Selenium and Arsenic Interaction in the Rat

Cecile Younan Andraos

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SELENIUM AND ARSENIC INTERACTION
IN THE RAT

by
CECILE YOUNAN ANDRAOS

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science
Major in Chemistry
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1984
SELENIUM AND ARSENIC INTERACTION
IN THE RAT

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Selenium was discovered by Berzelius and Gahn in 1817 while they were examining the sediment from a sulfuric acid plant at Gripsholm, Sweden. However, it was not until 1929 that the greatest interest in selenium and its compounds was aroused in relation to its toxic effects in the animal body. At that time, Franke (1) at the South Dakota Experiment Station and other workers (2-3) discovered that selenium was the toxic agent causing "Alkali disease", a chronic poisoning that affects livestock raised in certain areas of the Great Plains of the United States.

It was found that certain plants could take up large amounts of selenium, when present in soil, and these plants could be highly toxic to farm animals. Since this discovery, many efforts have been made to find suitable means of control and protection against selenium toxicity.

The discovery of one of the first protective factors was made by Moxon in 1938. He demonstrated the remarkable ability of arsenic to protect against selenium toxicity (4). From this original observation, a wealth of experimentation has been generated including the
demonstration that arsenic exerts its effect by increasing the biliary excretion of selenium (5).

The studies reported here are an attempt to investigate more thoroughly this metabolic antagonism between arsenic and selenium, and to characterize the forms of selenium in the bile of rats treated with arsenic.
Selenium biochemistry has been of general interest since the discovery of selenium as the toxic agent in certain plants which, when ingested by animals, cause a definite disease syndrome known as alkali disease.

Although Japha proved as early as 1842 that selenium was definitely toxic (6), it was not associated with general livestock poisoning (alkali disease) until 1931 (7). Probably the first authentic written record of selenium poisoning in livestock is the report written in 1856 by T. C. Madison, an army surgeon, stationed at Fort Randall, Territory of Nebraska (8). He described a fatal disease among the horses which had grazed in a certain area near the fort. The horses lost the long hair from the mane and tail, and their feet became so sore that they were unable to move about in search of food.

In 1891, farmers associated the disease that Madison had described with alkali seeps and water of high salt content, and thus they called it the "alkali disease". In 1912 and 1913, workers at the South Dakota Experiment Station proved that the suspected water did not cause the malady (9, 10). Even so, the name alkali disease is still often associated with the livestock
problem which is now known to be "chronic selenium poisoning".

In 1929, Franke and co-workers (1) started a series of investigations with the cooperation of the U.S. Department of Agriculture, that led to the discovery of selenium as the causative agent of the alkali disease. They concluded that soils, which have been derived from certain geological formations, contain relatively high levels of selenium. The element is partially available and absorbed by plants. In some instances, these plants are toxic when consumed by animals.

The chronic type of selenium poisoning is predominant in South Dakota and in other states when seleniferous soils, containing up to approximately 25.0 ppm of selenium, are farmed extensively. Dullness and lack of vitality are general signs of selenium toxicity. The animals become emaciated, stiff, lame, and fail to respond to good care and selenium-free feed. A prominent sign in horses and mules is the loss of the long hair from the mane and tail and hence the name, "bobtailed disease". In many species, soreness of the feet occurs with swelling at the coronary band. In severe cases, a gradual separation of the wall of the hoof occurs below the coronary band, and a new growth of the hoof starts. During the time the animals are sloughing the old hooves
and growing new ones, they are very lame and often are in severe pain. Usually they do not move about much, and unless feed and water are within easy reach, death may result from starvation and thirst (3, 11). Also, as a result of placental transmission of selenium (12), animals may be born with hooves which show the effects of selenium poisoning (3, 13).

In the acute type of poisoning, there is usually a slight impairment of vision. The animal has difficulty in judging nearness of objects in its path and it may stray from the herd. Later on, the blindness usually becomes more pronounced and is accompanied, in most cases, by a depressed appetite and the desire to chew wood, bone, metal objects, etc. There is also a greater tendency to wander, often aimlessly, in circles. The last stage is characterized by various degrees of paralysis. There is evidence of abdominal pain and death results from respiratory failure (11, 14, 15).

Since the discovery of the toxic effects of selenium, several investigators have studied the metabolism of selenium in the body and the possible means to protect against its toxicity.

**Selenium metabolism:**

Little is known about the metabolism of selenium in the body. In 1936, Dudley (16) fed NaHSeO₃ or plants
containing selenium to hogs, calves, and horses and found that selenium is found mainly in their liver and kidneys in the acute cases, and in the same tissues plus the spleen in the chronic cases. It appeared that all tissues had some selenium in them. In 1937, Moxon (3) fed dogs a seleniferous corn diet and found a decreasing concentration of selenium in the following tissues: liver, kidney, pancreas, heart, spleen, lungs, muscle, intestines, brain, bone, and blood cells.

Selenium was known to be excreted mainly through the kidneys (17). In 1940, Schultz and Lewis (18) reported the lungs as a main excretory pathway, since 17-52% of a single dose of subcutaneously injected selenium as sodium selenite was excreted within 8 hours as volatile compounds in the respiratory gases. Bile was not considered a significant pathway of selenium excretion. Only 1.3-2.1% of injected selenate appeared in the bile of dogs after 7 hours (19).

Although interest in selenium metabolism has extended over several decades, very few metabolites have been identified from mammalian systems. In 1952, McConnell and Portman (20) published substantial proof that dimethyl selenide is one of the volatile products exhaled, in large amounts, by rats injected with high levels of Na₂SeO₃. In 1969, Palmer et al. (21) isolated
a major selenium metabolite from the urine of rats injected with \(^{75}\text{Se}\) selenite. The metabolite was identified as a trimethylselenonium ion in agreement with the results of Byard (22). Thirty to fifty per cent of urinary selenium in a 24 hour sample was excreted as trimethylselenonium ion after a single injection of selenite. Palmer et al. (21), suggested that it is a normal excretory product of selenite selenium since it is excreted at both high and low levels of selenium and when the selenite is administered either orally or by injection. Later it was shown that trimethylselenonium ion was a major excretory product not only from selenite but also selenate, selenomethionine, selenocystine, Se-methylselenocysteine, and seleniferous wheat (23). A second major unidentified urinary metabolite was observed and accounted for 11-28% of the total urinary selenium (23).

**Arsenic as a protective agent:**

In 1938, Moxon, studied the toxicity of selenium in combination with tellurium, arsenic, vanadium, nickel, tungsten, and molybdenum. He discovered that the addition of 5 ppm of arsenic (NaAsO\(_2\)) to the drinking water gave full protection against liver damage in rats caused by 15 ppm of selenium in the form of seleniferous wheat (4).
Many other forms of arsenic have been tested for their ability to counteract selenium poisoning with varying results. Moxon and Dubois found that sodium arsenite and sodium arsenate were equally effective in counteracting selenium toxicity, while arsenic sulfides such as $\text{AsS}_2$ and $\text{AsS}_3$ were not (24). Organic arsenicals, neoarsphenamine, and sulfarsphenamine (formerly used as antisyphilitic drugs) were shown to be partially protective (25). In an attempt to find other forms of arsenic that would provide full protection against selenosis but be less toxic than sodium arsenite, two arsenical growth stimulants, 3 nitro-4-hydroxyphenylarsonic acid and arsanilic acid were tried. Both arsenicals provided partial protection (26). On the other hand, Hendrick et al. (27), found no protective effect from sodium methyl arsenate and calcium methyl arsenate against chronic selenium toxicity in the rat. In 1956, Leitis, et al. (28), added several organic arsenicals at a level of 15 ppm (As) to seleniferous diets. They found that triphenylarsine, arsenomethane As-1,2 disulfide, p-hydroxyphenylarsonic acid, and arsanilic acid, all protected against selenium poisoning in albino rats. The order of their effectiveness appears to be as listed above with triphenylarsine being the most effective. Dodecylamine p-chlorophenylarsonate was
shown to give some protection against selenium poisoning, but its own toxicity masked this effect (28).

Arsenic compounds have also been shown to protect against a variety of different forms of selenium. Sodium arsenite, for example, has been shown to be active against selenium as seleniferous wheat, sodium selenite, and selenocystine (24), as well as selenomethionine (29).

The protective effect of arsenic has been demonstrated in a wide variety of species including rats (4, 30), hogs (31), dogs (32), cattle (33), and in poultry (34-35).

The arsenic-selenium interaction has been also observed under a variety of conditions and doses. Moxon first showed that the administration of sodium arsenite in the drinking water (5 ppm) protected rats against the chronic toxicity of selenium (15 ppm) in the feed (4). In 1940, he proved that sodium arsenite in the diet (10 ppm) is even more effective in counteracting selenium poisoning than in the drinking water (5 ppm) when selenium (14 ppm) was fed to the rats as seleniferous wheat (24). Organic arsenicals (up to 15 ppm) added to the feed have been found to alleviate the chronic effects of selenium (10 ppm) in the rats (26-28).

There are conflicting reports on the efficacy of inorganic arsenic added to the diet of turkeys. Carlson,
et al. (36), found that arsenic as sodium arsenite was not effective either as a cure or as a prevention when added to the diet of turkeys, however, adding arsenic to the water counteracted the toxic effects of selenium in chickens (35-37). On the other hand, Frost (38), has reported that 3, 6, or 20 ppm of arsenic as arsenate in the drinking water caused a marked increase in mortality in rats administered 6 ppm of Se as selenate in the water. A similar increase in toxicity has been shown when 10 ppm of selenium as selenate and 50 ppm of arsenic as arsenate were administered in the water (39).

Inorganic arsenic (sodium arsenite) injected subcutaneously is also effective against sodium selenite administered parenterally (40-41).

Cabe, et al. (39), suggested that arsenic and selenium may be antagonistic in acute administrations and synergistic in chronic dosing. But Palmer et al. (42), suggested otherwise, "since chronic selenosis has been alleviated under such a wide variety of conditions by low levels of arsenic, it is possible that, within limits, the protective effect can be demonstrated under a wide variety of conditions, but that once the sum of the two elements exceeds these limits, their toxicities become additive, if not synergistic".

The mechanism by which arsenic detoxifies
selenium in the body has been the subject of several studies. It was first suggested that arsenic combined with selenium in the gastrointestinal tract and decreased its absorption (43). This was soon disproved by Moxon, et al. (40) based on the fact that regardless of the route of administration of either of the elements, arsenic was effective as a detoxicant. They also concluded that arsenic had no effect on exhalation, urinary or fecal excretion, or on the deposition of selenium in tissues of the rat when 1.4 mg of sodium selenite per kg body weight was administered by stomach tube.

During another study on the As-Se antagonism, where acutely toxic doses of selenite Se were used, it was noted by Kamstra and Bonhorst (41) that the breath of rats injected with selenium had the usual garlic odor associated with selenium compounds, while those injected also with arsenic did not. This led to a study where they showed that rats injected with both sodium arsenite and sodium selenite exhaled much less selenium than animals injected with sodium selenite alone (41). This was confirmed later by Ganther and Baumann (44), and Olson, et al. (45).

In 1950, it was reported (46) that at chronic toxic levels (10.3 ppm Se in corn diet), the absorption and retention of selenium did not seem to be influenced
by protective amounts of arsenite.

In 1957, Palmer and Bonhorst (47) found that arsenic definitely delays the entry of selenite into the liver with an accompanying high blood selenium level. They speculated that, since arsenite prevents exhalation of volatile selenium, it is reasonable to expect an increase in excretion by other routes, for instance urinary excretion of selenium should be increased. However, Ganther and Baumann (44) found that when selenium levels of 1.5 or 2.0 mg per kg of body weight were injected, urinary excretion of selenium was not affected by arsenite. Arsenite did markedly increase the excretion of selenium into the gastrointestinal tract from 5-15% of the injected dose in the controls to 30-40% in the aresenic treated group. Kidney levels were also increased, while levels of selenium in the blood, liver, carcass, and the expired air were decreased by arsenite.

In 1966, Levander and Baumann (48) confirmed the increased elimination of selenium into the gut in the presence of arsenic, under a variety of conditions including varied doses, forms of arsenic, and time intervals between the injections. They also demonstrated that selenite stimulated the gastrointestinal excretion of arsenic, suggesting the possibility of a detoxication conjugate involving both elements. Further, Levander and
Baumann (5) showed that the increased gastrointestinal excretion of selenium in the presence of arsenic could be accounted for by the increased biliary excretion of selenium when appropriate compounds of arsenic were also injected. These data confirmed those of McConnell and Martin (19) and Smith, et al. (49) that the rat generally excretes only minor amounts of selenium into the bile in the absence of As. The effect of arsenic on the biliary excretion of selenium has been demonstrated with several forms of selenium and arsenic, but sodium arsenite was by far the most active form of arsenic tested compared to arsenate and organic arsenicals (5). Administration of sodium dehydrocholate caused no changes in the distribution or biliary excretion of selenium in animals receiving only selenite even though bile flow was markedly enhanced. But in animals receiving both arsenite and selenite, dehydrocholate and certain other choleretics inhibited the usual increase in the biliary output of selenium brought about by arsenic (5).

In an attempt to identify selenium metabolites in the bile of arsenized rats, it was found that about 75% of the selenium in the undiluted bile could be precipitated by the addition of an equal volume of 10% trichloroacetic acid, whereas less than 5% was extractable with chloroform or ether. Dialysis of
diluted bile from rats receiving both selenium and arsenic indicated that only 10-15% of the biliary selenium was dialyzable against distilled water. The fraction of biliary selenium dialyzable was increased by raising the ionic strength (only 15% dialyzable against distilled water vs. 29% against 0.1 M NaCl) or the pH (27% dialyzed against 0.1 M phosphate buffer, pH = 6.7, vs. 68% against 0.1 M phosphate buffer, pH = 12.4) of the dialysis medium. Additional increments of biliary selenium could be rendered dialyzable by the incorporation of certain sulfhydryl compounds into the dialysis medium at a concentration of $10^{-3}$ M: control (0.1 M NaCl, pH = 7), $36.5 \pm 5.8\%$ of the selenium in bile dialyzed; control plus BAL (2, 3-dimercaptopropanol), $55.2 \pm 7.3\%$; control plus reduced glutathione, $72.8 \pm 3.1\%$ (values expressed as means of four samples ± standard error). The differences in behavior toward dialysis seen under these various conditions suggest that the selenium in bile from arsenized rats may exist in several forms, some of which are macromolecular, although their exact nature is unknown (5).

The objective of this work has been to examine more thoroughly the metabolic antagonism between selenium and arsenic and to characterize the forms of selenium in the bile of arsenized rats.
MATERIALS AND METHODS

**Apparatus:**

The following instruments and apparatus were used in this research: 1) Beckman gamma 4000 counting system; 2) Bausch and Lomb spectronic 88 spectrophotometer; 3) Waters, model 5000, high performance liquid chromatograph with U6K injector and equipped with a refractory index and ultraviolet detector; and 4) Refrigeration for Science freeze-drier.

**Materials:**

1. $\text{Na}_2\text{SeO}_3^{75}$ was purchased from ICN Chemical and Radioisotopes Division, 2) Sodium selenite and trimethylselenonium chloride were prepared in this laboratory, 3) All other reagents were analytical grade and were used as purchased, 4) Sephadex G-10, G-75, and G-200 were obtained from Pharmacia Fine Chemicals, Inc., 5) Silica gel G thin layer chromatography plates were used for all thin layer chromatography, 6) Anion exchange resin AGLX8, 200-400 mesh, was obtained from Bio-Rad Laboratories, 7) Male albino Sprague Dawley rats were used, unless otherwise indicated and were purchased from Sasco, Inc., Omaha, NE.

**Special Procedures:**

1. Care of the animals: Male albino Sprague
Dawley rats were used unless otherwise indicated. They were kept in stainless steel cages, in a temperature controlled room at 23°C and fed a regular diet of laboratory chow. Food and water were replenished every 48 hours.

2. Cannulation of the bile duct of the rat: In order to collect the bile of rats, a cannulation procedure was performed. Anaesthesia was induced by intraperitoneal injection of sodium phenobarbital (40 mg/kg body weight). The abdomen of the rat was shaved, and an incision was made along the linea alba. The common bile duct was then exposed by pulling on the duodenal loop downward and to the left. A ligature of the common bile duct was done about 7 mm from its bifurcation. A transverse cut across half of its diameter was then done above the ligature with the points of a straight fine scissors. A bevelled polyethylene tube, 0.775 mm (0.031") outside diameter, 0.275 mm (0.011") internal diameter, and 10-15 cm long, was then introduced into this opening and pushed in 1-2 cm without undue force. Bile usually filled the tube immediately. In order to anchor the catheter in the bile duct, a ligature was tied around the segment of the duct containing the catheter. The operation was then terminated by closing the peritoneum, muscle, and skin.
The tube was allowed to protrude from between two stitches.

Bile was collected at half-hour intervals for a period of at least 3 hours, during which the rat was maintained under anesthesia and was immobilized by wrapping in wire mesh. At the end of the experiment, the rat was killed by ether inhalation.

Rats were cannulated at the rate of 2 to 3 per day. Consequently, the results for a single group of five rats were obtained over a 2 to 3 day period.

3. Selenium analyses: All selenium analyses were done according to either the modification of the official fluorometric method (50) or a modification of the digesting block method of Koh and Benson (51).

4. Protein analyses: Protein was determined by the method of Lowry, et al. (52).

5. Statistical analyses: The data were analyzed statistically by analysis of variance and the means were compared using either Tukey's \( \omega \) procedure or the "t" test (53).

**Experimental Designs:**

In order to study the effect of arsenic on the biliary excretion of selenium, radioactive selenium in the form of \( \text{Na}_2^{75}\text{SeO}_3 \) (0.5 mg Se/kg) was injected subcutaneously in the hind flanks of the rats. Arsenic,
when administered, was injected subcutaneously as NaAsO₂ (1 mg As/kg), into the opposite hind flanks. Arsenic was injected 10 minutes after selenite injection unless otherwise indicated. Bile was collected, as previously described, directly into the scintillation tubes and the 75Se activity was determined. Bile samples were then kept frozen for later use.

**Experiment 1. Effect of time variation between selenium and arsenic injections on Se excretion in the bile.** Preliminary work in this laboratory showed that the percentage of selenium excreted in the bile after 3 hours was much more when arsenic was injected 10 minutes after selenium than when it was injected 10 minutes before selenium. Thus it seemed important to study the effect of timing, between selenium and arsenic injections, on the biliary excretion of selenium.

For this reason, a study was conducted using 5 groups of rats (5 rats/group). All groups were injected with Na₂⁷⁵SeO₃. The first group served as a control. The other 4 groups were injected with arsenite solution at -10, +10, +60, and +120 minutes from the selenite injection. Bile was then collected at half-hour intervals and the amount of selenium excreted was determined for a period of 3 hours.

**Experiment 2. Effect of strain and age on the**
biliary excretion of Se in arsenized rats. Results from the first experiment indicated that the amounts of selenium excreted in the bile of arsenized rats was lower than what had previously been reported by Levander and Baumann (5). It appeared that the difference in results might be attributed to either a strain or age effect. To determine whether strain might affect biliary excretion of selenium, 10 male Holtzman rats (292 to 403 g in weight) and 10 male Sprague Dawley rats (223 to 280 g) were injected with Na$_2^{75}$SeO$_3$. Ten minutes later, five rats of each strain were injected with NaAsO$_2$. Bile was then collected as previously described.

The potential effect of age was examined by injecting 10 mature male Sprague Dawley rats (365 to 560 g; 4 to 6 months old) with selenium. Ten minutes later, five of the 10 rats were injected with arsenic. The selenium excretion of these rats was compared with that of the young Sprague Dawley rats (2 to 3 months old) used in the study on strain effects.

Experiment 3. Effect of arsenic on the excretion of seleno-organic compounds. Selenium is mainly found in plants in the form of selenoamino acids and since selenomethionine and selenocystine are the forms available for animals in plants, we decided to check the effect of arsenic on their biliary excretion. Biliary
excretion of trimethylselenonion ion, a normal urinary excretion product, was also determined with or without arsenic. The study was done on 3 groups of rats (10 rats/group). Each group was injected with one of the following: 0.5 mg Se/kg as selenomethionine, 0.5 mg Se/kg as selenocystine, or 0.9 mg Se/kg as trimethylselenonion chloride. Five rats of each group were injected with 1 mg As/kg as NaAsO₂. Bile samples were collected, frozen, and analyzed later for selenium content.

**Experiment 4. Effect of delayed administration of arsenic on selenium toxicity.** Preliminary work in this laboratory showed that delayed administration of arsenic would increase selenium toxicity (54). Thus, it seemed reasonable to examine the effect of delayed administration of arsenic on selenium toxicity in greater detail and to compare this with the biliary excretion of selenium (from the first experiment).

Male albino rats weighing 209 to 300 grams were used for this study. They were kept in separate stainless steel cages and fed a stock diet ad libitum. They were fasted overnight before injection to ensure that the injection dosage was based on true body weight. Both selenite and arsenite solutions were prepared in aqueous solutions and injected intraperitoneally on
opposite sides of the linea alba. Concentrations were adjusted so that the injected volume in ml was determined by dividing body weight by 1,000. Six groups of rats were used; 3 groups were injected with Na$_2$SeO$_3$ (3 mg Se/kg) while the other 3 groups served as controls and were injected with physiological saline. One group of controls and one selenite treated group were each injected with NaAsO$_2$ (4 mg As/kg) 10 minutes before either saline or selenite injection. Another control group and selenite treated group were injected with NaAsO$_2$ 60 minutes after either saline or selenite injection. Death within 24 hours was used as the criterion of toxicity.

In order to obtain a reasonable number of rats per group (10 rats/group), animals were reused. This necessitated randomization of the animals in each experiment. In the first experiment, 6 groups of rats (6 rats/group) were used. In the second one, 6 groups of rats again were used, but this time with only 4 rats/group, to adjust for the death losses and exclusion of animals which showed signs of toxicity (decrease in weight gain). Also, the groups were reordered so that, as much as possible, new groups contained one rat from each of the previously treated groups.
Characterization of forms of selenium in the bile of rats injected with both Na$_2^{75}$SeO$_2$ and NaAsO$_2$.

a. Solubility--In an attempt to determine the solubility of the selenium compounds excreted in the bile of arsenized rats, radioactive bile samples (0.10 and 0.55 ml) were freeze-dried and the residues were extracted with 1 ml methanol or 21 ml chloroform, respectively. The amount of radioactivity in the solvent was determined and used as a measure of solubility.

b. Stability

1. Effect of acid--The stability of bile selenium compounds in acid was assessed by determining the $^{75}\text{Se}$ activity in samples of bile (20 µl and 220 µl) before and after the addition of HCl to give a final normality of 1.46 N and .26N, respectively. In the last case, the material was evaporated to dryness by blowing with nitrogen and counted again.

2. The effect of freeze-drying and of the addition of methanol--Freeze-drying the bile was used to concentrate the bile selenium compounds, thus it was important to assess the stability of selenium compounds to the freeze-drying procedure. A sample of the bile (100 µl) was counted before and after freeze-drying. One ml of methanol was then added to the residue in the scintillation tube and again the $^{75}\text{Se}$ activity was
determined.

3. The effect of temperature--Bile was collected from rats at room temperature (≈22°C), counted, and frozen immediately for later use. Samples of bile were usually recounted before use. One sample was counted, left at room temperature for 24 hours, counted again, and finally heated in a boiling water bath for a few minutes. It was recounted again to determine the effect of temperature on the selenium compounds.

4. The effect of silica gel--Since some preliminary studies showed poor recoveries from thin layer chromatography using silica gel plates, it was necessary to determine the stability of bile selenium compounds on silica gel plates. A 20 μl bile extract in chloroform was counted. Another 20 μl from the same extract was applied to a silica gel plate. The silica gel was then scraped off the plate and counted.

c. Dialysis--Dialysis was done to study the chemical nature of the selenium compounds excreted in the bile of arsenized rats. A radioactive bile sample (0.02 ml) diluted with 4 ml of 0.01 M KCl was dialyzed against 10⁻³ M reduced glutathione (GSH). After 24 hours, the GSH was replaced by another solution of GSH for another 24 hours. The amount of radioactivity in the GSH solutions was used to determine the percentage of
selenium compounds that passed through the membrane.

d. Gel filtration—An attempt was made to fractionate the radioactivity in the bile using gel filtration. Sephadex G-10, G-75, and G-200 were used successively in the order mentioned. Gels were prepared by heating with excess water in a boiling waterbath for 3 hours. The swollen gel was then packed in a column 1 X 75 cm. Blue dextran was used to determine the void volume of these columns. The columns were developed at room temperature. Fractions were collected every 2-4 minutes by means of a fraction collector attached to the column. They were then counted to determine $^{75}$Se activity. Operating conditions are shown in Table I.

e. Thin layer chromatography (TLC)—In a further attempt to fractionate and characterize the forms of selenium in the bile of arsenized rats, thin layer chromatography was used.

1. Two silica gel plates were spotted, one inch from the bottom, with both 5 and 10 μl of the bile (3,672 cpm and 7,343 cpm, respectively). The one dimensional thin layer chromatograms were developed in solutions of chloroform:methanol (3:1) and (5:1). When the solvent front reached 14.6 cm, the experiment was ended and the plates were air-dried and examined under the U.V. light. The plates were then divided into
Table I. Conditions for fractionation of bile by gel filtration

<table>
<thead>
<tr>
<th></th>
<th>Sephadex G10</th>
<th>Sephadex G75</th>
<th>Sephadex G200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bed dimensions:</td>
<td>1 x 65 cm</td>
<td>1 x 65 cm</td>
<td>1 x 66 cm</td>
</tr>
<tr>
<td>Solvent:</td>
<td>Distilled water</td>
<td>Distilled water</td>
<td>Distilled water</td>
</tr>
<tr>
<td>Flow rate drop/min:</td>
<td>8.8</td>
<td>9.5</td>
<td>a) 5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) 6</td>
</tr>
<tr>
<td>Sample*:</td>
<td>0.1 ml bile</td>
<td>0.1 ml bile</td>
<td>a) 0.1 ml bile</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(35,022 cpm)</td>
</tr>
<tr>
<td></td>
<td>(41,253 cpm)</td>
<td>(38,199 cpm)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b) 0.1 ml bile</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(114,870 cpm)</td>
</tr>
</tbody>
</table>

*Blue dextran solution (10 mg/ml) was used to determine the void volume of each column.
sections and the silica gel was scraped off the plates and counted for activity.

2. Because of the possibility that selenium might be attached to bile acids (bile salts), we decided to compare the selenium compounds in bile with sodium glycocholate, a bile salt, using TLC. A bile sample (0.55 ml-231,862 cpm) was freeze-dried. The residue was extracted with 21 ml of chloroform. Three mls (30,000 cpm) of this extract was evaporated to dryness with nitrogen and redissolved in 100 μl of chloroform. Twenty microliters of the chloroform was subjected to TLC with 20 μl of a 1% solution of sodium glycocholate. The plate was developed in petroleum ether:diethylether (87.5:12.5), air-dried, and examined under U.V. light. The 75Se activity was determined by scraping the gel off the plate from the region that contained the bile extract. The remainder of the plate was sprayed with 50% H₂SO₄ and heated to visualize the sodium glycocholate.

e. High performance liquid chromatography (HPLC)--A Water's high performance liquid chromatography fitted with an Alltech C₁₈ reversed phase column was used. Conditions were: Column: Alltech C₁₈; Detector: U.V. detector (λ = 254 nm, range = 0.32); Solvent: 80% filtered methanol (using a 0.45 micron filter); Flow rate: 1 ml/min; Chart Speed: 0.5 inch/min.
### Table II. Typical conditions for fractionation of bile metabolites on HPLC

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Extraction solvent</th>
<th>Volume applied to the HPLC</th>
<th>Amount of $^{75}\text{Se}$ applied to the HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CHCl$_3$:CH$_3$OH (2:1) and methanol</td>
<td>10 µl</td>
<td>524 cpm</td>
</tr>
<tr>
<td>2</td>
<td>Methanol</td>
<td>100 µl</td>
<td>1,903 cpm</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sample #1 after being extracted with chloroform:methanol (2:1) solution was evaporated at 40°C, redissolved in methanol, filtered, and injected into the HPLC.
Radioactive bile samples were freeze-dried in order to get the samples concentrated enough for HPLC. The residues were extracted with different solvents. Different volumes of these solutions were applied to the HPLC (see Table II). Fractions eluted corresponding to different peaks were collected manually and the $^{75}\text{Se}$ activity was determined.

f. Ion exchange chromatography--An anion exchange resin Ag 1 X 8, 200-400 mesh was used. An excess of 4.0 N HCl was first added to the column. The column was eluted with several volumes of distilled H$_2$O. A sample of radioactive bile (0.05 ml) was then applied to the column and it was eluted successively with 60 ml each of distilled water, 0.05 NHCl, 1.5 NHCl, and finally 4NHCl. Fractions eluted were collected and counted for radioactivity.
RESULTS AND DISCUSSION

Experiment 1. Effect of time variation between arsenic and selenium injections. The matter of the effect of time of arsenic injection on selenium metabolism has not been totally examined. Palmer and Bonhorst (47) injected arsenic 10 minutes before selenium in a study of the effect of arsenic on selenium toxicity and distribution. Levander and Baumann (5) injected arsenic 10 minutes after selenium in their study of the effect of arsenic on biliary excretion of selenium. Preliminary work in our laboratory showed that the amount of Se excreted in the bile, when arsenic was injected 10 minutes after Se, was more than the amount excreted, when arsenic was injected 10 minutes before selenium. So it seemed necessary to examine the problem systematically.

Rats, injected with 0.5 mg Se/kg, were divided into groups of five and injected with 1.0 mg As/kg at -10, +10, +60, or +120 minutes after the selenium. Bile was collected in 30 minute intervals from the time of the selenium injection. The results in Table III show that animals receiving no arsenic excrete only 3.9% of the injected selenium into the bile in three hours. When arsenic was injected, selenium excretion increased at least four-fold. The most effective time for arsenic
Table III. The effect of time of arsenic injection on Se excretion in the bile

<table>
<thead>
<tr>
<th>Time of arsenic injection</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
<th>150 min</th>
<th>180 min</th>
<th>210 min</th>
<th>240 min</th>
<th>Total %b of Se excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>No arsenic</td>
<td>0.3±0.1</td>
<td>0.5±0.2</td>
<td>0.7±0.3</td>
<td>0.7±0.4</td>
<td>0.7±0.3</td>
<td>0.9±0.2</td>
<td>---</td>
<td>---</td>
<td>3.9±1.61e</td>
</tr>
<tr>
<td>10 min before Se</td>
<td>6.1±2.9</td>
<td>7.7±2.3</td>
<td>3.3±1.4</td>
<td>2.2±0.7</td>
<td>1.4±0.3</td>
<td>1.4±0.5</td>
<td>---</td>
<td>---</td>
<td>22.2±7.0II</td>
</tr>
<tr>
<td>10 min after Se&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.7±2.1</td>
<td>9.4±0.8</td>
<td>5.9±1.7</td>
<td>3.5±0.7</td>
<td>2.4±0.7</td>
<td>1.9±0.4</td>
<td>---</td>
<td>---</td>
<td>32.8±3.6III</td>
</tr>
<tr>
<td>60 min after Se&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.4±0.2</td>
<td>0.7±0.2</td>
<td>13.3±2.3</td>
<td>11.3±1.2</td>
<td>5.3±1.8</td>
<td>3.2±0.7</td>
<td>1.6±0.4</td>
<td>1.5±0.3</td>
<td>34.2±3.1III</td>
</tr>
<tr>
<td>120 min after Se&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.3±0.1</td>
<td>0.6±0.3</td>
<td>0.6±0.3</td>
<td>0.6±0.3</td>
<td>9.1±2.6</td>
<td>6.9±2.1</td>
<td>3.8±0.6</td>
<td>2.0±0.4</td>
<td>18.1±5.4II</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of Se excreted of total injected per half hour intervals, mean of 5 animals ± standard error.

<sup>b</sup> Percentage of Se excreted of total injected after 3 hours of selenium injection, mean of 5 animals ± standard error.

<sup>c</sup> Since the percentage of Se excreted in the 10 min difference between Se and As injection was low (0.04±0.03), it was ignored and bile was counted as half hour intervals after As injection.

<sup>d</sup> The total percentages of selenium excreted of total injected after 4 hours from selenium injection are 37.4±2.9 and 24.1±5.9 for the +60 and +120 groups, respectively.

<sup>e</sup> Values with different Roman numerals are significantly different (P<0.05) by Tukey's w procedure.
The effect of time of arsenic injection on selenium excretion in the bile.
The numbers by each curve represent the time of arsenic injection (in minutes) relative to the selenium injection.

Fig 1
injection was within the first hour, since the groups receiving arsenic 10 minutes or 60 minutes after selenium excreted significantly higher (P<0.05) amounts of selenium 32.8% and 34.2%, respectively, than the group receiving arsenic before selenium (22.2%).

The amount of selenium excreted increased immediately after arsenic administration as shown from both the results in Table III and the graph in Fig. I. However, the rate of excretion in the first two 30 minute intervals after arsenic injection was greatest when arsenic was delayed 60 minutes compared to 10 minutes, although the overall excretion in three hours was almost the same. These data indicate that the selenium is apparently metabolized to some intermediate which accumulates in the system and which is capable of reacting with arsenic rather rapidly. This intermediate remains in the system for a considerable period of time after which it may be excreted or metabolized into another form which is not reactive with arsenic. This is shown from the results of the +120 group in which selenium excretion increased immediately after arsenic but at a slower rate than the +60 group and the amount excreted in 3 hours was lower than the other groups (P<0.05).

In any event, the data are conclusive that the
maximum response from arsenic injection occurs within the first hour after selenium injection. In the following studies, all arsenic injections were made 10 minutes after the selenium.

Experiment 2. Effect of strain and age on biliary excretion of selenium. Although the time of injection of arsenic did affect the level of selenium excreted in the bile, the maximum obtained (34% of injected dose) was still not as high as the 40% reported by Levanter and Baumann (5). Another obvious difference in the design of the two experiments was the strain of animals. Sprague Dawley rats were used in our studies versus Holtzman rats in the study of Levanter and Baumann. A study was designed to evaluate the effect of strain and also the effect of age on selenium biliary excretion.

To evaluate the effect of age, Sprague Dawley rats were maintained on stock diets an additional 2-3 months (total of 4-5 months) and then the cannulation experiment was performed. Holtzman rats were purchased to study the effect of strain.

The effect of varying the age and strain of rat on biliary excretion are shown in Table IV. The total excretion of selenium in the bile by Holtzman and Sprague Dawley rats of approximately the same age were
Table IV. Effect of strain and age on the biliary excretion of Se in arsenized rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Se excreted as percent of total injected&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sprague Dawley rats</th>
<th>Holtzman rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Young&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Old</td>
</tr>
<tr>
<td>Selenite only&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.90 ± 1.4</td>
<td>[223-280 g]</td>
<td>[365-560 g]</td>
</tr>
<tr>
<td>Selenite + arsenite&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.81 ± 3.57</td>
<td>24.69 ± 4.23&lt;sup&gt;e&lt;/sup&gt;</td>
<td>32.28 ± 8.45</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of selenium excreted of total injected after 3 hours from the last injection made. Mean of five animals ± standard error.

<sup>b</sup> Selenite dose was 0.5 mg Se/kg body weight.

<sup>c</sup> Arsenite was injected 10 minutes after selenite in a dose of 1.0 mg As/kg body weight.

<sup>d</sup> Same data are reported in the "10 minutes after Se" group in Experiment I, Table III.

<sup>e</sup> Significantly different (P < 0.05) from other values in the row by "t" test.
essentially the same (32.8% and 32.3%, respectively). Strain does not seem to be an important factor in determining the effect of arsenic on biliary excretion of selenium.

Comparing the effect of arsenic on the excretion of selenium in the bile of 2 to 3-month-old Sprague Dawley rats with that from 4 to 5-month-old animals shows that the old rats excrete significantly less (P<0.05 by "t" test) than the young rats. This is a factor that should be considered when comparing data between different workers.

It appears then from the results of Experiments 1 and 2 that young rats, injected with arsenic within the first hour after selenium infection, excrete a major amount of selenium in the bile and this agrees with the work of Levander and Baumann (5). Under the conditions of these studies, including optimization of age and time of arsenic injection, it still was not possible to obtain the level of excretion obtained by others (5).

Experiment 3. Effect of arsenic on the excretion of seleno-organic compounds. Most of the naturally occurring forms of selenium in edible plants are thought to be selenoamino acids (55). Since two of these selenoamino acid compounds, selenomethionine and selenocystine, have toxicities similar to that of
Table V. Effect of arsenic on the biliary excretion of seleno-organic compounds

<table>
<thead>
<tr>
<th>Type of methionine</th>
<th>Se excreted as percent of total injected&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Trimethyl-selenonium chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Se only</td>
<td>3.46 ± 0.99</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>Se + As&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.01 ± 3.83</td>
<td>0.38 ± 0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of Se excretion of total injected after 3 hours from last injection made. Mean of five animals ± standard error.

<sup>b</sup> Arsenite dose was 1.0 mg As/kg. Arsenite injection was 10 min. after selenium injection.
selenite [minimal lethal dose (MLD) of 4.25 and 4.0 mg Se/kg vs. 3.25-3.5 mg Se/kg, respectively] (56), it seemed reasonable to investigate the effect of arsenic on their excretion in the bile. The biliary excretion of selenium from these compounds along with that from the non-toxic urinary excretory product, trimethylselenonium ion, was studied. The results are shown in Table V.

As in the case of selenite, selenium from the two amino acids was excreted in the bile in small amounts in the absence of arsenic (less than 4%). The injection of arsenic, 10 minutes after selenomethionine or selenocystine, increased the level of selenium in the bile in three hours to 21% and 21.8%, respectively. These data indicate that at least part of the detoxification of organic selenium by arsenic is due to increased biliary excretion of selenium just as suggested for inorganic forms of the element.

Trimethylselenonium ion was excreted in only trace amounts when administered alone. The injection of arsenic increased the excretion two-fold (to 0.38%) which would seem biologically unimportant. These data support the data of Tsay, et al. (57) which demonstrated the apparent biological unavailability of the trimethylselenonium moiety.

Experiment 4. Effect of delayed administration
of arsenic on selenium toxicity. Arsenic is known to counteract toxicity of selenite selenium (24, 47). On the other hand, administration of arsenic with various methylated forms of selenium appears to potentiate the toxicity of selenium (54, 58). Preliminary experiments from this laboratory indicated that delaying arsenic administration, when selenite had been injected, would also increase selenium toxicity. Since results already reported in this thesis indicate that arsenic is effective in increasing selenium excretion in bile (detoxification) when administered up to 2 hours after selenium, it seemed important to examine further the effect of delayed arsenic administration on selenium toxicity.

The results of the toxicity experiments are shown in Table VI. No deaths were observed in the saline or saline + arsenic injected controls, and no deaths occurred in the animals injected with 3 mg Se/kg as selenite. This selenite dose is less than the reported MLD of 3.25-3.5 mg Se/kg (56) and was selected so that any potentiation effect by arsenic could be clearly seen.

Among animals injected with arsenic 10 minutes prior to selenium, two deaths occurred out of a total of 10 animals, whereas the injection of arsenic 60 minutes after selenium caused deaths in nine out of ten animals.
Table VI. Effect of delayed injection of arsenic on selenium toxicity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline groups(^a)</th>
<th>Selenite groups(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No As</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>
| 2. As\(^c\) was injected
| 10 min before Se   | 0/10                 | 2/10                  |
| 3. As\(^c\) was injected
| 60 min after Se    | 0/10                 | 9/10                  |

\(^a\) Saline groups were injected with saline instead of selenite.

\(^b\) Selenite groups were injected with 3 mg Se/kg.

\(^c\) Arsenite dose was 4 mg As/kg.
This represents a great increase in toxicity of selenium at the level of 3 mg Se/kg. These data indicate that selenium is apparently being metabolized to forms which in turn can interact with arsenic and bring about greater toxicity than that exhibited by selenite alone. This supports an earlier report, given only in abstract form (54), that delayed arsenic administration increased the toxicity of selenite. Further, it suggests that the selenium intermediates from selenite may be various methylated derivatives since arsenic is known to increase their toxicity (54, 58). It also provides concrete evidence that arsenic must be administered early to be an effective antagonist of selenium.

The deaths occurring in the group receiving arsenic 10 minutes before selenium were unexpected since previous work has shown complete protection by arsenic at this level of selenium (45). At this point, we cannot explain the toxicity at this level.

Characterization of forms of selenium in the bile. As reported, the administration of arsenic after selenium results in the excretion of over 30% of an injected selenium dose in the bile. This presents a biological system with adequate selenium to permit potential isolation and identification. Using bile from animals injected with $^{75}$Se-selenite and arsenite, several
types of characterization were attempted.

a. **Solubility**--Appropriate aliquots of bile were freeze-dried and the residue extracted with either chloroform or methanol. $^{75}\text{Se}$ activity was determined in the extracts and used as a measure of solubility. Ninety-five percent of the selenium was found to be soluble in chloroform while ~33% was soluble in methanol. This would indicate that the selenium was present in a relative non-polar form.

b. **Stability**--Since poor recoveries were obtained in several procedures, attempts were made to determine if various treatments would cause volatilization of selenium.

1. Effect of acid--Preliminary work showed poor recovery from an anion exchange column eluted with HCl solutions of different concentrations. Stability of selenium compounds in the bile was then assessed in HCl by counting a sample of bile before and after the addition of HCl. Selenium compounds seemed to be stable to the addition of either 0.26 N or 1.46 N HCl.

2. Effect of freeze-drying and addition of methanol--The freeze-drying procedure was thought to be the cause of poor recovery of radioactivity from a high performance liquid chromatography involving freeze-drying as a preliminary step which was done in order to
concentrate the bile selenium compounds. This was disproved after counting a sample of bile before and after freeze-drying it in its scintillation tube. The addition of methanol to this bile sample did not change the amount of radioactivity in the sample indicating that the methanol at least did not cause volatilization of the sample.

3. Effect of temperature--The $^{75}\text{Se}$ activity in the bile was determined before and after freezing of a bile sample at $-10^\circ\text{C}$, standing at room temperature for 24 hours, and heating it in a boiling waterbath for 20 minutes. The selenium compounds in the bile appeared to be stable over a wide range of temperature from $-10^\circ\text{C}$ to $100^\circ\text{C}$.

4. Effect of silicic acid--Due to poor recovery from a thin layer chromatography (TLC) using silica gel plates, an aliquot of chloroform extract was counted, another one was spotted on a silica gel plate, the gel was then scraped off the plate in sections and counted. Since no loss of activity occurred, it was concluded that selenium compounds in the bile are stable to silicic acid.

c. Dialysis--Samples of radioactive bile (0.02 ml) were diluted with 4 ml of 0.01 M KCl and dialyzed against $10^{-3}\text{M}$ reduced glutathione (GSH) for 24 hours at
4°C. The GSH solution was changed and dialysis continued for an additional 24 hours. In the first 24 hours, 46% of the activity passed through the membrane and an additional 19% passed through in the second 24 hours for a total of 65%. This is comparable to the results of Levander and Baumann (5), who observed that 73% of the activity was easily dialyzable. This would seem to indicate that the selenium was in the form of small molecules or at least it was very loosely bound to large molecules.

d. Gel filtration--In an attempt to isolate selenium metabolites or at least to find a means of fractionating the radioactivity, samples of bile were applied to columns containing various Sephadex gels. The specific column conditions are discussed in the Experimental section (Table I). On the basis of the dialysis data, it was assumed that the selenium was in the form of small molecules and fractionation was first attempted with gels having low exclusion limits. In succession, G-10, G-75, and G-200 were used (Table VII). For G-10, a single sharp peak of activity was eluted representing 57.0% of the total activity applied. The recovery from this column was 60.9%. Since only a single peak of activity was eluted, Sephadex G-75 was tried. A sharp peak (65.3%) of the activity was obtained at the
Table VII. Gel filtration results

<table>
<thead>
<tr>
<th>Sephadex</th>
<th>% of activity in the 1st peak</th>
<th>% of activity in the 2nd peak</th>
<th>Total recovery of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>G10</td>
<td>57.0</td>
<td>---</td>
<td>60.9%</td>
</tr>
<tr>
<td>G75</td>
<td>65.3</td>
<td>15.2</td>
<td>86.8%</td>
</tr>
<tr>
<td>G200</td>
<td>64.2</td>
<td>23.7</td>
<td>95.2%</td>
</tr>
</tbody>
</table>
void volume (17 ml) with Sephadex G-75 and a smaller one was also eluted at 44 ml representing 15.2% of the total activity applied. Total recovery of activity was 86.8%. When separation was then performed on G-200 (200,000 exclusion limit), a sharp peak was again obtained (64.2% of the activity) slightly after the void volume (26.4 ml) at 32.4 ml. Another broader peak (23.7%) was eluted at 100.8 ml. Total recovery from the column was 95.2% (see Fig. II).

Protein content of the bile was determined to be 3 mg/ml. When 1 ml was applied to the G-200 columns, 0.042 mg of protein were recovered in the first major peak (14% of the total protein). The protein determination may be somewhat inaccurate since a precipitate formed during the analysis and considerable radioactivity (40%) was associated with the precipitate.

These data indicated that a large portion of the selenium in bile is at least associated with large molecules. This appears somewhat contradictory to the dialysis data, unless the selenium is very loosely bound to the protein. The identity of the smaller peak was not further examined.

e. Thin layer chromatography (TLC)--Bile was first subjected to separation on silica gel plates using chloroform:methanol (3:1 or 5:1) as the developing
Gel filtration

Sephadex G200
Void Volume: 26.4 ml
1st peak: 32.4 ml
2nd peak: 100.8 ml

Fig II

75Se Activity (cpm x 10^-3)
Figures IIIa and IIIb. Silica gel plates were spotted with 5 and 10 µl of bile (3,672 cpm and 7,343 cpm, respectively). They were developed in chloroform:methanol (3:1) in Figure IIIa and (5:1) in Figure IIIb. Spots viewed under UV light are represented by circles. Percentage of total activity recovered shows up on both sides of each figure.
Fig IIIa

Fig IIIb
solvent. Three to four spots were visualized under ultraviolet light as shown in Figs. IIIa and IIIb. When the plates were divided into regions and the silica gel scraped off, radioactivity was not necessarily associated with the ultraviolet visible spots. Of the radioactivity recovered, ~60.3% moved near the solvent front and ~32.5% remained on the origin. The total radioactivity recovered was about 62%. The distribution was essentially the same for both the 5:1 and 3:1 chloroform-methanol solvent systems.

Because of the non-polar nature of a major part of the radioactivity as indicated by TLC, it seemed possible that the selenium might be conjugated to the bile acids (bile salts). As a result, the extract of bile was chromatographed with sodium glycocholate a bile salt. The developing solvent was petroleum ether diethylether (87.5:12.5). No spots were visualized under UV light, except at the origin. The region of the plate containing the bile extract was scraped off in sections and counted as before (see Fig. IV). All the radioactivity moved away from the origin and the highest amount was near the solvent front (43.4%) of the recovered activity. Total recovery was 89.5%. The standard was visualized by spraying the plate with 50% H₂SO₄ and heating. The sodium glycocholate moved very
Figure IV. Ten and 20 µl of chloroform extract of the bile residue were spotted on TLC, also 20 µl of sodium glycocholate. The plate was developed in petroleum ether:diethyl ether (87.5:12.5). Spots viewed under UV light after spraying the plate with 50%, H₂SO₄ are represented by circles. Percentages of total activity recovered appear on the right side of the figure.
close to the origin and a similar spot was visible in the bile extract. Since most of the activity moved away from the origin and essentially none was found in the region where the bile salt was expected, it was concluded that the selenium excreted in the bile is probably not attached to bile salts. No further bile acid derivatives were investigated.

**f. High performance liquid chromatography (HPLC)**—Reversed phase liquid chromatography was then tried. In order to get the sample concentrated enough for HPLC, bile was freeze-dried and the residue extracted with CHCl₃:CH₃OH (2:1). Seventy-three percent of the original radioactivity was recovered in the solvent. Another 50% of the original count was lost after evaporating the HCl₃:CH₃OH extract in a glass flask evaporator at 40°C. Only 23% was redissolved in methanol and applied to the HPLC. Total recovery from the HPLC was only 33% of the applied activity. This radioactivity was not associated with any of the peaks detected by the UV detector.

Poor recovery in general was not explainable. Freeze-drying appeared not to be the cause, nor did the addition of the solvent.

When another sample (61,152 cpm) was freeze dried in its scintillation tube and 1 ml methanol was added to
it, no radioactivity was lost. However, when the tube was left for 1 hour and an aliquot of 100 μl was taken from the supernatant, a count of 2,000 cpm was obtained, indicating that only about 1/3 of the original activity was soluble in methanol. Subjection of this 100 μl aliquot to HPLC resulted in elution of 45% of the applied activity in the first fraction (6 ml). The column was then flushed with the solvent (methanol) an additional 48 minutes, but no more activity was eluted. This means about 55% was bound tightly to the column.

It appears that, under the conditions of this study, HPLC is not a very useful technique since so much of the $^{75}$Se activity sticks to the C$_{18}$ column.

g. Anion exchange chromatography--A further attempt to fractionate the selenium compounds in the bile of the arsenized rats was done using an anion exchange chromatography. A sample of 0.05 ml of radioactive bile (37,163 cpm) was applied to the column. Fractions of eluted wash were counted for $^{75}$Se activity and the data are shown in Table IX. Of the added activity, 7.5% was eluted with 0.05 N HCl and 0.2% with 1.5 N HCl. This could represent selenium present as selenite and selenate, respectively (59), and indicate that only small amounts of inorganic selenium are excreted in the bile. No further attempts were made to characterize these
fractions. An additional 3.0% was eluted with 4.0 N HCl and represent very strongly bound selenium (high negative charge or large molecules).

Since only low amounts of activity were recovered in the eluted fractions (12%), the column was removed in sections (∼0.5 cm) and counted. An additional 50% was recovered from the column with a large proportion of it in the top section (see Table VIII). This implies that Se in bile may be present as a lipoprotein or a protein that would adhere to the top of the column or at least in a form that would strongly bind to an anion exchange resin.

Total recovery from the column and eluted fractions was only 62%. Reasons for the low recovery are unclear. Handling of the column sections may not have been as quantitative as desired. There is also the possibility of decomposition and volatilization of the sample, however, previously reported data on the stability of bile-selenium in acid tends to negate this speculation. In any event, a large portion of the material remains bound to the column and more work is required to determine if it can be further fractionated.

It might also be noted that if the selenium in the bile was to be present as a selenium-arsenic conjugate as suggested by Levander and Baumann (5, 48),
one would have expected it to be more easily removed from the column than what was observed in these works.
Table VIII. Recovery of selenium metabolites in bile from ion exchange chromatography

<table>
<thead>
<tr>
<th>Fraction #</th>
<th>Solvent</th>
<th>Column eluent's activity (cpm)</th>
<th>% Activity of total applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H₂O</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>0.05 NHCl</td>
<td>2,782</td>
<td>7.5</td>
</tr>
<tr>
<td>3</td>
<td>0.5 NHCl</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>1.5 NHCl</td>
<td>780</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>4.0 NHCl</td>
<td>1,112</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Column's sections (0.5 ml each)</th>
<th>Ion exchange column's activity (cpm)</th>
<th>% Activity of total applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Top)</td>
<td>7,500</td>
<td>20.2</td>
</tr>
<tr>
<td>2</td>
<td>4,882</td>
<td>13.1</td>
</tr>
<tr>
<td>3</td>
<td>1,889</td>
<td>5.1</td>
</tr>
<tr>
<td>4</td>
<td>1,637</td>
<td>4.4</td>
</tr>
<tr>
<td>5</td>
<td>602</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>481</td>
<td>1.3</td>
</tr>
<tr>
<td>7</td>
<td>348</td>
<td>0.9</td>
</tr>
<tr>
<td>8</td>
<td>462</td>
<td>1.2</td>
</tr>
<tr>
<td>9</td>
<td>182</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>139</td>
<td>0.4</td>
</tr>
<tr>
<td>Rest of column</td>
<td>568</td>
<td>1.5</td>
</tr>
</tbody>
</table>
SUMMARY AND CONCLUSIONS

Selenium is an element essential for life processes, but it can also cause severe toxicity in excessive amounts. Arsenic is known to be an antagonist (detoxicant) for selenium and it is thought to exert its protection by increasing biliary excretion of selenium.

The objectives of this work were to examine further the effect of arsenic on biliary excretion of selenium and to attempt the characterization of the forms of selenium excreted in the bile. On the basis of the work presented here, several conclusions can be deduced:

1. Arsenic is most effective in increasing biliary excretion of selenium when it is administered within one hour after the selenium.

2. Arsenic administration increases the excretion of selenium from organic forms such as selenomethionine and selenocystine, at least six fold. This has not been shown before and it indicates that the mechanism of detoxification is the same for organic and inorganic forms of selenium.

3. The known metabolic endproduct, trimethylselenonium ion, is excreted in only small amounts in the bile and arsenic has little meaningful effect on its biliary excretion.
4. Timing studies indicate selenium is metabolized to a form which accumulates in the system up to at least one hour and which reacts rapidly with arsenic, resulting in its excretion in the bile. The reaction product of selenium and arsenic appears to be more toxic than either element alone. This was shown by the increase in toxicity obtained by the delayed administration of arsenic with selenium levels more closely approximating the MLD.

5. Age of the rats was found to decrease the effect of arsenic on excretion of selenium in the bile, but strain did not have an effect.

6. Se compounds excreted in the bile of the arsenized rats seem to be more in a non-polar form. The majority of selenium appears to be in the form of small molecules loosely bound to large molecules probably a protein or a lipoprotein. More work needs to be done on the characterization of selenium compounds in the bile.
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