodium periodate, urethane, and Molselect G-10 were purchased from Reanal. BSP was obtained from Fluka, DBSP from Société d'Etudes de Recherches Biologiques, methylselenol from Acros Chimica N.V., and tetrapentylammonium bromide from Sigma. Standards for HPLC analysis of BSP metabolites (e.g. the cysteine, *N*-acetylcysteine and glutathione conjugates of BSP) were kindly provided by Takashi Uesugi, Meiji College of Pharmacy, Tokyo, Japan. Sodium selenite labelled with [^{75}Se] (7.78 Mbq/μmol Se) was purchased from Radioisotope Centre. BSP-SG was synthesised by the method of Whelan *et al.* [15] and purified by gel filtration on Molselect G-10. PAD was prepared by incubation of adenosine with sodium periodate according to the method of Hoffman [18]. Immediately before use, a saturated aqueous solution of PAD was prepared and its PAD concentration was determined spectrophotometrically as described by Tandon *et al.* [19].

Animal Experiments

Female Wistar rats (LATI Gödöllö, Hungary) weighing 220–260 g were used for the study of the biliary excretion of selenium. Anaesthesia and surgery of the animals have been described [17]. Sodium [^{75}Se]selenite (10 μmol/kg) dissolved in saline was injected into the left saphenous vein in a volume of 3 mL/kg. The radioactivity injected was 7–15 μCi/kg when the excretion of total selenium was to be quantified; however, it was 120 μCi/kg when selenium metabolites were to be analysed by HPLC. BSP (50 μmol/kg) was injected into the right saphenous vein 1 min after selenite administration, and collection of bile samples in 20-min periods were started. Doses and times of administration of the BSP derivatives (i.e. DBSP and BSP-SG) and methylation inhibitors (i.e. ethionine and PAD) are given in the legends for Figs. 2 and 5, respectively.

Male Wistar rats weighing 260–320 g were used for the study of the biliary, urinary and pulmonary excretion of selenium. To induce urine production, these rats were hydrated by gavage of 30 mL/kg of saline containing 10 mM potassium chloride and anaesthetised by i.p. injection of a mixture of fentanyl, midazolam, and droperidol (0.045, 4.5, and 5.5 mg/kg, respectively) in a volume of 4 mL/kg. The left carotid artery was cannulated with polyethylene tubing (PE-50). A tracheotomy was performed, and the perpen-

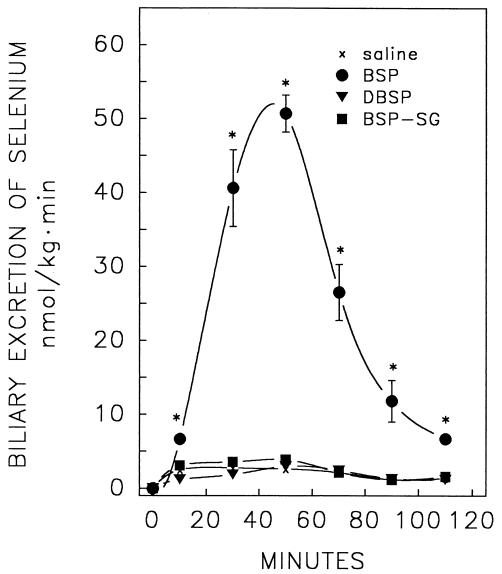

FIG. 2. Effect of BSP, DBSP, and BSP-SG on the biliary excretion of selenium. Urethane-anaesthetised, bile duct-cannulated female rats were injected with sodium [^{75}Se]selenite (10 μmol/kg, 7–15 μCi/kg) in the left saphenous vein. One minute later, saline (3 mL/kg), BSP (50 μmol/kg), DBSP (50 μmol/kg), or BSP-SG (50 μmol/kg) was injected into the right saphenous vein and collection of bile samples in 20-min periods was started. Symbols represent means ± SEM for five to six rats. Asterisks indicate values significantly different ($P < 0.05$) from the respective values of the saline-injected rats.

dicular portion of a Y-shaped cannula was inserted into the trachea and tightly secured by ligation. One of the outer branches of this trachea cannula remained open to permit inspiration of air, whereas the other branch was connected with 10-cm in length PVC tubing (inner diameter 3.0 mm, outer diameter 6.0 mm) to an apparatus constructed for entrapment of exhaled dimethyl selenide (see below) and kept under suction. Subsequently, the urinary bladder was exteriorised through a lower midline abdominal incision, and the bile duct was cannulated through an upper midline abdominal incision as described above. The rats thus prepared were administered 10 mL/kg of 10% mannitol in saline via the carotid cannula to promote urine flow and were subsequently injected with sodium [^{75}Se]selenite (10 μmol/kg, 10 μCi/kg) into the left saphenous vein and 1 min later with BSP (100 μmol/kg) into the right saphenous vein. Bile and urine samples were then collected in 20-min periods into preweighed 1.5-mL microcentrifuge tubes. To obtain urine, the urinary bladder was gently compressed manually when full and at the end of each collection period. To maintain urine flow at rates of 130–180 μL/kg · min, 3 mL/kg of 10% mannitol in saline was injected via the carotid cannula every 20 min. Throughout the experiment (100 min), the air expired by the rat was continuously aspirated from the trachea cannula through three serially connected, perpendicularly kept closed glass tubes (150 × 12 mm inner diameter, 16 mm outer diameter), each containing 5 mL of 8 M HNO_3 solution. The last of these tubes was connected to a water-jet aspirator pump, and the suction was set so that air aspirated from the trachea cannula should bubble rapidly into the HNO_3 solutions in the tubes. Nitric acid can trap the dimethyl selenide as nonvolatile dimethylhydroxyselenonium nitrate [20] and has been used to collect this volatile metabolite of selenite exhaled by animals [21, 22]. The procedure for maintenance of urinary flow and continuous urine collection during anaesthesia has also been extensively used in urinary excretion studies in rats [23].

Reaction of BSP with Selenite Metabolites In Vitro

To investigate whether possible nucleophilic metabolites of selenite react with BSP *in vitro*, BSP was incubated either with a thiol (i.e. glutathione, cysteine or *N*-acetylcysteine) plus selenite, or with synthetic methylselenol, a known metabolite of selenite.

For incubation of BSP with glutathione and selenite, 500 μL of 0.24 M glutathione solution (pH 7.4) in 0.05 M phosphate buffer (pH 7.4) and 500 μL of 0.012 M BSP in 0.05 M phosphate buffer (pH 7.4) were mixed in a 1.0-mL vial. The obtained pale violet solution was saturated with oxygen-free argon gas for 10 min. To this solution was added 200 μL of 0.06 M $Na_2SeO_3 \cdot 5H_2O$ in 0.05 M phosphate buffer (pH 7.4), which had been saturated with oxygen-free argon gas for 10 min prior to use. The vial was closed tight and kept at 37° for 1 hr. The incubation was also carried out using cysteine or *N*-acetylcysteine instead of glutathione. After incubation, the solutions were diluted appropriately with water, briefly centrifuged to remove any selenium particles formed, and the supernatant was analysed by HPLC for BSP and reaction products.

For incubation of BSP with methylselenol, 10 μL of 0.1 M aqueous BSP solution was mixed with 500 μL of 0.1 M NaOH in a 1.0 mL V-vial. The obtained solution was placed into an ice bath and saturated with oxygen-free argon gas for 10 min. Then, approximately 2 mg of methylselenol frozen in liquid nitrogen was added, and the vial was closed tight and kept at room temperature. Three hours later, the cap of the vial was removed to allow the unreacted methylselenol to escape, after which the solution was stored at 4°. Before analysis for BSP and reaction products by HPLC, the solution was diluted appropriately with water, and its pH was adjusted to approximately 8.0 with 0.05 M HCl.

Analysis

The total amount of selenium excreted in bile, urine, and exhaled air was determined by measuring the radioactivity of the collected bile and urine samples, as well as the radioactivity of the tubes containing the exhaled dimethyl selenide entrapped in 8 M HNO_3 in a well-type γ scintillation counter. Standard solutions containing a known amount of [^{75}Se]selenite were also counted to calculate the dosimetry.

Metabolites of BSP and selenite were analysed by a modified HPLC procedure of Van't Klooster *et al.* [24] using a Nova-Pak C18 (4 μm, 3.9 × 150 mm) analytical column (Waters), an absorbance detector for detection of BSP metabolites, and a Flo-One Beta series A-100 radio-chromatographic detector equipped with a γ C flow cell (300 μL) (Canberra-Packard) for detection of radioactive selenite metabolites. Gradient elution with a combined flow rate of 1 mL/min was performed using eluent A containing 45% acetonitrile and 2 mM tetrapentylammonium bromide in water and eluent B containing 60% acetonitrile, 2 mM tetrapentylammonium bromide, and 10 mM sodium sulfate in water. The elution was started with 100% A, but by minute 5 the proportion of eluents was changed linearly to 75% A, 25% B, which was maintained until minute 20. Subsequently, the proportion of B was increased linearly to 100% by minute 25, which was maintained until minute 30, when the initial condition was reestablished. A 10-min re-equilibration period was maintained between analyses. Detection of BSP metabolites by absorbance at 578 nm was made possible by alkalinisation of the column effluent, which converts these compounds into their purple quinoidal forms. This was achieved by pumping a solution containing 0.05 M NaOH and 40% acetonitrile in water with a Waters 510 pump at a rate of 0.2 mL/min into the stream of effluent coming from the column via a tee. Mixing was facilitated with a 20-cm long mixing coil after the tee. The alkalinised effluent was first directed into the absorbance detector, then into the radioactivity detector.

The delay between the detectors was 0.4 min. To deproteinise bile samples before HPLC analysis, they were mixed with 9 vols of methanol, briefly vortexed and sonicated in an ultrasonic bath, and centrifuged. The supernatant was saved and the precipitate resuspended in 20 volumes of 90% methanol and centrifuged. After removal of the supernatant, this procedure was repeated. The combined supernatants were evaporated to dryness using a SpeedVac (Savant Instruments), and the residue was stored at −20° until analysis, when it was redissolved in 10 volumes of water. The major biliary BSP metabolites were identified by comparing their elution times with those of synthetic standards.

Statistics

Data were analysed by ANOVA followed by Duncan's test with $P < 0.05$ as the level of significance.

RESULTS

Effect of BSP and its Congeners on Biliary Excretion of Selenium

To elucidate some of the structural requirements for BSP to enhance the biliary excretion of selenium, the effect of BSP on the hepatobiliary transport of selenium in rats was compared with that of its two congeners that are also readily excreted into bile, namely DBSP and BSP-SG. As shown in Fig. 2, BSP (50 μmol/kg, i.v.), injected 1 min after sodium [^{75}Se]selenite (10 μmol/kg, i.v.), significantly increased the excretion of selenium throughout the 2-hr long experiments, with the maximal selenium excretory rates in selenite plus BSP-injected rats exceeding those in selenite plus saline-injected animals as much as 20-fold. In contrast, under identical experimental conditions, neither DBSP nor BSP-SG significantly influenced the biliary excretion of selenium in rats receiving selenite (Fig. 2).

Biliary Metabolites of BSP and Selenite

To determine whether BSP-induced increase in biliary selenium output was related to altered biotransformation of BSP and/or selenite, bile was analysed for BSP and/or [^{75}Se] following injection of [^{75}Se]selenite or BSP alone and [^{75}Se]selenite and BSP in combination. Figure 3 demonstrates a representative HPLC analysis, for both BSP and [^{75}Se], of a bile sample collected from a rat 20–40 min after receiving both radioactive selenite (10 μmol/kg, 120 μCi/kg) and BSP (50 μmol/kg) i.v.

The upper chromatogram in Fig. 3 was recorded by an absorbance detector at 578 nm and represents BSP metabolites. The largest peak eluted at 6.8 min represents BSP-SG, whereas the two minor peaks that were eluted before and after the glutathione conjugate of BSP at 6.0 and 8.8 min correspond to the cysteine and cysteinylglycine conjugates of BSP, respectively. The peak that appears immediately before BSP-SG, at 6.5 min remained unidentified. It may represent a positional isomer of BSP-SG, as it has been shown that such an isomer is the second most prominent BSP metabolite in rat bile [25]. BSP, the unchanged parent compound, was eluted at 20.9 min in peak B (Fig. 3). BSP and the peaks eluting before BSP were also observed in the chromatogram of the bile sample of rats that were injected with BSP alone without selenite (not shown). However, while the analysis of bile from rats receiving BSP alone indicated no BSP metabolites eluting after BSP, the chromatogram of the bile sample collected from the rat injected with both sodium [^{75}Se]selenite and BSP revealed the presence of at least two BSP metabolites that were eluted after BSP at 22.2 and 26.2 min and are labelled X and Y, respectively (Fig. 3, upper panel).

The lower chromatogram in Fig. 3 was recorded by a radioactivity detector and represents metabolites of sodium [^{75}Se]selenite in the bile of a rat injected with radioactive selenite plus BSP. No unchanged selenite (that eluted at 2.0 min) was detected in any of the bile samples analysed. While the HPLC analysis of the bile of a rat receiving sodium [^{75}Se]selenite (10 μmol/kg, 120 μCi/kg, i.v.) alone without BSP revealed only the presence of a minor [^{75}Se] metabolite eluting at 3.2 min (not shown), similar analysis of the bile of a rat injected with both sodium [^{75}Se]selenite and BSP indicated the presence of at least two prominent [^{75}Se] metabolites eluting late in peaks X and Y (Fig. 3, lower panel). These peaks were detected by the radioactivity detector at 22.6 and 26.6 min, respectively. Because compounds were detected by the radioactivity detector 0.4 min later than by the absorbance detector, peaks X and Y in the upper and lower chromatograms in Fig. 3 represent co-eluting compounds. Thus, peaks X and Y detected by both the absorbance and the radioactivity detectors in the bile of rats receiving both radioactive selenite and BSP (Fig. 3) are indicative of the presence of selenium-containing biliary BSP metabolites.

Reaction of Selenite Metabolites with BSP

Reaction of nucleophilic selenite metabolites with BSP was tested *in vitro* in two experimental arrangements, and the reaction products were separated from BSP by HPLC and detected by absorbance under the conditions employed for analysis of biliary BSP metabolites.

The chromatogram of the incubate of synthetic methylselenol and BSP is shown in Fig. 4, upper panel. This chromatogram reveals the presence of residual BSP in the incubate that eluted in peak B as well as the appearance of two new BSP-derived products. The major product eluted immediately after BSP in the peak labelled X. When an aliquot of the incubate was mixed with an aliquot of a bile sample collected from a rat injected with selenite and BSP, the peak representing the major reaction product of methylselenol and BSP became superimposed on peak X of the bile sample (see Fig. 3, upper panel).

As will be discussed later, thiols react with selenite to form selenium-containing metabolites and hydrogen se-

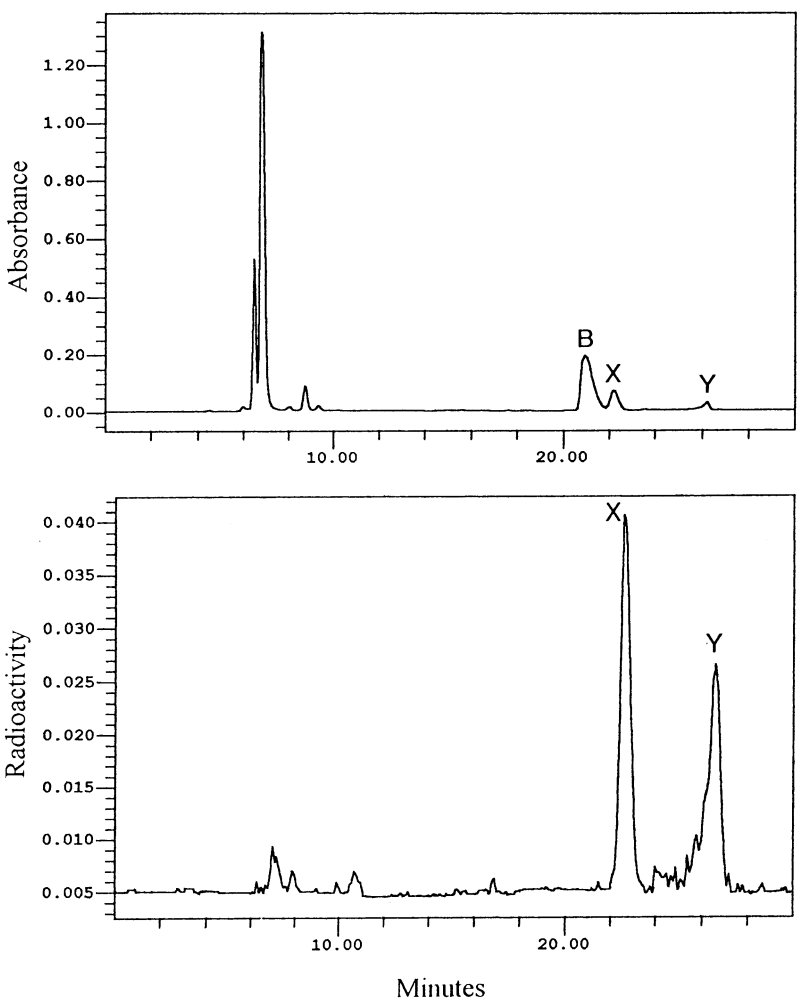

FIG. 3. Representative HPLC analysis of the bile of a rat injected with sodium [^{75}Se]selenite and BSP for BSP metabolites with absorbance detector (upper panel) and for [^{75}Se]-containing metabolites with radioactivity detector (lower panel). The female urethane-anaesthetised, bile duct-cannulated rat was injected with sodium [^{75}Se]selenite (10 μmol/kg, 120 μCi/kg) into the left saphenous vein and 1 min later with BSP (50 μmol/kg) into the right saphenous vein. The analysed bile sample was collected 20–40 min after administration of BSP. Peak B represents BSP, whereas peaks X and Y represent co-eluting compounds detected by both detectors. The largest peak appearing at 6.8 min (upper panel) represents BSP-SG. The analytical conditions are described under Materials and Methods.

lenide. To test whether these selenite metabolites are reactive with BSP, sodium selenite was incubated with BSP in the presence of a thiol, either glutathione, cysteine, or *N*-acetylcysteine. The chromatogram of the incubate of selenite, BSP, and glutathione is shown in Fig. 4, lower panel. In this chromatogram, the small peak B indicates the presence of some residual BSP, and the two large peaks indicate formation of BSP metabolites. The first of these peaks that appears at 6.8 min represents BSP-SG, which is also formed when BSP is incubated with glutathione in the absence of sodium selenite. The large peak labelled Y appeared only if the incubation mixture contained selenite as well. Peak Y was also detected when the incubation mixture contained cysteine or *N*-acetylcysteine instead of glutathione (not shown). In these latter cases, however, HPLC analysis of the incubate indicated formation of BSP-cysteine and BSP-*N*-acetylcysteine conjugates, respectively, instead of BSP-SG. Peak Y shown in the chromatogram of the incubate of BSP, selenite, and a thiol (Fig. 4, lower panel) and peak Y appearing in the chromatogram of the bile collected from a rat injected with sodium selenite and BSP (Fig. 3, upper chromatogram) had identical elution times (26.2 min) and similar asymmetric shapes.

Effect of Methylation Inhibitors on BSP-induced Increase in Biliary Selenium Excretion

Because biotransformation of selenite involves methylations, we studied the effect of PAD and ethionine, inhibitors of *S*-adenosylmethionine-dependent methylations, on the ability of BSP to enhance the biliary excretion of selenium in selenite-injected rats. In some of these animals, we also analysed the bile for BSP and selenite metabolites paying special attention to metabolites eluting in peaks X and Y, which are specific for rats receiving both selenite and BSP.

Figure 5 demonstrates the biliary excretion of selenium in control rats and in rats pretreated with either PAD or ethionine, all receiving sodium selenite (10 μmol/kg) and BSP (50 μmol/kg) i.v. Neither methylation inhibitor influenced the maximal BSP-induced increase in biliary excretion of total selenium. While PAD did not affect the time course of the BSP-stimulated selenium excretion, ethionine pretreatment slightly prolonged it, as in the declining phase of selenium excretion; i.e. 40–80 min after injection of BSP, ethionine-treated rats excreted significantly more selenium into bile than the control animals (Fig. 5).

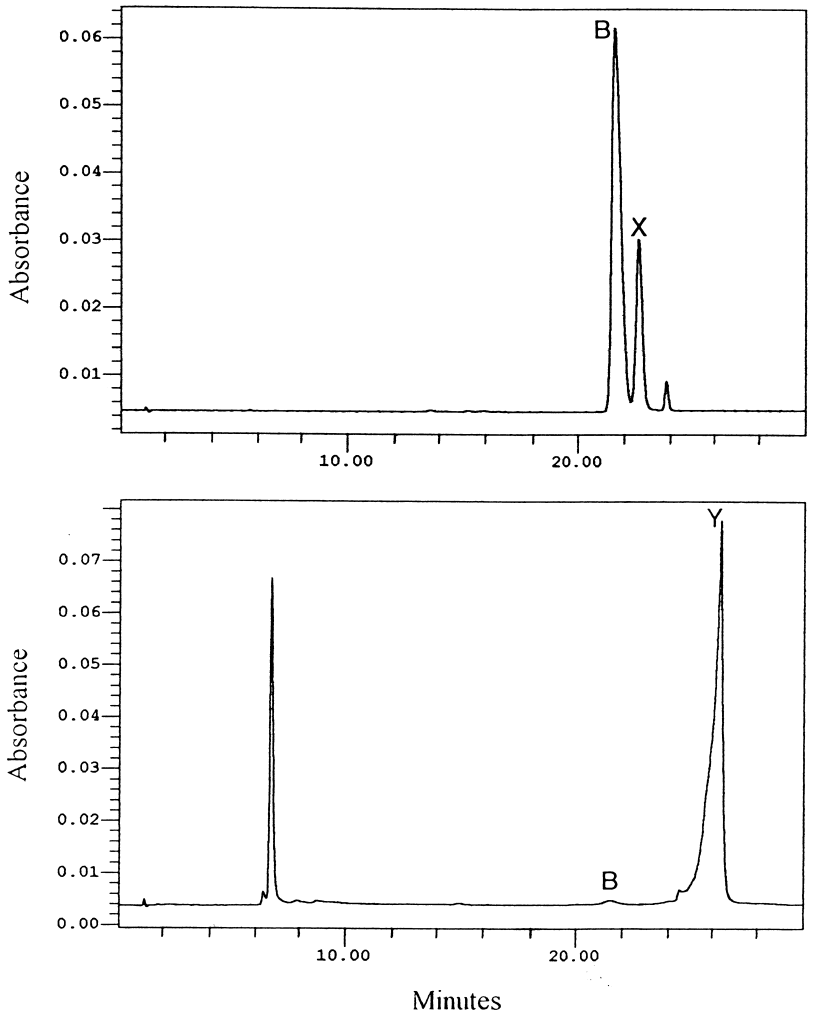

FIG. 4. HPLC analyses of the incubate of BSP and methylselenol (upper panel) and the incubate of BSP, sodium selenite and glutathione (lower panel). The conditions of incubations and HPLC analysis with absorbance detector at 580 nm are described under Materials and Methods. Peak B represents BSP, whereas peaks X and Y represent compounds that co-elute with those in the sodium [^{75}Se]selenite plus BSP-injected rat bile labelled with X and Y, respectively (see upper panel in Fig. 3). The peak appearing at 6.3 min (lower panel) represents BSP-SG.

HPLC analysis for BSP and selenite metabolites of bile from rats pretreated with PAD or ethionine and injected with sodium selenite plus BSP is demonstrated in Figs. 6 and 7, respectively. These representative chromatograms are to be compared with those showing a similar analysis of bile from a control, nonpretreated rat that are presented in Fig. 3. In all these analyses, bile extracts representing 2 μL of undiluted bile sample were injected into the HPLC; thus, corresponding peak sizes are directly comparable and are proportional to the biliary concentration of metabolites.

With the exception of peaks X and Y, other peaks corresponding to BSP and its biliary metabolites excreted by control rats remained apparently unchanged in response to pretreatment of rats with the methylation inhibitors. However, both PAD and ethionine pretreatments resulted in dramatic changes in the biliary concentration of metabolites specific for BSP plus selenite-injected rats. Compared to the control (Fig. 3), both methylation inhibitors markedly decreased the size of peak X, both in the chromatogram recorded from the absorbance detector, which specifically detects BSP metabolites (Figs. 6 and 7, upper panels), and in the chromatogram recorded from the radioactivity detector, which specifically detects sodium [^{75}Se]selenite metabolites (Figs. 6 and 7, lower panels). For example, peak X detected by absorbance in analyses of bile from PAD- and ethionine-treated rats became barely noticeable (Figs. 6 and 7, upper panels), as the areas of peak X in the chromatograms derived from these animals were diminished to 13% and 8%, respectively, of the representative control presented in Fig. 3, upper panel. In contrast, the size of peak Y, as detected either by absorbance or by radioactivity, was increased substantially after PAD treatment (Fig. 6) and was not influenced significantly after treatment with ethionine (Fig. 7) as compared to controls (Fig. 3). However, analysis of the bile of ethionine-pretreated, [^{75}Se]selenite- and BSP-injected rats revealed the appearance of a new metabolite that eluted in peak E, which was simultaneously detected by both the absorbance and the radioactivity detectors (Fig. 7). In fact, in ethionine-pretreated rats this biliary metabolite predominated among those found specifically in the bile of rats co-injected with selenite and BSP.

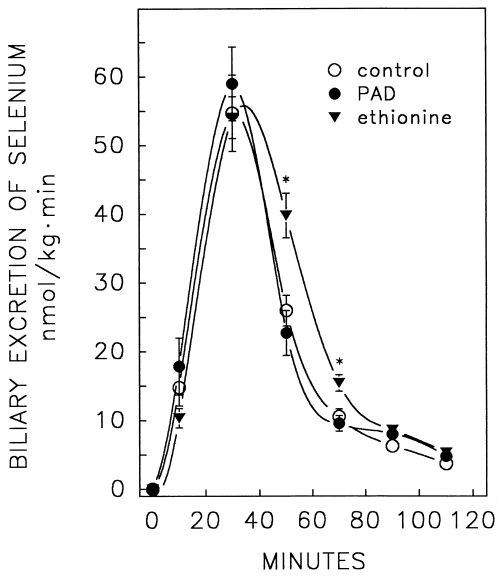

FIG. 5. Effect of the methylation inhibitors PAD and ethionine on the BSP-induced increase in biliary excretion of selenium. Urethane-anaesthetised, bile duct-cannulated female rats were injected with sodium [^{75}Se]selenite (10 μmol/kg, 7–15 μCi/kg) into the left saphenous vein. One minute later, BSP (50 μmol/kg) was injected into the right saphenous vein and collection of bile samples in 20-min periods was started. Rats received saline (5 mL/kg; control), PAD (50 μmol/kg), or ethionine (500 mg/kg) i.p. 30, 30, and 120 min before selenite administration, respectively. Symbols represent means ± SEM for five rats. Asterisks indicate values significantly different ($P < 0.05$) from the respective values of control rats.

Effect of BSP on Biliary and Urinary Excretion of Selenium and Exhalation of Dimethyl Selenide

To investigate whether BSP also affects the urinary excretion of selenium and especially formation of the volatile dimethyl selenide, separate experiments were carried out on male rats injected with sodium [^{75}Se]selenite (10 μmol/kg, i.v.) and BSP (100 μmol/kg, i.v.) to collect bile and urine for selenium determination and to entrap the exhaled dimethyl selenide.

This study confirmed that BSP increases both the biliary excretion rate (Fig. 8, left upper panel) and the cumulative biliary excretion of selenium several-fold (Fig. 8, right panel) and also demonstrated that it enhances the cumulative total (i.e. biliary plus urinary plus pulmonary) excretion of this metalloid by 70% (Fig. 8, right panel). In contrast, BSP affected neither the urinary excretion rate (Fig. 8, left lower panel) nor the cumulative urinary excretion of selenium (Fig. 8, right panel), while it lowered the exhalation of selenium by 36%, purportedly in the form of dimethyl selenide (Fig. 8, right panel).

DISCUSSION

This study was aimed at exploring the mechanism whereby BSP enhances the biliary excretion of exogenous selenium, a dramatic and unexpected effect reported in the preceding paper [17]. The first hint as to the nature of this mechanism was given by the observation that neither BSP-SG nor DBSP, analogs of BSP that are also rapidly excreted into bile, shares the potency of BSP to promote biliary selenium excretion (Fig. 2). An important property of BSP, that is also not shared by these congeners, is its enhanced reactivity toward nucleophilic reactants, whereby it can spontaneously react with thiol nucleophiles (e.g. glutathione) [25]. In these reactions, the thiols are thought to substitute for the bromine atom indicated in Fig. 1. In BSP-SG, substitution of the bromine atom by glutathione substantially decreases its reactivity with a second nucleophile, whereas in DBSP, the lack of two bromines precludes formation of a sufficiently electrophilic carbon atom with enhanced reactivity toward nucleophiles. Indeed, DBSP is excreted into bile unchanged [26]. Based on the observation that the electrophilic BSP can, but its nonelectrophilic congeners cannot, enhance biliary excretion of selenium in selenite-injected rats (Fig. 2), we formulated the hypothesis that reaction of BSP with nucleophilic selenite metabolites may underlie the mechanism of the BSP-induced increase in hepatobiliary transport of selenium.

Biotransformation of selenite (Fig. 9) includes glutathione-dependent reductions leading to sequential formation of diglutathionyl selenide, GS-Se-H, and H-Se-H, as well as *S*-adenosylmethionine-dependent methylations that form in sequence CH_3-Se-H, dimethyl selenide, and trimethylselenonium ion [27, 28]. Formation of H-Se-H from selenite occurs nonenzymatically in the presence of a large excess of glutathione [27]; however, this process may be facilitated *in vivo* by glutathione reductase [29, 30] and thioredoxin reductase [31] at the expense of NADPH. Thus, three highly nucleophilic metabolites are formed from selenite, namely GS-Se-H, H-Se-H and CH_3-Se-H, which are reactive with electrophiles. In fact, this property of these metabolites, i.e. the reactivity of GS-Se-H with iodoacetate [29] as well as that of H-Se-H and CH_3-Se-H with 1-fluoro-2,4-dinitrobenzene [32, 33], has been exploited for their *in vitro* entrapment in stable and analytically quantifiable forms. Therefore, the hypothesis that nucleophilic selenite metabolites react *in vivo* with BSP, another organic electrophile, was not unfounded.

To find experimental evidence for an *in vivo* reaction of BSP with selenite metabolites and involvement of such a reaction in enhancement of the hepatobiliary transport of selenium by BSP, the bile of rats injected with selenite and BSP was analysed. The analytical system permitted selective detection of the metabolites of BSP (by absorbance at 578 nm) and those of selenite (by radioactivity) simultaneously. The chromatograms revealed biliary compounds, eluted in peaks X and Y, that were detected simultaneously by both detectors (Fig. 3). Because these compounds were found only in the bile of rats receiving both selenite and BSP and because they were detected simultaneously as both BSP metabolites and selenium-containing metabolites, compounds eluted in peaks X and Y are thought to represent selenium-containing BSP metabolites.

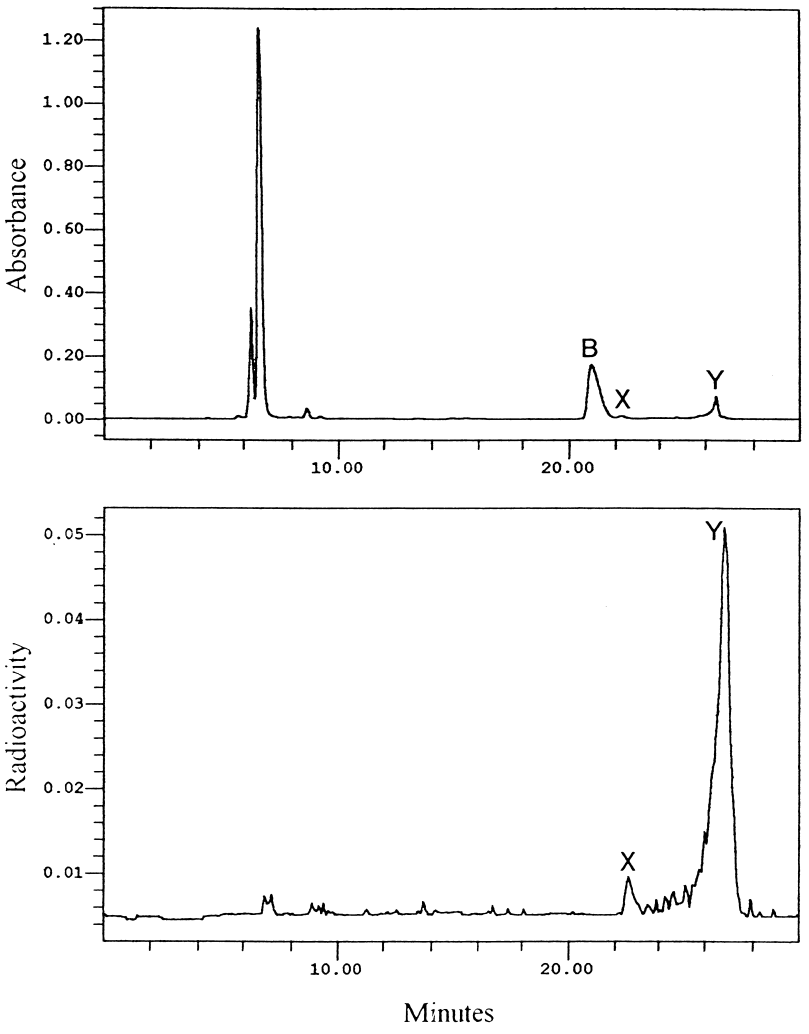

FIG. 6. Representative HPLC analysis with absorbance detector (upper panel) and radioactivity detector (lower panel) of the bile of a PAD-pretreated rat injected with sodium [^{75}Se]selenite and BSP. These chromatograms are to be compared with those in Fig. 3 showing a similar analysis of a bile sample from a control rat and where labelling of peaks are described. Experimental conditions were similar to those described under Fig. 5 with the exception of the radioactive dose of sodium [^{75}Se]selenite, which was 120 μCi/kg.

The joint metabolites of BSP and selenite formed *in vivo* could apparently be produced *in vitro* in reactions of BSP with selenite metabolites (Fig. 4). A compound with chromatographic properties identical to those of compound X in the bile was formed when BSP was incubated with CH_3-Se-H (Fig. 4, upper panel), suggesting that X in the bile of selenite- and BSP-injected rats is a product originating from reaction of the injected BSP and selenite-derived CH_3-Se-H *in vivo*. The results of experiments with inhibitors of methylation, i.e. PAD and ethionine, further support this assumption. PAD inhibits *S*-adenosylhomocysteine (SAHC) hydrolase, which eliminates SAHC produced from *S*-adenosylmethionine in methylation reactions, and results in hepatic accumulation of SAHC, a very potent inhibitor of methyltransferases [18]. Pretreatment with PAD has been shown to markedly decrease the exhalation of dimethyl selenide in selenite-injected mice [22] and rats [19], indicating that it does inhibit selenium methylation. Ethionine, an analogue of methionine, is a false substrate for *S*-adenosylmethionine synthetase [34]. Administration of ethionine to rats results in hepatic accumulation of *S*-adenosylethionine as well as SAHC and depletion of *S*-adenosylmethionine [35]. All these changes are expected to counteract selenium methylation. Pretreatment of rats with PAD or ethionine lowered the biliary concentration of the selenium-containing BSP metabolite eluted in peak X by as much as 90% (Figs. 6 and 7). This observation indicates that *S*-adenosylmethionine-dependent methylation is involved in formation of this metabolite and corroborates the finding that compound X is a reaction product of BSP and methylselenol (Fig. 4, upper panel).

Some information has also been obtained about the quality of the selenium-containing biliary BSP metabolites that eluted in peak Y (Fig. 3). The asymmetric appearance of this peak suggests that it most certainly represents elution of more than one BSP-selenium metabolite; however, their chromatographic separation have not been successful. Nevertheless, compounds with chromatographic behaviour similar to those of the biliary BSP-selenium metabolite eluting in peak Y were formed during *in vitro* incubation of BSP with sodium selenite in the presence of glutathione (Fig. 4, lower panel). Under these conditions, reaction of selenite and glutathione could produce the first three metabolites of selenious acid shown in the metabolic chart (Fig. 9). Of these, both GS-Se-H and H-Se-H could have reacted with BSP to produce glutathione- and seleni-

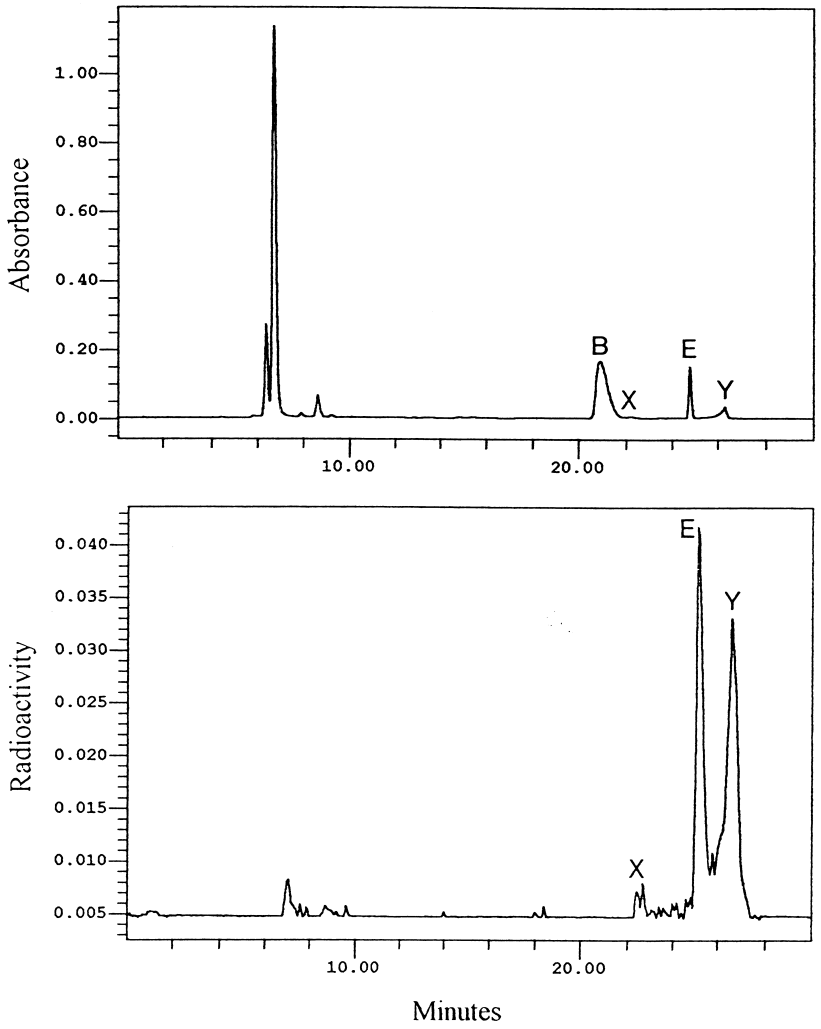

FIG. 7. Representative HPLC analysis with absorbance detector (upper panel) and radioactivity detector (lower panel) of the bile of an ethionine-pretreated rat injected with sodium [^{75}Se]selenite and BSP. These chromatograms are to be compared with those in Fig. 3 showing similar analysis of a bile sample from a control rat and where labelling of peaks are described. Peak E represents a compound prominent only in the bile of ethionine-pretreated rats injected with selenite and BSP. Experimental conditions were similar to those described under Fig. 5 with the exception of the radioactive dose of sodium [^{75}Se]selenite, which was 120 μCi/kg.

um-containing or only selenium-containing products, respectively. However, when the glutathione in the incubation mixture was replaced with other thiols, namely cysteine or *N*-acetylcysteine, HPLC analysis of the incubates indicated the presence of peak Y, with a retention time and shape identical to that obtained when glutathione was the thiol in the reaction mixture. This finding indicates that compounds eluted in peak Y (Fig. 4, lower panel) are formed irrespective of the nature of thiol included in the incubation. Therefore, it is unlikely that a thiol moiety is also present in the selenium-containing BSP metabolites of peak Y. It is also unlikely that the biliary compounds eluted in peak Y (Fig. 3) are joint metabolites of BSP and a methylated form of selenium because inhibition of selenium methylation by PAD or ethionine failed to lower but rather increased or did not influence, respectively, the biliary concentration of selenium-containing BSP metabolites of peak Y (Figs. 6 and 7). Thus, methylation of selenium does not facilitate, but rather counteracts formation of these metabolites in rats from selenite and BSP.

In ethionine-pretreated rats receiving selenite and BSP, a third and very prominent selenium-containing BSP metabolite, labelled with E, appeared in bile (Fig. 7). Although the identity of this metabolite is unknown, its formation may be related to accumulation in the liver of *S*-adenosylethionine following ethionine administration [35]. *S*-adenosylethionine may give rise to ethylselenol, which in turn may react with BSP. Further experimentation is required to substantiate such a scenario.

It is interesting to note that while the methylation inhibitors markedly altered the relative amount of selenium-containing biliary BSP metabolites in selenite plus BSP-injected rats, PAD did not change and ethionine only slightly increased the output of total selenium into bile in these animals as compared to the controls (Fig. 5). A likely explanation for this observation is that decreased formation and biliary excretion of one selenium-containing BSP metabolite (e.g. metabolite X) becomes compensated for by increased formation and excretion of other selenium-containing BSP metabolites (e.g. metabolites Y and E). This also suggests that the relative amounts of BSP-reactive selenite metabolites formed *in vivo* (e.g. methylselenol, ethylselenol, and hydrogen selenide) determine the quantities of the individual selenium-containing BSP metabolites produced.

Reaction of the *in vivo* formed selenite metabolites with

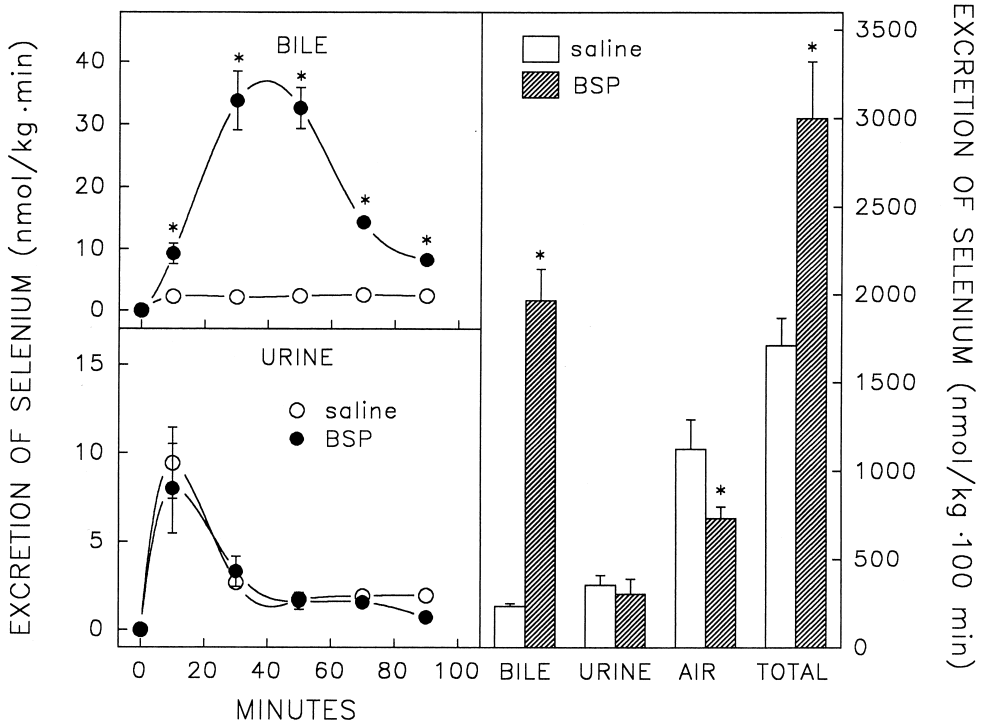

FIG. 8. Effect of BSP on excretion of selenium via bile, urine and expired air. Male bile duct-cannulated rats anaesthetised with a combination of fentanyl, droperidol, and midazolam and equipped with a trachea cannula connected to a device for entrapment of expired dimethyl selenide were injected with sodium [^{75}Se]selenite (10 μmol/kg, 10 μCi/kg) into the left saphenous vein. One minute later, saline (3 mL/kg) or BSP (100 μmol/kg) was injected into the right saphenous vein, and collection of bile and urine in 20-min periods was started. Other experimental details are described under Materials and Methods. Symbols and bars represent means ± SEM for six to eight rats. Asterisks indicate values significantly different ($P < 0.05$) from the respective values of saline-injected rats.

the injected BSP and biliary excretion of the resultant BSP-selenium metabolites seems to explain some other findings presented in this and the preceding paper [17]. For example, the observation that BSP significantly diminished exhalation of dimethyl selenide in selenite-injected rats (Fig. 8, right panel) can be accounted for by consumption of some precursors of dimethyl selenide, e.g. H-Se-H and CH_3-Se-H, in their reaction with BSP. On a similar basis, BSP would be expected to diminish formation of trimethylselenonium ion, provided this metabolite is directly formed from dimethyl selenide as indicated in Fig. 9. Because trimethylselenonium ion has been reported to be a prominent metabolite in the urine of rats collected for 24 hr following selenite administration [36], it could be expected that BSP reduces the urinary excretion of selenium. However, this did not happen (Fig. 8, left lower panel). To explain this apparent discrepancy, one may assume that early after selenite administration only a little selenium is excreted into urine as trimethylselenonium ion.

In the previous paper, we demonstrated that BSP increased the biliary excretion of selenium much more in rats injected with larger than smaller doses of selenite [17]. It has also been reported that rats given a large amount of selenite exhale disproportionately more dimethyl selenide than the animals receiving a small amount of this compound [19, 37, 38]. This latter finding indicates that production of the dimethyl selenide precursors and/or their availability for formation of dimethyl selenide is dose-dependent and is restricted at low selenite doses. Because these precursors are also thought to be the reaction partners of BSP when forming the biliary selenium-containing BSP metabolites, it is understandable that at low selenite doses the yield of such joint metabolites is lower than at high doses. The preceding paper [17] also described an unusual dose-response relationship, namely the gradual diminution of the BSP-induced initial increase in the biliary excretion of selenium at high BSP doses. It is very likely that this phenomenon reflects inhibition of the hepatobiliary transport of BSP-selenium metabolites by BSP. Because BSP is a very potent inhibitor of the hepatobiliary transport of its glutathione conjugate [16], it may also inhibit, in a dose-dependent manner, the biliary excretion of its selenium-containing metabolites, causing reduction of initial selenium excretion at higher BSP doses.

Reaction of selenite metabolites *in vivo* with inorganic electrophiles, including heavy metal ions [6], alkylmercurials [19], and the platinum complex cisplatin [39], as well as compounds of trivalent arsenic and antimony [40, 41], has been demonstrated. A similar reaction *in vivo* between metabolites of exogenous selenite and an electrophilic organic xenobiotic, however, is apparently unknown. Organic electrophiles include a number of industrial chemicals, antitumor drugs, and metabolites of numerous xenobiotics which may be mutagenic, carcinogenic, or otherwise

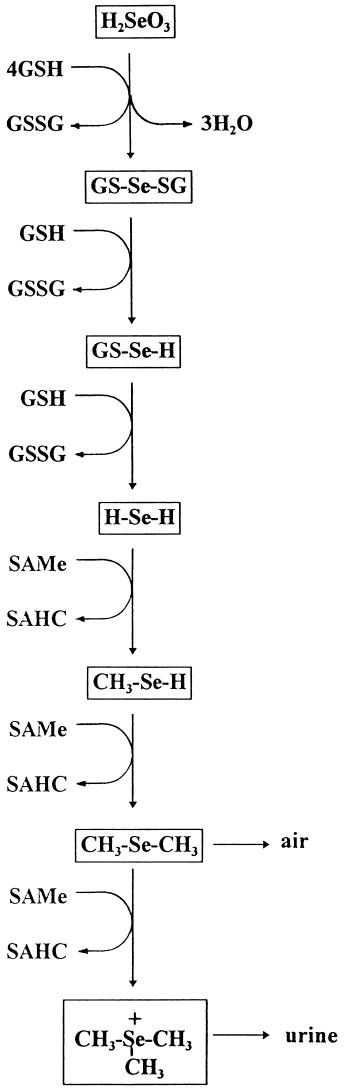

FIG. 9. Biotransformation of selenious acid (H_2SeO_3). GSH = GSH, GSSG = glutathione disulfide, SAMe = *S*-adenosylmethionine, SAHC = *S*-adenosylhomocysteine.

injurious to cells. Our observation that BSP (an organic electrophile) reacts with selenium raises the possibility that electrophiles in general are "selenophiles" and that their *in vivo* reaction with endogenous or exogenous selenium may affect the fate and/or the action of either reaction partner.

In summary, this study provides evidence that BSP, a model electrophile, reacts with nucleophilic selenite metabolites and forms selenium-containing compounds in selenite-injected rats. In these animals, BSP dramatically increases the biliary excretion of selenium, because the joint metabolites of BSP and selenite are readily transported into bile. Studies are in progress to identify the selenium-containing BSP metabolites and to determine whether the phenomenon analysed in this report can be generalised to electrophilic xenobiotics with potential toxicological significance.

This publication is based on a study supported by the Hungarian National Scientific Research Fund (OTKA T-016542), the Hungarian Ministry of Social Welfare (ETT 371/1996), and the Hungarian Academy of Sciences (AKP 97-69 3,2). The authors thank Angéla Schön for technical assistance and Katalin Gyulai and Ildikó Nagy for typing the manuscript.

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