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EFFECTS OF SELENITE AND SELENOMETHIONINE

The Ohio State University

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EVALUATION OF SELENIUM TOXICITY ON CELLULAR METABOLISM:
EFFECTS OF SELENITE AND SELENOMETHIONINE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Pamela Ann Toy-Manning, B.S., M.S.

* * * * *

The Ohio State University

1980

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Biochemical Pharmacology. Drs. D. Couri and J. Bianchine

Autonomic Pharmacology. Dr. S. Tjioe

Radioisotopes Methodology. Drs. L. Malspeis and D. Feller

Cellular Control Mechanisms. Dr. T. Webb

Immunology. Dr. R. Lang

Neurochemistry. Dr. L. Horrocks

Advanced Mammalian Organ Systems. Physiology Staff
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INTRODUCTION

A. General Aspects

1. Natural Occurrence of Selenium

Selenium is unevenly distributed over the earth's surface, the soil having a mean concentration of 0.1-0.2 ppm (Lakin and Davidson, 1967). There are areas where soils are deficient, partially due to leaching and run-off by rainfall, and other areas where the amount of selenium in the soil is large enough to make vegetation toxic to animals. Geographically, soils with high selenium concentrations, seleniferous soils, exist in the Western United States and Great Plain States, including South Dakota, Wyoming, Nebraska, Kansas and Colorado, and in various parts of Ireland and Israel, while soils of low selenium concentrations exist in the Pacific Northwest, much of the Eastern United States, and in New Zealand and Australia (Moxon, 1976).

Selenium is present in the soil in several forms. In seleniferous soils, which are usually alkaline and dry, selenate which is soluble and thus available to plants, is the prevalent form of selenium. In contrast, most of the soil in the Eastern U.S. is acidic and wet, forming a reducing environment. Most of the selenium is thus in the form of either elemental selenium or selenide, and is therefore much less soluble and available to plants (Olson, 1967).
River and ground water usually contain less than 0.02 ppm selenium, and in general water is a poor source of selenium except when seleniferous soils are irrigated. However concentrations of selenium as high as 9.0 ppm have been found in springs and wells in Colorado, Utah and other areas of the Western United States (Lakin and Davidson, 1976). The safe upper limit for selenium in drinking water has been set at 10 ug/L (0.01 ppm) by the U.S. Public Health Service (1962). Selenium has been found in the air over both cities and suburban areas, however, average concentrations are very low (Hoshimoto and Winchester, 1967).

Many plants can accumulate measurable amounts of selenium when grown in seleniferous soils. These seleniferous plants have been divided into 3 groups according to their ability to accumulate selenium (Rosenfeld and Beath, 1964). Primary accumulator or indicator plants form Group 1. Some of the primary accumulator species include Astragalus, Xylorhiza and Stanleya. Indicator plants contain 1000-10,000 ppm of selenium, present in water-soluble, low molecular weight organic compounds, and can grow only in seleniferous soils. In addition, some non-accumulator species of Astragalus exist which contain only traces of selenium. Accumulator plants have been found to synthesize primarily Se-methylselenocysteine from selenate and selenite while non-accumulator species synthesize primarily Se-methylselenomethionine (Virupaksha and Shrift, 1965). Consumption of primary accumulators by livestock results in a toxicity manifested either as blind staggers or acute selenium poisoning. Secondary accumulators or absorbers, which contain only a few hundred ppm, form Group 2. Some species of this group include Aster, Atriplex, Comandra and Grayia; their growth does not
appear to be restricted to seleniferous soils. Much of the selenium in these plants occurs as inorganic selenate, and to a lesser degree, an organic form. Ingestion of these plants by livestock can result in acute or chronic selenosis. Group 3 consists of most cultivated plants and grains including wheat, corn, rye, barley, oats, and onions, and native grasses. These tend to accumulate low concentrations of selenium, usually less than 30 ppm, which is mainly associated with plant proteins. The selenoamino acid, selenomethionine, is a major constituent of these proteins (Olson et al., 1970). Alkali disease occurs in livestock when toxic amounts of selenium are ingested from these plants.

2. Uses of Selenium

a. Industrial

In 1873, the photoelectric properties of selenium were discovered, and it is now used in the electronic industry for rectifiers, photocells, solar batteries, television cameras and xerography (Lucovsky and Tabak, 1974). The largest use of selenium is as a decolorizer for glass and ceramics. Selenium in the form of cadmium selenide, is the ruby red pigment in the glass of traffic signals, automobile tail lights and tableware (Hares, 1974). Selenium is also used extensively in rubber and steel manufacturing, and is employed as an anti-oxidant in lubricating oils and other oils, as a solvent for synthetic resins, and as a paint, varnish and glue remover (Mueller, 1974).
b. **Medicinal and Agricultural**

Selenium has been used as an anti-dandruff agent, as an insecticide, and for the control of dermatitis and mange in dogs (National Academy of Science Committee on Selenium, 1976). In combination with tocopherol, selenium has also been used to treat cataracts and nuclear sclerosis in dogs (Brooksby, 1979). Recently, the use of selenium as either sodium selenite or selenate has been approved by the U.S. Food and Drug Administration, as an additive to selenium deficient animal feeds. Selenium may be added to the diets in concentrations up to 0.1 ppm for chickens, swine and dairy cattle, and up to 0.2 ppm for turkeys (U.S. Department of H.E.W., 1974; Halsey, 1979).

3. **Se Content in Food and Human Nutritional Requirements**

A study of the selenium content of foods representing a cross-section of the American diet was conducted by Morris and Levander (1970). Most fruits and vegetables were found to contain less than 0.01 ug/g (0.01 ppm) of selenium. However, garlic, mushrooms and radishes tend to contain between 0.04-0.25 ug/g selenium. Grain products varied in their selenium content; cornflakes contained 0.025 ug/g, rice contained 0.332 ug/g, rice cereal contained 0.029 ug/g, barley cereal contained 0.660 ug/g, white flour had 0.192 ug/g, while whole wheat flour contained a higher value of 0.636 ug/g. Among dairy products, whole milk had a concentration of 0.012 ug/g, skim milk contained 0.050 ug/g, dried milk contained between 0.090 ug/g and 0.240 ug/g, egg yolks contained approximately 0.18 ug/g, and egg whites contained 0.05 ug/g. Meats ranged from 0.1 ug/g for chickens and up to 1.9 ug/g.
for pork kidney. However, most of the values for beef, lamb and pork fell between 0.2-0.5 ug/g. The selenium content of seafood was generally higher than that of meat and ranged from 0.4 ug/g up to 0.5 ug/g. The minimum dietary requirement for humans has been estimated to be 20-30 ug/day (Stewart et al., 1978), and the average daily intake in a standard diet has been estimated to be 62 ug/day (Schroeder et al., 1970), suggesting that the levels of selenium in the average American diet are adequate for good nutrition.

B. Chemistry of Selenium

Selenium, atomic number 34, was discovered in 1817 by J.J. Berzelius, while searching for a source of tellurium in sulfuric acid. Selenium, sulfur and tellurium are group VIA elements. Many of the chemical and physical properties of selenium and sulfur are similar, including the sizes of the atoms in both the covalent and ionic states, bond energies, ionization potentials, electron affinities, electronegativities and polarizabilities. However, they do not substitute universally for one another in vivo, since metabolically, selenite tends to undergo reduction while sulfite tends to undergo oxidation (Levander, 1976).

Selenium exists in nature in the -2, 0, +4, and +6 oxidation states. In the -2 oxidation state, it occurs in the form of either hydrogen selenide (H₂Se), or heavy metal selenides. In the 0 oxidation state, elemental selenium is insoluble, fairly stable to both oxidation and
reduction, and is therefore relatively non-toxic. In the +4 oxidation state, selenium occurs as inorganic selenites (SeO\textsubscript{3}\textsuperscript{2-}), and when further oxidized to the +6 oxidation state, it occurs as inorganic selenates (SeO\textsubscript{4}\textsuperscript{2-}). Hydrogen selenide and soluble forms of selenite and selenate tend to be highly toxic (National Academy of Sciences Committee on Selenium, 1976).

C. Disposition and Metabolism

1. Absorption and Distribution of Selenium

The disposition of selenium has been studied extensively under a variety of experimental conditions in several different species. Absorption of both selenite and selenomethionine in monogastric animals is fairly complete, as demonstrated in the rat where absorption was greater than 90% (Thompson and Stewart, 1973), whereas absorption in ruminant animals has been shown to be lower, possibly due to the reduction of selenium to insoluble forms in the rumen (Cousins and Cairney, 1961).

Distribution has been studied in selenium deficient, adequate and chronically poisoned animals. Smith et al. (1938), chronically poisoned cats with inorganic (selenite and selenate) and organic (wheat gluten) selenium compounds. The highest concentrations of selenium were present in liver, kidney, spleen, pancreas, heart and lung, and overall tissue retention was greater in animals receiving the organic form of selenium. In deficient animals, uptake of a dose of \textsuperscript{75}Se-selenite was also high in liver and kidney (Lopez et al., 1969). However, the brain
and testis retained the $^{75}\text{Se}$ for longer periods of time (Brown and Burk, 1973; Trapp and Millam, 1975). Hopkins et al. (1966), studied the distribution of a single dose of $^{75}\text{Se}$-selenite in rats following feeding of 0, 0.1, 1.0 and 5.0 ppm selenium as selenite in the diet for two weeks. Twenty four hours after administration of the $^{75}\text{Se}$, the amount of radioactivity present in the kidney, blood and carcass decreased and the amount excreted in the urine increased with increasing dietary selenium. However, the amount of $^{75}\text{Se}$ in liver was relatively constant, decreasing only slightly in the group which received 5.0 ppm. In a similar study in lambs, the concentration of $^{75}\text{Se}$ in various tissues twelve days after the administration of the $^{75}\text{Se}$-selenite was also found to be inversely related to the dietary selenium level. In addition, kidney and liver tissue contained higher concentrations of $^{75}\text{Se}$ compared to all other organs (Lopez et al., 1969). Jacobson (1966) compared the uptake of $^{75}\text{Se}$-selenium as selenite, selenomethionine, and selenocystine in normal sheep, and found that tissue uptake was similar, except that a greater amount of $^{75}\text{Se}$ was present in the pancreas when selenium was administered as the selenoamino acids. This finding was confirmed in the mouse by Hanson and Jacobson (1966). In summary, all results indicated that the highest concentrations of selenium were located in the liver and kidney, regardless of the experimental animal or the form of selenium used.
2. **Metabolism and Excretion**

It has been proposed that methylation is the primary mechanism for the detoxification of selenium. McConnell and Portman (1952) reported the presence of a volatile metabolite, dimethyl selenide, in rats given selenite as $^{75}$Se-selenite. It was subsequently shown to be much less toxic than selenite (McConnell and Portman, 1952). Ganther (1966) described the enzymatic synthesis of dimethyl selenide $\left((\text{CH}_3\right)_2\text{Se}\right)$ from sodium selenite in mouse liver extracts. There was an absolute requirement for glutathione and NADPH. S-adenosylmethionine was identified as the methyl donor in the formation of $\left((\text{CH}_3\right)_2\text{Se}\right)$. Coenzyme A, ATP and $\text{Mg}^{+2}$ were also required for optimal activity. The conversion of selenite to dimethyl selenide involves a six-electron reduction in addition to two separate methylation reactions.

The extent of excretion of this volatile metabolite by the lung is dependent upon the amount of protein and methionine in the diet, and upon the amount of selenium in the diet (Ganther et al., 1966). Production and excretion of dimethyl selenide is an extremely important route of excretion following acute toxic doses of selenite (Hoffman, 1977), and much less important in chronic states of toxicity (Ganther and Baumann, 1970).

A major urinary metabolite, trimethylselenonium ion, $\left(\text{CH}_3\right)_3\text{Se}^+$, has also been identified (Palmer et al., 1969; Byard, 1969). S-adenosylmethionine again appears to be the methyl donor. It accounts for approximately 30-50% of the urinary selenium excreted and appears to be
a normal excretory product. Trimethylselenonium ion has been found to be a major excretory product of selenium metabolism, regardless of the amount or form of selenium administered (Palmer et al., 1970; Burk et al., 1972). Burk's group postulated that a dietary threshold for urinary excretion of selenium existed in the rat which was less than 0.05 ppm (Burk et al., 1973). Excretion of a tracer dose of $^{75}$Se-selenite over a ten day period has been shown to be directly related to the dietary selenium level (Burk et al., 1972).

Fecal excretion of selenium appears to be a minor route of elimination. McConnell (1941) showed that only 3-6% of a single, subtoxic dose of selenate was excreted in the feces. Burk (1972) later reported that regardless of the dietary content of selenium ranging from 0.004 to 1.0 ppm as selenite, fecal excretion of a tracer dose of $^{75}$Se-selenite was approximately 10% for a ten day period.

Ganther (1974) has proposed a scheme for the metabolism of selenium from the $+4$ oxidation state (selenious acid) to the $-2$ oxidation state (hydrogen selenide), the form required for the formation of the methyl metabolites of selenium (Figure 1). In this scheme, selenious acid reacts with 4 glutathione molecules to form a selenotrisulfide (GSSeSG) and oxidized glutathione. The selenotrisulfide is reduced by glutathione reductase to form a selenopersulfide (GSSeH), which is then further reduced enzymatically to form hydrogen selenide ($H_2Se$). Hydrogen selenide can then be transmethylated to either dimethyl selenide or trimethylselenonium ion.
PATHWAY FOR CONVERSION OF SELENIDES FROM SELENITE

\[
\begin{align*}
H_2SeO_3 + 4GSH & \rightarrow GSSeSG + GSSG + 3H_2O \\
(\text{Se Oxidation State} +4) & \quad (\text{Se Oxidation State} +2) \\
\end{align*}
\]

\[
\begin{align*}
GSSeSG + \text{NADPH} + H^+ & \rightarrow \text{GSSeH} + \text{GSH} + \text{NADP} \\
(\text{Se Oxidation State} +2) & \quad (\text{Se Oxidation State} 0) \\
\end{align*}
\]

\[
\begin{align*}
\text{GSSeH} + \text{NADPH} + H^+ & \rightarrow \text{H}_2\text{Se} \\
(\text{Se Oxidation State} 0) & \quad (\text{Se Oxidation State} -2) \\
\end{align*}
\]

\[
\begin{align*}
? & \quad \text{SAM} \\
\text{Methyl Transferase} & \quad \text{H}_2\text{Se} \\
\end{align*}
\]

\[
\begin{align*}
\text{Acid-Labile Protein Se}^{2-} & \quad (1) \ (\text{CH}_3)_3\text{Se}^+ \\
& \quad (2) \ (\text{CH}_3)_2\text{Se} \\
\end{align*}
\]

FIGURE 1. Pathway for the conversion of selenides from selenite, from Diplock (1976), as proposed by Ganther.
D. Biological Effects of Selenium

1. Selenium Deficiency

In 1957, Schwarz and Foltz first demonstrated that selenium was an essential trace element and that it was the active component of Factor 3, a factor present in baker's yeast, which was capable of preventing dietary liver necrosis in rats, as were Factors 1 and 2, cysteine and vitamin E. Trace amounts of selenite or purified Factor 3 prevented dietary liver necrosis in rats fed a vitamin E deficient diet. Factor 3 was also found to prevent exudative diathesis in chick, as was a diet containing 0.1 ppm selenium as sodium selenite (Scott et al., 1957).

Following the discovery of selenium as an essential trace element, selenium deficiency was described in a number of species, and was identified as the cause of various disease states in different species. Although vitamin E and selenium are related in several deficiency syndromes, many of these have been found to be totally selenium responsive.

Selenium deficiency is characterized by dietary liver necrosis in the rat, which can be partially alleviated by vitamin E, and by decreased growth rate, lack of hair growth and failure to reproduce in the second generation (McCoy and Weswig, 1969). Ewan (1976) has also reported a decrease in growth rate, a decrease in total growth hormone in the anterior pituitary, a decrease in food consumption, and a reduced utilization of energy and nitrogen in selenium deficient rats, adequately supplemented with vitamin E. Testicular weight in second generation selenium deficient rats was found to be approximately 40% lower than in rats which
received 0.1 ppm selenium. There was also a decrease in spermatogenesis coupled with an impaired function of the epididymal ducts. In selenium deficient females, follicular development appeared to be altered (Wu et al., 1969). In recovered sperm, motility was found to be poor and the majority of sperm exhibited breakage in the midpiece region of the tail (Wu et al., 1973). In 30-day deficient rats, a single tracer dose of $^{75}\text{Se}$-selenite accumulated in the testes-epididymal complex and was localized in the midpiece of the sperm (Brown and Burk, 1973).

In the chick, selenium deficiency is characterized by poor feather growth, pancreatic fibrosis and exudative diathesis (Nesheim and Scott, 1968; Thompson and Scott, 1970). Both selenite and selenomethionine are effective for the prevention of exudative diathesis in the chick (Cantor et al., 1975), however, selenomethionine has been found to be approximately four times more effective than either selenite or selenocysteine in the prevention of pancreatic fibrosis (Cantor et al., 1975).

In the mouse, selenium deficiency is characterized by multiple necrotic lesions of the heart, liver, kidney and skeletal muscle, and by atrophy of the pancreas and testicular degeneration. Death usually results from cardiac failure (Diplock, 1976).

A myopathy, termed white muscle disease, occurs in selenium deficient lambs and calves (Muth, 1963). Sudden death in calves attributed to myocardial degeneration has also been associated with selenium deficiency (Cawley, 1978). In addition, embryonic mortality and unthriftness occur in sheep and cattle as manifestations of selenium deficiency (Andrews et al, 1968; Horton and Jenkins, 1978).
Pigs develop both necrotic liver degeneration (hepatosis dietetica) and a cardiac myopathy (mullberry heart) when given diets deficient in both selenium and vitamin E (Eggert et al., 1957). Vitamin E has been shown to prevent the development of the Se/Vit E deficiency syndrome (Simesin et al., 1979).

There are no reports of selenium deficiency per se in man. However, children with kwashiorkor, characterized by a severe malnutrition with slow body weight gain recovery, showed a marked increase in body weight gain following selenium treatment (Schwarz, 1965). Low serum selenium levels have also been associated with several disease states including cirrhosis of the liver (Sullivan et al., 1979) and gastrointestinal cancer and various other carcinomas, excluding reticuloendothelial carcinoma (McConnell et al., 1975).

2. Selenium Toxicity

a. Chronic Toxicity

Selenium toxicosis, although the cause was unknown, was first observed by Marco Polo. During his travels he noted that his horses, which were grazing on seleniferous plants, developed a strange disease characterized by the shedding of their hooves (Gissel-Nielsen, 1976). In 1934, Franke reported that high levels of selenium in the grasses and grains of South Dakota were responsible for the toxicity, termed alkali disease, which was manifested in cattle and other livestock. Clinically, alkali disease in horses, cattle, sheep and pigs is characterized by lack of vitality, deformities and shedding of hooves,
loss of hair and arthritic disorders of the joints resulting in lameness (Franke and Potter, 1935). Levels of selenium from 5-30 ppm produce chronic selenium toxicity which has been classified into three categories by Rosenfeld and Beath (1964). These include blind staggers, caused by ingestion of organic selenium compounds from seleniferous accumulator plants; alkali disease, resulting from ingestion of grasses and grains containing protein bound selenium; and chronic selenosis, usually occurring in laboratory animals fed diets with excess inorganic selenium, either selenate or selenite.

Blind staggers occurs in grazing sheep and cattle, and its severity depends on the species of accumulator plant that is consumed. Early in the disease process, the animal wanders about, usually in circles, and may stumble and walk into objects. Food and water consumption decrease and vision may be impaired. As the disease progresses, the front legs weaken and food and water consumption are minimal, leading to emaciation. Prior to death, the tongue and muscles involved in swallowing become paralyzed, blindness often occurs, severe abdominal pain develops, respiration is labored and accelerated, and body temperature is subnormal. Death results due to respiratory failure. In sheep suffering from blind staggers, congenital malformations may occur. Pathologically, the liver exhibits necrosis with cirrhosis, the kidney exhibits subacute and chronic nephritis, the heart becomes soft and flabby, and the intestinal tract becomes impacted (Draize and Beath, 1935: Rosenfeld and Beath, 1964).
The characteristics of alkali disease occurring in horses, cattle, sheep and pigs, were previously mentioned. Although tissue levels of selenium are generally lower in alkali disease than in blind staggers, the pathological lesions which occur in alkali disease tend to be more chronic and more progressive. The most severe changes occur in the liver and heart. The heart is atrophied with petechial hemorrhages on the epicardium. The liver is atrophied and cirrhotic, and the surface is pitted and scarred with connective tissue. Chronic nephritis occurs in the kidney, which is often shrunken and hemorrhagic. The hooves are almost always deformed. Unlike blind staggers, the intestinal tract does not appear to be involved. Reproductive performance usually declines (Rosenfeld and Beath, 1964).

Chronic selenosis occurs in either livestock or laboratory animals following exposure to inorganic selenium. The animals show a decrease in food intake, leading to growth inhibition and emaciation, and possible neuromuscular dysfunction. In addition, there is liver necrosis, sometimes associated with fatty infiltration and cirrhosis. The kidney may become nephritic and the intestinal tract may ulcerate (Rosenfeld and Beath, 1964). Relatively low amounts of selenite (2.5-7.5 ppm) administered to rats in drinking water produce decreased fertility in successive generations (Rosenfeld and Beath, 1954). Anemia due to hemolysis has also recently been shown to occur in rats chronically exposed to 5-15 ppm selenium as selenite (Halverson et al., 1970).

Historically, levels of 5 ppm and above have been considered to be chronically toxic. However, several more recent studies have been
conducted at lower exposure levels. Schroeder and Mitchener (1971) reported that 2 ppm selenium as selenite administered in drinking water was extremely toxic compared to 2 ppm as selenate, the former resulting in weight loss and mortality. This study has been criticized for several reasons, i.e. the level of selenite was later reported to be 3 ppm instead of the 2 ppm originally reported. In a more well controlled study, Palmer and Olson (1974) reported that the overall toxicities of both forms of inorganic selenium administered in drinking water over a nine week period were similar. Levels of 2-3 ppm as selenite or selenate produced a small decrease in body weight in 4-6 weeks as a toxic response to selenium, but no mortality occurred. When 6-9 ppm as either selenite or selenate were given, considerable mortality and pathological changes in organs occurred. Continuous exposure to selenium as selenite at low concentrations (< 2 ppm and > 1 ppm) has recently been shown to be toxic to Xenopus laevis embryos (Browne and Dumont, 1979). At levels of 2 ppm and greater, severe developmental abnormalities and increased mortality occurred. Exposure to both 0.5 and 1.0 ppm lowered the mortality rate below the control (selenium-free water), consistent with the trace element requirement for selenium.

b. Acute Toxicity

Acute selenium poisoning occurs following the ingestion or administration of a single, high dose of either seleniferous plants or inorganic selenium. Movement and posture are abnormal and a characteristic stance with head lowered and ears drooped is often assumed. A rise in body temperature, a rapid and weak pulse rate and labored respiration
occur. The animal develops diarrhea, becomes bloated, and urine output increases. Death results due to cardiac failure. Pathological changes occur in all organs, which are largely hemorrhagic in nature. Passive congestion, hemorrhage, and parenchymal degeneration and necrosis occur in liver; and degeneration, hemorrhage and nephritis occur in kidney (Rosenfeld and Beath, 1964).

c. Toxicity in Man

Both acute and chronic toxicities in man due to selenium exposure (industrial, environmental or intentional) have been reported. Acute selenium toxicity in a 15 year-old girl due to self poisoning with selenate sheep drench was reported by Civil and McDonald (1978). The symptoms included a garlic odor of the breath due to the production of dimethyl selenide, diarrhea, elevated liver enzymes, and irritability and nervousness. Industrial exposure, both acute and chronic, usually occurs through absorption of selenium compounds through the lungs via dust or fumes, or through the skin, while chronic environmental exposure in seleniferous areas occurs either due to ingestion of food or water from these areas (Beath, 1962; Cooper and Glover, 1974). Following acute exposure, symptoms include irritation to the tracheobronchial mucosa, pulmonary edema, gastrointestinal problems, chronic dermatitis, nervousness and a garlic odor of the breath (Cooper and Glover, 1974). In addition to these symptoms, chronic exposure in man leads to "rose cold" or hypersensitive lung disease, a metallic taste in the mouth, brittle and discolored fingernails, and fatigue (Diskin et al., 1979).
d. Teratology

Robertson (1970) reported that selenium may be a teratogen. In an industrial situation following routine exposure of eight female workers to sodium selenite, out of five pregnancies, four aborted. In the one which went to term, the infant had bilateral club foot. However, in an epidemiological study, Shamberger (1971) reported that there was an inverse relationship between neonatal deaths and high, medium and low selenium areas in the U.S. He found no evidence of teratogenicity, and rather suggested that at physiological levels, selenium was necessary for the proper growth and development of the human embryo.

e. Role of Methylation in Selenium Toxicity

As was previously mentioned, selenium is metabolized and detoxified by the formation of the methylated derivatives, dimethyl selenide and trimethylselenonium ion, which are excreted by the lung and kidney, respectively. S-adenosylmethionine (SAM) has been shown to be the methyl donor in the formation of these metabolites (McConnell and Portman, 1952; Palmer et al., 1969; Byard, 1969). SAM has been found to be an important methyl donor in a wide variety of reactions in mammalian and bacterial systems. Both DNA and tRNA exist as methylated species, especially in tissues where rapid growth occurs (Cox et al., 1977; Munns and Sims, 1975; Nau, 1976). Several different ribosomal proteins are methylated at various stages of the cell cycle (Cox et al., 1977), and it has been suggested that methylation is important in the functioning of the ribosomal assembly (Kruiswijk et al., 1978). Methylation is also involved in the metabolism of many small molecules
including serotonin, catecholamines and histamine (Brown et al., 1959), and carboxymethylation of membrane proteins has been shown to be essential for the chemotaxic response of bacteria and the migration of human monocytes (Pike et al., 1978). In addition, it has been shown that the enzymatic methylation of phosphatidylethanolamine to phosphadidylcholine increases membrane fluidity. The increase in fluidity appears to increase \( \beta \)-adrenergic receptor number and its coupling to adenylate cyclase (Hirata and Axelrod, 1978; Hirata et al., 1979; Strittmatter et al., 1979).

S-adenosylmethionine is formed from methionine and ATP by the enzyme methionine adenosyl transferase (Cantoni, 1953; Mudd and Cantoni, 1957), as shown in figure 2. Upon donation of its methyl group, catalyzed by a methyltransferase, S-adenosylhomocysteine (SAH) is formed (De la Haba and Cantoni, 1959), as would occur in the methylation of selenium to its excretory products. In an alternative pathway, SAM can be decarboxylated and its propylamino group is then cleaved and used in the synthesis of polyamines. 5-methylthioadenosine (MTA) is the product formed after decarboxylation and subsequent cleavage (Ferro, 1979). SAH, an extremely potent inhibitor of all methyltransferase enzymes (Borchardt et al., 1978; Kerr, 1972), is cleaved by SAH hydrolase to homocysteine and adenosine. Homocysteine may be either remethylated to methionine and thus recycled, or channeled into the sulfur pathway.

Hoffman (1975, 1977) reported that acute toxic doses of selenite altered normal methyl metabolism. Administration of selenite resulted in a depression of liver SAM concentrations, an elevation of liver SAH concentrations and an inactivation of methionine adenosyl transferase.
FIGURE 2. Synthesis and degradation of S-adenosylmethionine and S-adenosylhomocysteine.
This depression continued after the production of dimethyl selenide had ceased. Hoffman suggested that the hepatotoxic effects produced from both excess selenite and from methionine deficiency may be linked biochemically, both involving a shortage of active methyl groups for normal metabolism. Changes in SAM and SAH concentrations in animals chronically exposed to selenium have not been reported. Kaiser et al. (1979) have recently conducted a study with trout chronically exposed to either low or high selenium containing waters. They found no differences in the methyl accepting abilities of mature tRNAs in an in vitro methylating system in this non-mammalian species.

3. Interactions with Other Elements

From a toxicological standpoint, several interesting interrelationships have been discovered concerning the toxicities of cadmium, mercury, and arsenic in combination with selenium.

In 1938, Moxon first reported that the toxicity produced by feeding 15 ppm selenium as seleniferous grains could be alleviated or prevented by the co-administration of arsenic as sodium arsenate in drinking water. It was later shown that biliary excretion of selenium was enhanced by arsenic in a dose-related manner relative to the arsenic concentration (Levander and Baumann, 1966), presumably by reacting in the liver to form a conjugate which was then excreted into the bile (Levander, 1976). This protective effect has also been shown to occur in cell culture with mouse fibroblasts (Rossner, 1977).

Parizek (1957) demonstrated that the selective damage to the testes induced by cadmium could be prevented by the administration of zinc.
Mason and Young (1967) reported that selenium also prevented the effects of cadmium induced injury to the testes, and to a greater degree than zinc, particularly on the vascular endothelial cell junction (Niewenhuis and Fende, 1978). Pretreatment with selenium shifted the binding of $^{109}$Cd in testes from an unstable 30,000 MW protein, thought to be associated with cadmium induced testicular injury, to a higher molecular weight protein (Chen et al., 1974). Selenium pretreatment also increased the biliary excretion of cadmium (Stowe, 1976).

Selenium has also been shown to have a protective effect on the toxicity of mercury (Parizek et al., 1974). The interaction between selenium and mercury does not appear to form an insoluble complex (Magos and Webb, 1976). Rather, pretreatment with selenium may induce the formation of a high molecular weight selenium protein in liver with a high affinity for mercury, thus decreasing the uptake into kidney (Komsta-Szumska et al., 1976). The induction of metallothionien-like proteins (approximately 10,000 MW) by selenium does not appear to be involved in the protective effect of selenium against mercury or against other heavy metals (Piotrowski and Szymanska, 1976; Komsta-Szumska et al., 1976; Piotrowski et al., 1977).

4. **Carcinogenicity**

The role of selenium in cancer is controversial. Selenium has been reported to be a carcinogenic substance (Nelson et al., 1943; Valgarev and Tscherkes, 1967). However, these studies have been criticized both on the basis of interpretation (i.e., the lack of separation between the toxic effects and the carcinogenic effects of the element)
and because of lack of proper controls (Schrauzer, 1976). In addition, reports from Oregon State University indicate that selenium does not significantly affect tumor production in rats (Tinsley et al., 1967; Harr et al., 1967). Finally other investigators have demonstrated inhibitory effects of selenium on carcinogenesis (Clayton and Bauman, 1949; Shamberger and Rudolph, 1966; Shamberger, 1970) resulting from exposure to m-methyl-p-dimethylaminoazobenzene, 7,12-dimethylbenzanthracene and benzo(a)pyrene. The epidemiological study of Shamberger and Willis (1971) also supported an anticarcinogenic effect of selenium. They found a negative correlation between forage crop selenium levels and blood selenium levels, and human cancer death rates. Although much evidence at the present time supports the no-effect or protective role of selenium, this remains a controversial topic.

5. **Immunological Responses**

Dietary selenium as sodium selenite has been shown to enhance the primary immune response (as has vitamin E) as measured by increases in the number of plaque forming colonies and the amount of sheep red blood cell (SRBC) agglutinating antibodies produced following a previous inoculation with SRBCs. The enhancement of the immune response occurred when greater than 0.1 ppm selenium was administered in the diet, the value generally accepted as nutritionally adequate. The immune response was maximal at 1.25 ppm dietary selenium, and declined above this level of supplementation, markedly at levels of 3.75 ppm and greater (Spallholz et al., 1973).
Prior to 1970, little was known about the specific biochemical role of selenium in mammalian systems. It was suggested that selenium may function in vivo as an antioxidant due to its nutritional inter-relationship with vitamin E (Tappel, 1965). Selenium was also found to be present in many proteins, either bound to sulfur amino acids (Cummins and Martin, 1967) or as analogs of sulfur amino acids (McConnell and Wabnitz, 1957).

Within the last decade, three enzyme catalyzed reactions have been identified which require selenium for activity. The selenium requiring enzymes are formate dehydrogenase and glycine reductase in bacteria (Stadtman, 1974) and glutathione peroxidase in mammalian erythrocytes and tissues (Rotruck et al., 1973). A common feature of these reactions is that they are all oxidation-reduction reactions. A fourth seleno-protein has been isolated from sheep skeletal muscle, and it has been suggested that this protein plays a role in the prevention of white muscle disease (Pederson et al., 1972; Black et al., 1978). It has been proposed that selenium, as selenide, plays a role in the nonheme iron proteins of the microsomal drug metabolizing system in liver (Diplock et al., 1971; Diplock, 1974).

In 1957, Mills demonstrated the presence of a glutathione peroxidase in erythrocytes which appeared to protect hemoglobin from oxidative breakdown by hydrogen peroxide \( \left( \text{H}_2\text{O}_2 \right) \), and in 1968, Little and O'Brien described a tissue peroxidase capable of destroying hydroperoxides. They suggested that this enzyme was the glutathione peroxidase (GSH-Px) of Mills. In 1972, Rotruck et al. demonstrated that in rat erythrocytes,
selenium deficiency increased autohemolysis, ascorbic acid induced hemolysis and hemoglobin oxidation in the presence of normal levels of glutathione (GSH). In 1973, they demonstrated that selenium was an integral component of the GSH-Px, previously identified by Mills and Little and O'Brien (Rotruck et al., 1973). After GSH-Px was purified from ovine erythrocytes by Flohe (1973), it was found to be a tetramer of 84,000 daltons, consisting of four similar subunits, each having a molecular weight of 21,000, and containing 4 gram-atoms of selenium per mole of enzyme. Selenium containing GSH-Px enzymes with similar properties have since been purified from ovine and human tissues (Oh et al., 1974; Awasthi et al., 1975; Awasthi et al., 1979). GSH-Px catalyzes the reduction of H$_2$O$_2$ and also the reduction of organic hydroperoxides, as shown in Figure 3. There is an absolute requirement for GSH, which undergoes oxidation. Reduced glutathione is maintained by glutathione reductase (GSSG-R). GSH-Px appears to function in the cell to reduce peroxides within the cell, thus protecting the tissue and the membranes from peroxidative damage (Diplock, 1976).

GSH-Px activity has been found in various tissues of animals and its activity varies with both the tissue and the amount of selenium fed in the diet. Lawrence et al. (1974) compared the effect of dietary selenium intake on glutathione peroxidase activity in various organs of the rat and lamb. In both species, the GSH-Px activity in liver in deficient animals was less than 1% of selenium adequate controls. GSH-Px activity was also significantly reduced in kidney, heart, lung, pancreas and erythrocytes of selenium deficient animals. Chow and Tappel (1974) also
FIGURE 3. Schematic of GSH-Px and related reactions.
demonstrated decreases in GSH-Px activity to varying degrees in different tissues of selenium deficient rats, followed by increases after repletion with selenomethionine. In rats fed diets ranging from 0.01-5.0 ppm as sodium selenite, both erythrocyte and liver GSH-Px activities increased with the amount of selenium fed. The exception was GSH-Px activity in liver in rats repleted with 5.0 ppm as selenite, in which activity began to decline after approximately two weeks of feeding. It remained depressed throughout the entire 20 week period of the experiment as compared to groups receiving lower amounts of selenium (Hafeman et al., 1974). Since the activity of GSH-Px in liver increased as non-toxic amounts of dietary selenium increased, Hafeman proposed that liver was a good index of selenium adequacy. GSH-Px activity was also found to decline in deficiency and increase with selenium supplementation in the chick (Omaye and Tappel, 1974) and pig (Chavez, 1979). Some of the above authors have suggested that the lack of GSH-Px activity in selenium deficiency and thus the failure to effectively destroy tissue peroxides may produce several of the symptoms characteristic of selenium deficiency.

In 1976, Lawrence and Burk described the presence of another form of GSH-Px in rat liver which did not contain selenium, which had a lower molecular weight (39,000 vs 84,000) than the selenium dependent (SD) form of GSH-Px, and which destroyed organic hydroperoxides effectively but had very little activity toward \( \text{H}_2\text{O}_2 \). They later established that glutathione S-transferase B activity contributed to this non-selenium dependent (NSD) form of GSH-Px, and that its activity persisted in deficiency (Lawrence et al., 1978). This NSD form of GSH-Px was shown to have the capacity to
remove tert-butyl hydroperoxide, but not $\text{H}_2\text{O}_2$ in perfused rat liver preparations (Burk et al., 1978).

Tappel and coworkers have recently identified the catalytic site of SD GSH-Px in rat liver as selenocysteine (Forstrom et al., 1978), and have determined that selenocysteine is located within the polypeptide chain in the native enzyme (Zabowski et al., 1978). Selenocysteine appears to be in the form of a selenol (SeH) in the reduced form of GSH-Px and in the form of a selenenic acid (SeOH) in the oxidized state after reacting with a peroxide (Forstrom et al., 1978; Forstrom and Tappel, 1979). This red-ox reaction between a selenol and a selenenic acid was previously proposed by Ganther et al. (1976), prior to the identification of selenocysteine as the form of selenium present in GSH-Px.
STATEMENT OF THE PROBLEM

The present drinking water standard for selenium set by the E.P.A. is 10 µg/L (0.01 ppm). Although selenium is an essential trace element, this value was purposely set at a very low level due to the element's potential carcinogenicity as well as its observable toxicity at levels in excess of 5-10 ppm.

Although river and ground water generally contain less than 0.02 ppm selenium, some springs and wells in seleniferous regions have been found to contain as much as 9 ppm. Additional selenium intake also occurs through the ingestion of foodstuffs which contain organic selenium compounds especially when grown on seleniferous soils. Selenium is also widely used in industrial settings, and inorganic forms are added to animal feeds to prevent the occurrence of deficiency symptoms in livestock. Finally, most recent evidence now suggests that selenium, in nutritionally adequate amounts, may aid in the prevention of cancer rather than to cause its occurrence. It is therefore necessary to determine the levels at which exposure to selenium produces toxic changes, preferably at the cellular level rather than as manifested by gross pathological alterations.

Experiments will be conducted to determine the biochemical effects of exposure to various levels of selenite, administered in drinking water, on rats over a ten week period, especially those on the selenoenzyme
glutathione peroxidase, in an attempt to determine the level at which selenium becomes toxic. Subsequent experiments will then be conducted to determine the effects of exposure to various levels of selenite, administered in water, in combination with various levels of selenomethionine (the major form of selenium present in grains), administered in food.

The purpose of these experiments is to determine the potency and toxicity of the organic form of selenium, selenomethionine, versus the inorganic form, selenite, and to simulate naturally occurring environmental exposure to the element. No experiments to date have reported the effects of combined exposure to differing chemical forms within the same animal.

Finally, experiments will be conducted to determine the effects of chronic toxicity on the concentrations of S-adenosylmethionine and S-adenosylhomocysteine, upon which the metabolism and thus detoxification of selenium depend.
METHODS

A. Animals

Holtzman male rats were used in all experiments in this study. They were obtained as either weanlings (21 days old) or slightly older, weighing 80-100 g, acclimated in the animal quarters for one week, and then housed in suspended wire cages. The animals were maintained under the following conditions: 55 percent humidity, 73-74°F, and a 12 hour light-dark cycle beginning at 6:00 a.m. Food and water were available ad libitum; specific feeding schedules are described below.

B. Preparation of Water

A stock solution of 100 ppm selenium (100 μg/ml or 10 mg/100 ml) as sodium selenite (Na₂SeO₃, Pfaltz & Bauer, Inc.) was prepared weekly. Since selenium (MW 78.96) is only one component of sodium selenite (Na₂SeO₃, MW 172.94) it was necessary to add 172.94/78.96 x 10 mg = 21.90 mg of selenite/100 ml of double distilled demineralized (DDD) water to achieve a 10 mg/100 ml or 100 ppm stock solution of selenium.

Working solutions were made from the 100 ppm stock diluted appropriately with DDD water to concentrations of 0.1, 0.5, 1.0, 1.5, 2.0 and 4.0 ppm. These solutions were made twice weekly and given ad libitum to the appropriate group of rats.
C. **Enzymatic Determinations**

1. **Glutathione Peroxidase (GSH-Px)**

Glutathione peroxidase (glutathione H$_2$O$_2$ oxidoreductase, E.C. 1.11.1.9) was measured according to the method of Paglia and Valentine (1967), which measures the decrease in absorbance at 340 nm as reduced nicotinamide adenine dinucleotide phosphate (NADPH) is oxidized to NADP in the presence of the enzyme, glutathione reductase (GSSG-R). The substrate, GSH, is maintained by the addition of exogenous GSSG-R and NADPH, which reduces any oxidized glutathione (GSSG) formed by the action of glutathione peroxidase:

$$2 \text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GSH-Px}} 2\text{H}_2\text{O} + \text{GSSG} \xrightarrow{\text{GSSG-R}} 2 \text{GSH}$$

a) **Tissue Preparation**

Animals were anesthetized with ether, and exsanguinated by cardiac puncture. Livers were perfused with 0.9% NaCl, excised and rinsed in cold NaCl. Testes and kidneys were excised and rinsed in cold 0.9% NaCl. All tissues were homogenized with a Polytron homogenizer (Tekmar Company, Cincinnati, Ohio) in four volumes of cold 0.05M sodium phosphate buffer containing 5 mM EDTA. Tissue samples were then filtered through cheesecloth, and either assayed immediately or refrigerated and assayed within four days, during which time no significant loss was found to occur.
b) **Assay Procedure**

All solutions were prepared fresh with deionized distilled water. The reaction mixture, total volume 28 ml. contained:

- NADPH, 8.4 mM, 1.0 ml (Sigma Chemical Co., St. Louis, MO)
- Na$_2$N$_3$, 1.125 M, 0.1 ml (Fisher Scientific Co., Fairlawn, N.J.)
- GSH, 0.15 M, 1.0 ml (Sigma Chemical Co., St. Louis, MO)
- Glutathione reductase, Type III from yeast, 0.1 ml (or the equivalent of 10 units per assay; Sigma Chemical Co., St. Louis MO)
- Sodium phosphate buffer, 0.05 M, 5 mM EDTA, pH 7.0, 25.8 ml

(or if the volume of GSSG-R is adjusted, an amount to yield a total volume of 28.0 ml).

A volume of 2.8 ml of the above reaction mixture was used for each assay. Preparing a large quantity of the reaction mixture allowed for more efficient analysis of large sample numbers. No variation in the outcome of the assay has been shown to occur (Paglia and Valentine, 1967).

Samples were diluted sufficiently to allow monitoring for at least three minutes and 0.1 ml was added to 2.8 ml of the reaction mixture and mixed well. The reaction was initiated by the addition of either 0.1 ml of 2.2 mM H$_2$O$_2$ (Mallinckrodt, Inc., Paris, KY) or 0.1 ml of 1.6 mM cumene hydroperoxide (K & K Rare Chemicals, Cleveland, OH). The selenium dependent form of GSH-Px was measured using H$_2$O$_2$, and total GSH-Px activity was measured using cumene hydroperoxide. Samples were run with 0.1 ml of buffer instead of tissue to provide a blank for nonenzymatic oxidation of NADPH. The reaction was monitored at 340 nm for 3-5 minutes at room
temperature against the blank using a Varian 635A dual beam spectrophotometer equipped with a recorder.

The final concentrations of substrates and reactants per assay were:

- NADPH, 0.28 mM
- NaN₃, 3.75 mM
- GSSG-R, 10 units
- GSH, 5.0 mM
- H₂O₂, 73.3 μM
- Cumene hydroperoxide 53.3 μM

Changes in absorbance were determined. GSH-Px activity was expressed as nanomoles of NADPH oxidized per minute per mg protein, based on the millimolar extinction coefficient of 6.2 for NADPH.

2. Gamma-Glutamyl Transpeptidase (γ GTP)

Gamma-glutamyl transpeptidase (E.C. 2.3.2.-) activity in rat plasma was determined by the kinetic spectrophotometric method of Szasz (1969). The substrate for the enzymatic reaction was L-γ-glutamyl-p-nitroanilide, which when cleaved by γ-GTP, formed the yellow colored product, p-nitroaniline, which absorbs at 405 nm. The acceptor for the γ-glutamyl group was glycylglycine, which subsequently formed γ-glutamyl-glycylglycine.

The reaction mixture consisted of 0.05M 2-amino-2-methyl-propane-1, 3-diol (Ammediol) buffer (Aldrich Chemical Co., Milwaukee, Wis.) adjusted to pH 8.2 with hydrochloric acid, 4.4 mM L-γ-glutamyl-p-nitroanilide (Sigma Chemical Corp., St. Louis, MO), 22.0 mM glycylglycine,
(free base, Sigma Chemical Corp., St. Louis, MO) and 11.0 mM magnesium chloride. To solubilize the substrate, it was necessary to stir the buffered solution for approximately 2 hours at room temperature prior to the assay, or to stir under low heat.

After the substrate was solubilized, 1 ml of buffered substrate was mixed well with 100 µl of rat plasma, obtained by cardiac puncture and subsequent centrifugation, and transferred to a microcuvette. All determinations were performed at room temperature in a Varian 635A dual beam spectrophotometer equipped with a recorder, with buffered substrate as the blank. The reaction was monitored at 405 nm for 5-10 minutes, and was stirred manually approximately once every minute to maintain adequate mixing of the enzyme-substrate solution. The average change in absorbance per minute was determined and the γ-GTP activity in mU/ml calculated according to the following equation:

\[
\text{mU/ml} = \Delta \frac{A}{\text{min}} \times \frac{10^6}{E} \times \frac{TV}{SV}
\]

\[
= \Delta \frac{A}{\text{min}} \times 1111
\]

where:

\(E\) (molar absorptivity of p-nitroaniline at 405 nm) = 9900 M\(^{-1}\)cm\(^{-1}\)

\(TV\) (total volume) = 1.1 ml

\(SV\) (sample volume) = 0.1 ml

All assays were performed with fresh plasma or with plasma stored at 0-4°C for no more than one week. No significant loss in activity has been shown to occur during this period when sample have been stored at temperatures of 20°C or lower (Szasz, 1969).
3. **Imidazole-N-Methyl Transferase**

Imidazole-N-methyl transferase (IMT) was measured in liver according to the method of Bayer (1977) with modifications. This enzyme, which is located 70-80% in the soluble fraction, preferentially methylates imidazole over a variety of other small molecules including 2-methylimidazole, 1-methyl-imidazole, imidazole acetic acid, histidine, 1-methylhistidine, and N-acetyl-histidine. S-adenosylmethionine (SAM) serves as the methyl donor for this enzyme.

a) **Tissue Preparation**

Animals were anesthetized with ether and exsanguinated by cardiac puncture. Livers were perfused with 0.9 % NaCl, quickly excised, rinsed in ice cold saline, blotted, and weighed. A portion was homogenized in nine volumes of 0.1 M sodium phosphate buffer, pH 7.9, using a polytron homogenizer. The crude homogenate was centrifuged at 10,000 x g for 20 minutes in a Sorvall RC-2B refrigerated centrifuge at 0-4° C. The resulting supernatant fraction, which contains the enzyme, was either used immediately or stored frozen at -25° C, and used within one week.

b) **Assay Procedure**

All glassware was washed with nitric acid prior to use. Incubations were carried out in 16 x 125 mm glass disposable test tubes at 37° C for one hour in a Dubnoff metabolic shaker. All reagents were made in 0.1 M phosphate buffer, pH 7.9. Each tube contained a 100 µl aliquot of the 10,000 x g supernatant fraction, 100 µl of 5 mM imidazole 200 µl of 16.2 µM S-(methyl-\(^{14}\)C)-adenosyl-L-methionine (S.A. 47 mCi/mmol,
New England Nuclear Corp.) containing 3.41 x 10^5 dpm, adjusted to a final volume of 1.0 ml with 0.1 M phosphate buffer. The final concentration of imidazole was 5 x 10^-4M and of ^14C-SAM was 3.24 x 10^-6M. Endogenous methylation was determined as above except the addition of substrate, imidazole, was omitted. Blanks contained all reagents except for the enzyme fraction from liver. The reaction was stopped with 200 µl of 0.8 M perchloric acid containing 1 mg/ml of 1-CH3-imidazole, 500 µl of 10 N NaOH was added, and the products were extracted with 10 ml of chloroform for 10 minutes using a mechanical shaker. After centrifuging at 250 x g for 10 minutes to ensure adequate phase separation, the aqueous phase was aspirated and discarded, and 5 ml of chloroform from each tube were transferred to liquid scintillation vials. Fifty microliters of 0.1 N HCl were added to each vial, and the chloroform was evaporated to dryness under room air. The residue was taken up in 1 ml of ethanol and 10 ml of a .4% PPO-.005% POPOP toluene cocktail (Liquifluor). Samples were counted in a Beckman Model 7000 liquid scintillation spectrometer. Radioactivity was determined and corrections applied from a quench curve, using H number as the standardization. All samples were run in duplicate.

4. **Protein Methylase I**

Protein methylase I, which methylates the guanidino group of arginine, was measured in liver and testes by the method of Paik and Kim (1968), as modified by Paik et al., (1971). Greater than 70% of the ^14C-methylated protein produced in this assay has been shown to be due to the activity of protein methylase I (Paik, et al., 1972).
a) **Tissue Preparation**

Rats were anesthetized with ether, exsanguinated, and livers were perfused with 0.9% NaCl and a portion excised and rinsed with several changes of cold water. The testes were also removed and rinsed with water. Livers and testes were homogenized in 4 volumes of cold, double distilled, deionized water using a Polytron homogenizer, and the homogenates were filtered through a double layer of cheesecloth. The protein concentrations in the whole homogenates were determined by the method of Bradford (1976), and the homogenates were diluted with water to concentrations of 10 mg protein per ml prior to the assay. This standardization was necessary because the activity of this enzyme has been shown to be dependent upon the amount of protein present in the assay incubation mixture (Paik and Kim, 1968; Paik et al., 1971).

b) **Assay Procedure**

All assays were performed in 16 x 125 disposable glass test tubes in a Dubnoff metabolic shaker at 37°C for one hour. The incubation mixture contained 0.1 ml of 0.5 M sodium-potassium phosphate buffer at pH 7.2, 0.1 ml of the homogenate, 0.1 ml (6 mg) of Type II A calf thymus histone (Sigma Chemical Co., St. Louis, MO) suspension, 0.1 ml (0.84 μg) of S-adenosyl-L-methionine-methyl-\(^{14}\)C (S.A., 47 mCi/mole, New England Nuclear Corp.) containing 0.1 μCi of radioactivity and 0.1 ml of water. The final assay volume was 0.5 ml. Liver and testes homogenates were boiled for 5 minutes, assayed under the above conditions, and used as the blanks. All values were corrected for the blank, and all assays were performed in duplicate.
The reaction was terminated with 0.5 ml of 30% trichloroacetic acid (TCA). The acid soluble fraction, nucleic acids, and phospholipids were removed using the sequence of washes described below (Allfrey, et al., 1957). Nine milliliters of 10% TCA were added to each tube, bringing the volume to 10 mls. Tubes were mixed well, centrifuged at 300 x g for 10 minutes, and the supernatant fractions discarded. The protein residue was washed three additional times with 10 ml volumes of 10% TCA to remove the acid soluble fraction. The residue was then taken up in 10 mls 10% TCA, heated to 90°C for 20 minutes, and centrifuged. The TCA, containing nucleic acids, was discarded. The protein residue was again washed with 10% TCA. The precipitate was next sequentially washed with 10 mls each of hot 95% ethanol, warm 2:2:1 mixture of ethanol:ether:chloroform, and ether (to remove phospholipids). The residue was transferred with ether to scintillation vials and air dried at room temperature.

The residue was dissolved in 1.0 ml of 1.5 N NH₄OH and 15 ml of an emulsifying type scintillation cocktail (Thrift Solv, Kew Scientific, Columbus, OH) added. Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3255. Disintegrations per minute were calculated from previously constructed quench curves.

D. Selenium Determination

Total selenium content in either tissue, food or water was measured by a modification of the fluorometric micromethod of Hoffman (1968). Selenium dioxide (SeO₂) powder was sublimed in a petri dish and the crystals removed and bottled for use as the primary standard. A stock
standard of 1 μg/μl Se was prepared by dissolving 140.53 mg

\[
\left( \frac{11.96 \text{ SeO}_2 \times 100}{78.96 \text{ Se}} \right)
\]

of sublimed SeO₂ in 100 ml of 0.1 N HCl. Prior to use, the stock standard was diluted 1:1000 in 0.1 N HCl, yielding a working standard of 1 μg/ml Se. A standard curve consisting of 0 (blank) 0.05, 0.10, 0.20, and 0.40 μg Se was prepared from the working standard, and taken through the entire digestion and extraction procedure for each set of determinations made. When measuring Se content from tissue homogenates, a 0.05 M phosphate buffer, pH 7.0, was used as the blank, otherwise water was used. All standards were adjusted to a final volume of 1.0 ml with either buffer or water. Standards and all samples were ≤ 1.0 ml total volume or ≤ 0.5 g dry weight.

All of the following procedures were performed in a fume hood and all glassware used was washed with nitric acid. Standards and samples were placed in 125 ml Erlenmeyer flasks containing 3 glass beads. Six milliliters of concentrated HNO₃ acid was added to the flasks and predigestion was allowed to occur overnight (at least 12 hours). Twelve to twenty-four hours later, 2 ml 70% HClO₄ and 5 ml concentrated H₂SO₄ were added, and the digestion was begun on low heat (hot plate). All acids were reagent grade. The brown fumes (NO₂) produced as the digestion proceeds were removed by blowing them off with air. The temperature was slowly increased during the digestion period. However, if the samples began to darken, indicating charring, additional HNO₃ was added as necessary in 1 ml aliquots. Heating was continued until the samples cleared
to a very light yellow color (standards have no color) and the fumes became white and could not be blown away with air, i.e., they immediately refilled the flask.

Flasks were individually removed from the heater and 1.0 ml 30% H₂O₂ carefully added. Since the reaction is violent, peroxide must be added very slowly to prevent sample loss. Flasks should not cool prior to the peroxide addition. Following the H₂O₂ addition, flasks were replaced on the heater and allowed to boil. H₂O₂ was added a total of three times for each flask. Following the third addition, all solutions were clear, and no brown fumes were liberated. Flasks were then returned to the heat and boiled for 5 minutes or until the solutions began to turn yellow. The temperature during boiling was kept as low as possible in order to minimize the possibility of explosion. Following boiling, the samples were removed from the heat and allowed to cool.

During the cooling period, the 2,3-diaminonaphthalene (DAN) solution was prepared. A small amount of a total volume of 200 ml 0.1 N HCl was added to 200 mg DAN (ICN Pharmaceuticals, Plainview, N.Y.) until a paste-like consistency was produced, then the remainder of the HCl was added and it was stirred until dissolved. The DAN solution was then extracted three times with 25 ml reagent grade cyclohexane in a 250 ml separatory funnel equipped with a teflon stopcock. The final aqueous phase was drawn off for subsequent use. All of the above steps were done in a darkened room.

After cooling, the contents of the flasks were transferred to 50 ml round bottom glass extraction tubes. Each flask was washed twice with a
5 ml volume of a 0.02 M disodium EDTA solution, which was then added to the appropriate extraction tubes. After carefully mixing, three 5 ml volumes of 11 M NH₄OH were slowly added to each tube with careful mixing by hand after each addition.

The remaining portion of the assay, the coupling of selenium to DAN and the fluorescence measurement, was done in a darkened room. A 5 ml aliquot of the DAN solution was added to each extraction tube; the tubes were then mixed and incubated for 45 minutes at 60° C in a water bath. After the tubes were cooled to room temperature, 5.0 ml of cyclohexane was added. The selenium complex was then extracted for 90 sec., the phases were allowed to separate, and the lower phase was aspirated and discarded. The organic phase was washed once with 30 ml 0.1 N HCl and transferred to 12 x 75 mm glass tubes. Fluorescence was measured on a Turner Model 110 fluorometer fitted with a #7-60 (365 nm) primary filter and a combination of a #58 (525 nm) and a #2A-15 (520 nm) with neutral density 2.00 as secondary filters. All readings were made at 30 X against a cyclohexane blank.

Standard curves were prepared by graphing fluorescence units vs. µg Se, the least squares regression lines were calculated, and unknowns determined from these. A representative standard curve is shown in Figure 4.

E. Protein Determination

Proteins were determined by the method of Lowry, et al., (1951) or by the method of Bradford (1976), which measures the binding of Coomassie Brilliant Blue G-250 to protein. Bovine serum albumin (BSA) was used as the standard for both methods. Samples were diluted appropriately with
STANDARD CURVE FOR SELENIUM DETERMINATION

$\text{Fluorescence Units}$

$\mu g \text{ Se}$

$\begin{array}{l}
r = 99.6 \\
m = 95.9 \\
y \text{ intercept} = -0.61
\end{array}$

FIGURE 4. Representative standard curve for the determination of selenium as described in methods, obtained by plotting relative fluorescence units vs $\mu g$ of selenium.
either buffer or water to concentrations within the linear range of the method. Absorbance was linear with respect to protein concentration from 50-1000 μg BSA with the Lowry method and from 5-50 μg BSA with the Bradford method.

F. Pathology

Tissues examined for pathological changes were fixed in 10% neutral buffered formalin for several days and sectioned into small pieces. Paraffin sections were made and stained with hematoxylin and eosin. Tissues were examined histologically using bright field microscopy.

G. Analysis of S-Adenosylmethionine and S-Adenosylhomocysteine by High Pressure Liquid Chromatography (HPLC)

S-adenosylmethionine and S-adenosylhomocysteine were measured from either liver or testes by a modification of the method of Hoffman (1975).

1. Column Preparation

Aminex A-6 cation exchange resin (sodium form, particle size 17.5 ± 2 μ, 8% crosslinked styrene divinylbenzene copolymer, packed density approximately 2.0 g/ml) (Bio-Rad Laboratories, Richmond, CA) was converted to the ammonium form with 1.0 N ammonium hydroxide and was equilibrated overnight in 1.0 M ammonium formate (NH₄CHO₂), adjusted to pH 4.65 with formic acid. A 25 cm x 5.3 mm stainless steel column (Applied Sciences Labs, State College, PA) was packed with the equilibrated resin under pressure, 4200–4800 psi, using a Serva HPLC unit and a Vydac slurry packer (Applied Sciences Labs). The buffer used for packing was 1.0 M NH₄CHO₂, pH 4.65. Packing time was approximately
one-half hour. The column was then equilibrated with 1.0 M NH₄CH₀₂ for one hour at 2500 psi prior to use, and was placed horizontally in a water bath to allow for column heating.

2. **Tissue Preparation**

Rats were anesthetized with ether, a midline incision was made and a portion of the liver was quickly removed and placed in liquid nitrogen to prevent the rise in S-adenosylhomocysteine levels which occur during tissue preparation at 0-4°C (Hoffman et al., 1979). The testes were then quickly removed and placed in liquid nitrogen, and the animal was killed by exsanguination. The tissues were weighed, pulverized in liquid nitrogen, and homogenized in 3 volumes of cold (0-5°C) 5% sulfosalicylic acid (SSA) using a Polytron homogenizer (Tekmar Co., Cincinnati, OH). Homogenates were centrifuged at 10,000 x g for 10 minutes at 0-4°C in a Sorvall RC-2B refrigerated centrifuge. The protein free supernatant was decanted and filtered sequentially through a 1.2μGF/C glass microfiber filter (Whatman Inc., Clifton, NJ) and a 0.2μ Metricel membrane filter (Gelman Instrument Co., Ann Arbor, MI) prior to column application. The filtered supernatant was either used immediately or stored frozen at -25°C and thawed and refiltered prior to use. Calculations were based on the assumption that 4 mls of filtrate represented 1 g original tissue (Hoffman, 1975).

3. **Apparatus and Operating Conditions**

All analyses were performed on a Serva HPLC unit (Applied Sciences Laboratories, State College, PA). Prior to each sample application, the column was equilibrated with 15-20 ml of 0.35 M NH₄CH₀₂,
adjusted to pH 4.65 with formic acid. The column was maintained at 55°C using a water bath.

Two milliliter aliquots of samples and standards, in 5% SSA, were applied to the cation-exchange column through a Rheodyne sample injection valve (Berkeley, CA), without prior neutralization. The column was eluted with a stepwise gradient of 0.35 M and 1.0 M NH₄CHO₂ at pH 4.65 for 14 and 40 minutes, respectively. The flow rate was 1.6 ml/min. The column effluent was monitored at 254 nm with a Pharmacia UV-2 dual path flow monitor and recorded on a Pharmacia Model 482 two channel recorder, generally at 0.1 and 0.5 absorbance units full scale. A typical chromatogram is shown in Figure 5. S-adenosylhomocysteine eluted with 0.35 M NH₄CHO₂ and S-adenosylmethionine with 1.0 M NH₄CHO₂.

The column effluent could also be connected to a fraction collector (Isco Model 1200, Lincoln, NE) when needed. Fractions were analyzed for radioactivity either directly in a Beckman 4000 gamma scintillation spectrometer or in a Packard liquid scintillation spectrometer, following the addition of scintillation cocktail to an aliquot of the fractions.

4. Quantitation

Standard curves of peak height vs. nanomoles were constructed using a series of standards added to tissue. This was necessary because retention times varied between non-extracted standards applied in 5% SSA and tissue plus standards extracted in 5% SSA. Retention times for extracted standards were shorter, presumably due to the presence of other cationic substances in the filtrate which would decrease the number of
FIGURE 5. Representative chromatogram of the adenosyl derivatives, inosine, adenosine, S-adenosylhomocysteine (SAH), 5-methylthioadenosine (MTA) and S-adenosylmethionine (SAM), in order of elution, determined by HPLC as described in methods.
available adjacent binding sites. Quantitation using tissue plus standards also corrected for losses in recovery both during tissue extraction and chromatographic procedures.

Livers were excised as previously described and 100, 50, 25 and 12.5 nmoles SAM and SAH/g (= 4 ml) were added to the homogenate and mixed well. These standards were then prepared as tissue samples. Calibration runs were carried out exactly as for tissue samples; 2 ml aliquots of the filtrate of the standards equivalent to 0.5 g original tissue, were applied to the column and eluted with a stepwise gradient of 0.35 M NH₄CHO₂ for 14 minutes and 1.0 M for 40 minutes. Values obtained for each standard were corrected for endogenous levels of SAH and SAM present in the original homogenate. Standard curves were linear for peak height vs, nanomoles for both compounds. Typical standard curves are shown in Figures 6 and 7; the correlation coefficients were 0.997 and 0.998 for SAH and SAM, respectively.

5. Peak Identification

Peaks from the HPLC chromatogram were identified in several ways.

a) Addition of authentic samples of SAH and SAM co-chromatographed with the endogenous peaks of SAH and SAM in the original homogenate.

b) Non-extracted standards containing 50 nmoles each of SAH and SAM were prepared to contain 0.1 μCi of S-(methyl-¹⁴C)-adenosyl-L-methionine prior to column application. They were eluted as previously described and 3 minute fractions collected. Aliquots from each fraction were analyzed for radioactivity using a Packard liquid scintillation counter. In 2 separate runs, the recovery of ¹⁴SAM was 91.7 and 94.1%. Of the
FIGURE 6. Representative standard curve for S-adenosylhomocysteine obtained by adding known amounts of standard to tissue, and extracting and analyzing by HPLC as described in methods.
FIGURE 7. Representative standard curve for S-adenosylmethionine obtained by adding known amounts of standard to tissue, and extracting and analyzing by HPLC as described in methods.
recovered radioactivity 91.5 and 89.8% were located in the fractions previously identified as SAM, 0.4% were located in the fractions previously identified as SAH, and 6.8 and 5.1% were in fractions probably resulting from slight on-column degradation due to the elevated temperature. SAM has been shown to be somewhat unstable at elevated temperatures (Schlenk, 1965).

c) The peaks corresponding to SAH and SAM were also identified using gas chromatography-mass spectroscopy. Fractions corresponding to these peaks and several other prominent peaks were collected and lyophilized. All mass spectroscopy was done with the technical assistance of Mr. James Ferguson and Mr. Robert Pegues.

Mass spectra were obtained using chemical ionization. The temperature used for chromatography was from 170° - 270° C at 10°/min. One mg of each standard was mixed with 0.1 ml of Sil-prep (Applied Sciences Laboratories, State College, PA), a commercial product for the preparation of trimethylsilyl derivatives. Vials were mixed well and allowed to stand for 5 minutes for the reaction to occur to completion. They were evaporated to dryness under N₂, and reconstituted with 50 μl of chloroform. Samples were derivatized in the same manner, except that 5 mg of lyophilized sample was mixed with 0.1 ml of Sil-prep. Both the SAH and SAM peaks were confirmed using this technique. In addition, peaks corresponding to inosine, adenosine, and 5-methylthioadenosine (MTA) were identified (see Figure 5). Both inosine and adenosine have been shown by Hoffman (1978) to chromatograph prior to SAH using an Aminex A-6 column eluted with 0.4 M ammonium formate.
Peaks corresponding to adenosine and MTA were also confirmed by adding authentic compounds to tissue samples. These spiked standards co-chromatographed with the endogenous peaks of adenosine and MTA. Standard curves were determined for each of these compounds using a series of added standards (Figures 8 and 9), as they were for SAH and SAM.

6. Reproducibility

Three separate runs were made from the same sample of tissue plus standard. Values for SAH and SAM were 60.8 ± 5.1 and 61.9 ± 4.8 (mean ± S.D.), respectively; the coefficients of variation were 8.4% for SAH and 7.8% for SAM.

H. Experimental Design

Experiments were conducted to determine the following:

1) The uptake of a single oral dose of $^{75}$Se over a 24 hour period into liver, kidney and testes.

2) The biochemical effects of exposure to selenite over a 10 week period, especially the effect on the selenoenzyme, GSH-Px.

3) The biochemical effects of exposure to a combination of an inorganic form of selenium, selenite, and an organic form, selenomethionine, over an 8 week period.

4) The biochemical effects of chronic toxicity from exposure to inorganic, and a combination of inorganic and organic forms of selenium, for 24 weeks, on S-adenosylhomocysteine and S-adenosylmethionine concentrations.
FIGURE 8. Representative standard curve for adenosine obtained by adding known amounts of standard to tissue, and extracting and analyzing by HPLC as described in methods.
FIGURE 9. Representative standard curve for 5-methylthioadenosine (MTA) obtained by adding known amounts of standard to tissue, and extracting and analyzing by HPLC as described in methods.
1. Uptake of Single $^{75}$Se Dose

Male Holtzman rats weighing from 200-250 g were given a single dose of 50 $\mu$Ci $^{75}$Se as Na$_2$SeO$_3$ (S.A. 63 mCi/mg, New England Nuclear Corp.) by intubation. At 1, 3, 6, 9, 12 or 24 hours after receiving the radioactivity, the animals were killed by decapitation. Livers, kidneys and testes were removed and homogenized in 4 volumes of phosphate buffer. Aliquots of the tissue homogenates were analyzed for radioactivity using a Packard gamma scintillation counter. Acid soluble and insoluble fractions of these homogenates were prepared by precipitation and washing with 10% trichloroacetic acid (TCA). They were also assayed for radioactivity. Protein concentrations were determined by the method of Bradford (1976).

2. Biochemical Effects of Prolonged Exposure to Selenite

a) Feeding Schedule

Twenty-one day-old male weanling rats of the Holtzman strain were housed in suspended wire cages in groups of 8 to 10 per cage following a one week acclimation period. Rats were divided into three groups, a food control group, a water control group, and a deficient group. All groups except the food control were fed a Torula yeast-based diet (Rotruck et al., 1972) deficient in selenium (<0.01 ppm) that contained: (in %) Torula yeast, 30; sucrose, 55.7; vitamin E free lard, 5; cod liver oil, 3; salt mix, 5; vitamin mix, 1; DL-methionine, 0.3; and 50 mg d-$\alpha$-tocopherol per kg of diet (Table 1). The group designated as the food control received the same diet supplemented with 0.5 ppm selenium as selenite (Na$_2$SeO$_3$) and selenium-free water. Of the groups fed the selenium
### TABLE 1

Composition of Basal Diet\(^1,2\)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% of Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torula yeast(^3)</td>
<td>30.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>55.7</td>
</tr>
<tr>
<td>Vitamin E-free lard</td>
<td>5.0</td>
</tr>
<tr>
<td>Cod Liver Oil</td>
<td>3.0</td>
</tr>
<tr>
<td>Salt mix(^4)</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mix(^5)</td>
<td>1.0</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.3</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^1\)The diet is similar to that described by Rotruck et al. (1972) but contains 50 IU/kg diet vitamin E and the addition of menaquinone (Vit K\(_2\)) rather than menadione (Vit K\(_3\)).

\(^2\)ICN Nutritional Biochemicals, Cleveland, OH 44138

\(^3\)Selenium content <0.01 ppm

\(^4\)Salt mix (g/kg): CaCO\(_3\), 543.0; MgCO\(_3\), 25.0; MgSO\(_4\), 16.0; NaCl, 69.0; KCl, 112.0; KH\(_2\)PO\(_4\), 212.0; FePO\(_4\)\(_4\)H\(_2\)O, 20.5; KI, 0.08; MnSO\(_4\), 0.35; NaF, 1.0; Al\(_2\)(SO\(_4\))\(_3\)\(\cdot\)K\(_2\)SO\(_4\), 0.17; CaSO\(_4\), 0.9.

\(^5\)Vitamin mix (per 100 g): glucose monohydrate, 88.54 g; thiamine·HCl, 40 mg; riboflavin, 25 mg; pyridoxine·HCl, 20 mg; calcium-DL-pantothenate, 200 mg; choline chloride, 10 g; niacin, 1 g; menaquinone, 50 mg; folic acid, 20 mg; biotin, 10 mg; vitamin B\(_12\) triturate (0.1% B\(_12\)), 100 mg. Gelatin coated vitamin A palmitate (to provide 2500 IU/100g of diet) and vitamin D\(_2\) (to provide 200 IU/100g of diet) were added to the vitamin mix prior to diet mixing.
deficient diet, one group was classified as the water control and received double-distilled, demineralized water containing 0.1 ppm selenium as Na$_2$SeO$_3$ (Pfaltz and Bauer, Inc.) *ad libitum*. All other animals were classified as deficient, receiving the selenium deficient diet and selenium free distilled water. After three weeks, three rats were randomly selected from the food control, water control and deficient groups and were handled as described below under *Experimental Treatment*. These nine rats formed the baseline or zero time sample group (Table 2). The food control group was not continued beyond the baseline time point. The remaining deficient rats were divided into three groups of six rats per group which were repleted with 1.0, 1.5 or 2.0 ppm selenium as sodium selenite in drinking water. The water control group was maintained as previously described. All groups continued to receive the selenium deficient diet *ad libitum*. After two weeks of repletion, three rats were removed from each group (0.1 ppm water control, 1.0, 1.5 and 2.0 ppm) for treatment as described below, forming the two week repletion sample group. After an additional eight weeks, the remaining three rats from each group were removed for treatment, constituting the ten week repletion sample group. Records were kept of the food and water consumed by each group during the entire feeding period.

b) *Experimental Treatment*

Rats removed for treatment received 20 μCi/kg body weight $^{75}$Se as Na$_2$SeO$_3$ (S.A. 8.82 μCi/μg, Amersham/Searle Corp.) in a constant volume of 0.1 ml by intubation. They were immediately housed in glass metabolic chambers for 24 hours, during which urine, feces and expired air were collected. Food and water were available *ad libitum*. 
TABLE 2

EXPERIMENTAL TIME COURSE FOR THE ADMINISTRATION OF SELENITE IN DRINKING WATER.

<table>
<thead>
<tr>
<th>TIME</th>
<th>TREATMENT</th>
<th>GROUPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Weeks</td>
<td>— Control groups established.</td>
<td>1) Food Control</td>
</tr>
<tr>
<td></td>
<td>— Deficient state established.</td>
<td>2) Water Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) Deficient</td>
</tr>
<tr>
<td>0 Weeks</td>
<td>— Sacrifice Baseline Rats</td>
<td>1) Water Control; 0.1 ppm</td>
</tr>
<tr>
<td>(Baseline)</td>
<td>— Begin repletion of Deficient Rats with Selenite.</td>
<td>2) 1.0 ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 1.5 ppm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4) 2.0 ppm</td>
</tr>
<tr>
<td>2 Weeks</td>
<td>— Sacrifice two week sample group.</td>
<td>Same as above</td>
</tr>
<tr>
<td>10 Weeks</td>
<td>— Sacrifice ten week sample group.</td>
<td>Same as above</td>
</tr>
</tbody>
</table>
Twenty-four hours after receiving $^{75}$Se, rats were killed by decapitation. Blood samples were collected, and plasma and packed cells were separated. Various tissues and organs were removed blotted and weighed, and portions counted in a 2 x 2, well type gamma scintillation spectrometer (Packard Model 3001) at 80% efficiency. Total volumes of urine and feces were recorded and aliquots taken for counting. Activated charcoal filters from the expired air lines were also analyzed for radioactivity.

Immediately following sacrifice, a portion of the median lobe of the liver and one kidney from each animal were homogenized separately in three volumes of ice cold (0 - 4° C) 0.05 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer containing 5 mM EDTA, pH 7.0. Glutathione peroxidase (GSH-Px) activities were determined in liver, kidney and plasma samples using H$_2$O$_2$ as the substrate by the method of Paglia and Valentine (1967), and selected tissues were assayed for total selenium content using the method of Hoffman et al. (1968). Protein concentrations were determined by the method of Lowry et al. (1951). Data were analyzed using unpaired Student's t-test, analysis of variance, and Duncan's new multiple range test (Li, 1964).

3. Biochemical Effects of Prolonged Exposure to Selenite and Selenomethionine

Experiments combining exposure to an inorganic form of selenium selenite, administered in water, and an organic form, selenomethionine, (SM), administered in food, were conducted. The purpose of these experiments was to determine the potency of the organic forms of selenium vs. the inorganic, and to simulate naturally occurring environmental exposure to the element.
Feeding Schedule

Male Holtzman rats weighing 80-100 g were housed in pairs in suspended wire cages following a two week acclimation period. All animals were made deficient for three weeks by feeding a Torula yeast diet containing < 0.01 ppm Se, described previously, and selenium-free water. Due to the large number of groups and animals, the study was divided into three parts, designated phase 1, 2 and 3. Each phase corresponded to one dietary concentration of selenomethionine, either 0.12, 0.60 or 1.20 ppm, and varying levels of selenium, as selenite, administered in water. Selenomethionine was added to the basal diet during preparation.

In phase 1, animals made deficient for three weeks were repleted with either 0.1, 0.5, 1.0, or 1.5 ppm Se as selenite in water and 0.12 ppm selenium as selenomethionine in food (Figure 10). In phase 2, animals were made deficient for three weeks, randomly placed in groups, and then repleted with 0.60 ppm selenium as selenomethionine and 0.0, 0.1, 0.5, 1.0, or 4.0 ppm selenium as selenite. In phase 3, animals were given tetracycline in water for one week prior to the initiation of the deficient state. This was to attempt to decrease the high incidence of pneumonia which had occurred during phase 2. Only 2% died as the result of pneumonia, confirmed by post-mortem examination, as compared to 20% in phase 2. In phase 3, the level of selenium as selenomethionine was 1.2 ppm, the highest amount of organic selenium used. Five levels of selenium as selenite, 0.0, 0.1, 0.5, 1.0, or 2.0 ppm were administered in drinking water combined with the selenomethionine in food. In addition, a control group, equivalent to the control used previously in the drinking water study, was maintained.
FIGURE 10. Experimental design for the combination selenite-selenomethione study. Non-stippled areas represent the groups which were tested, stippled areas represent the groups which were not tested (N.D. = not done).
on 0.1 ppm selenium as selenite in drinking water and on the selenium deficient diet. Food and water were available ad libitum.

The highest level of selenite in phase 1 was 1.5 ppm. This was increased in phase 2 to 4.0 ppm in an attempt to produce a more severe toxic response. However, water consumption decreased significantly in this group of rats. The level of selenite was therefore decreased to 2.0 ppm, the highest level given in the previous drinking water study, to restore water consumption to normal.

In each phase after animals were made deficient for three weeks, a baseline or zero time group was randomly chosen and handled as described below, and the remaining animals were then randomly assigned to the above mentioned groups. They were killed at two, four, and eight weeks following repletion with selenite and selenomethionine (Figure 11). There were four rats per group per time point.

Animals were weighed weekly. Records were kept of the food and water consumed by each group of rats during the entire feeding period.

b) Experimental Treatment

Rats removed for treatment received 20 µCi/kg body weight $^{75}$Se as Na$_{2}$SeO$_{3}$ (S.A. 63 mCi/mg, New England Nuclear Corp.) in a constant volume of 0.1 ml. Three hours after receiving $^{75}$Se, they were anesthetized with ether and exsanguinated by cardiac puncture. Blood was collected using heparinized vacutainer tubes. A portion of the blood collected was centrifuged, and plasma and packed cells separated. Livers were perfused with 0.9% saline, excised, rinsed several times with ice cold saline and
At 0, 2, 4, and 8 Weeks following repletion, four rats per group were sacrificed. The Livers and Testes were assayed for GSH-Px and Protein Methylase Activity. The Kidneys were assayed for GSH-Px Activity only.

FIGURE 11. Experimental time course for selenite-selenomethionine study. Rats were made deficient for 3 weeks and were then repleted with selenomethionine in food and selenite in water. Four rats from each group were sacrificed at 0, 2, 4, and 8 weeks following repletion.
weighed. Each liver was divided into three parts for various measurements described below. Kidneys and testes were also removed, rinsed in saline, blotted and weighed.

A portion of liver, the kidneys, and one testis were homogenized separately in 4 volumes of cold (0-4°C) 0.05 M phosphate buffer, pH 7.0, containing 5 mM EDTA. Both selenium-dependent (SD) and total glutathione peroxidase activities were determined in liver, kidney and testes at all time points by the method of Paglia and Valentine (1967), previously described in detail. Hydrogen peroxide was used as the substrate to determine the activity of the selenium dependent form of the enzyme, while cumene hydroperoxide was used to determine total GSH-Px activity, both the SD and the non-selenium dependent (NSD) forms. An estimate of the activity of the NSD form was obtained by subtracting the activity of the SD from the total activity.

An aliquot of homogenate from each of the above organs and of blood and plasma was analyzed for radioactivity using a Beckman 4000 gamma scintillation spectrometer. The soluble and insoluble fractions of the tissue homogenates were also analyzed for radioactivity.

A second portion of liver was homogenized in nine volumes of phosphate buffer, pH 7.9. A 10,000 x g supernatant fraction was prepared and analyzed for IMT by the method of Bayer (1977).

The remaining portion of liver and testis were rinsed several times in ice cold distilled water and homogenized separately in 4 volumes of ice cold water. Protein methylase I activities were measured by the method of Paik and Kim (1968).
Gamma-glutamyl transpeptidase (\(\gamma\)GTP) activity (Szasz, 1969) was measured in plasma at all levels of repletion at all time periods, to monitor liver damage. Both red and white blood cell counts were done on all animals. Proteins were by the method of Bradford (1976). Data were analyzed using unpaired Student’s t-test, analysis of variance, and Duncan’s new multiple range test (Li, 1964).

4. Chronic Toxicity: Effects on S-Adenosylhomocysteine and S-Adenosylmethionine

As stated in the introduction, Hoffman (1977) reported that acute toxic doses of selenite resulted in a depletion of liver SAM, an elevation of liver SAH, and inactivation of methionine adenosyltransferase. The purpose of this experiment was to determine whether the changes in methylating capacity accompanying acute toxicity also occurred chronically resulting in accumulation of selenium and alterations in normal methyl metabolism.

Rats were maintained on 0.1, 0.5, 1.0 and 2.0 ppm selenium as selenite in water in combination with 1.2 ppm selenium as selenomethionine in food. Controls received no selenite in water (0 group) and 1.2 ppm selenium as selenomethionine. An additional group of rats was maintained on 2.0 ppm selenium as selenite alone, with no selenomethionine added to the basal deficient diet. The control for the 2.0 ppm selenite group received 0.1 ppm selenium as selenite and a selenium deficient diet. All the animals received the regimens for a period of 24 weeks.
Animals were anesthetized with ether, and one testis and a portion of liver immediately removed and frozen in liquid nitrogen. They were then exsanguinated by cardiac puncture. Frozen tissues were prepared for analysis of SAH and SAM by HPLC as previously described. The remaining liver was then removed, rinsed in 0.9% saline, divided into three parts and homogenized in the appropriate buffers for the measurement of GSH-Px, IMT, and protein methylase I. The remaining testis was homogenized in 4 volumes of water and assayed for protein methylase I activity. In addition, rats from each group were killed, autopsied, and their tissues prepared for histological evaluation.
RESULTS

A. Uptake of Single $^{75}\text{Se-Na}_2\text{SeO}_3$ Dose into Liver, Kidney and Testes

Untreated rats were given a single dose of 50 μCi $^{75}\text{Se}$ as sodium selenite by intubation and were killed at various times from 1-24 hours following dosing. The purpose of this experiment was to determine suitable time periods after dosing for the labelling of liver, kidney and testes in subsequent experiments.

In all of the above organs, uptake of $^{75}\text{Se}$, standardized by protein concentration, increased with time from 1 to 24 hours (radioactive uptake was determined at 1, 3, 6, 9, 12 and 24 hours following dosing). After three hours, uptake in liver and kidney was greater than 50% of the 24 hour uptake, and in testes it was approximately 20% of the 24 hour value. The lower value seen with testes at 3 hours was not unexpected, since a time-dependent uptake in testes has previously been shown to occur for approximately 15 days after a single dose of $^{75}\text{Se-Na}_2\text{SeO}_3$ (Trapp and Millam, 1975).

The amount of radioactivity associated with the acid insoluble (protein) and with the acid soluble phases was also determined. Three hours following oral administration of $^{75}\text{Se}$, 53% of the radioactivity present in liver, 66% of that in kidney and 75% of that in testes was associated with the protein fraction. However, by 24 hours, 94%, 89% and 86% of the radioactivity in liver, kidney and testes, respectively, was
associated with the protein fraction. Three and 24 hours after dosing were chosen as the time periods for labelling of organs in future experiments. An interval of 24 hours after the administration of the $^{75}\text{Se}$ tracer was acceptable for metabolic studies in which urine, feces and expired air are collected. At this time point, uptake was maximal in the organs assayed. After three hours, although uptake was not maximal, it was sufficient. In addition, the amount of $^{75}\text{Se}$ associated with the insoluble phase was lower than after 24 hours. Therefore, it would be possible to detect changes in the amount of $^{75}\text{Se}$ associated with proteins in animals of differing selenium states, should this occur.

B. Biochemical Effects of Prolonged Exposure to Selenite

Rats were made deficient for 3 weeks, and then repleted with 1.0, 1.5 or 2.0 ppm selenium as sodium selenite in drinking water for 10 weeks. Controls received 0.1 ppm selenium as selenite in water for the entire period. The purpose of this experiment was to determine the level at which selenium administered in drinking water becomes toxic, as indicated by biochemical indices rather than by weight loss, liver disease, and death.

1. Food and Water Consumption and Liver to Body Weight Ratios

There were no apparent differences in food consumption between selenite treated and control animals. Control rats, which received 0.1 ppm selenium as selenite in drinking water, generally consumed a greater quantity of water than treated rats, but patterns of consumption were similar in all groups. The group receiving a 2.0 ppm as selenite consumed
approximately 10% less water than the control group. Groups receiving 1.0 and 1.5 ppm as selenite consumed a quantity of water intermediate between the 2.0 ppm group and the control. None of these differences were statistically different.

Liver to body weight ratios were calculated. At baseline the ratios for the water control group were significantly higher (P < 0.01) than the selenium deficient group (0.053 ± 0.001 vs 0.037 ± 0.002). At 2 and 10 weeks after repletion, there were no differences between the ratios of the controls and any treated group at each time point. However, liver to body weight ratios decreased from a mean of all groups 0.045 at 2 weeks to a mean of 0.032 at 10 weeks, which occurs normally with increasing age. There were no significant differences in body weights between groups at each time point.

2. GSH-Px Determinations

Selenium-dependent GSH-Px activity was determined in liver, kidney and plasma at baseline and at 2 and 10 weeks after repletion with selenium supplemented drinking water. In the tissue samples, (at baseline), the enzymatic activity of the water control group (0.1 ppm) did not differ significantly from the food control, which was given 0.5 ppm as selenite in food and selenium-free water for the three weeks prior to the baseline time point. This level of selenium in food has previously been established as a control diet (Hafeman et al., 1974). This amount of selenium supplemented in drinking water, 0.1 ppm as selenite, was therefore a comparable control to feeding studies of others and was used as the control group for this study.
Baseline (0 weeks) liver GSH-Px activities in 3 week deficient animals were significantly depressed (P < 0.01) compared to the 0.1 ppm control (Figure 12). Two weeks following repletion, there was a significant dose effect (P < 0.01), analyzed by analysis of variance. The significant differences between doses were evaluated using Duncan's new multiple range test. In those animals repleted with 2.0 ppm selenium, GSH-Px activity was maximal and significantly elevated above both the 0.1 ppm control and the 1.0 ppm group (P < 0.01). The activity in the 1.5 ppm group was also elevated above the 1.0 ppm group (P < 0.05). There were no other significant differences between groups. Two week GSH-Px activities were, therefore, increased in a dose-related manner with increasing concentrations of selenium. This increase in enzymatic activity with increasing dietary selenium has previously been shown to occur in non-toxic states (Hafeman et al., 1974). After ten weeks of repletion, there was also a significant difference between animals receiving different selenium doses (P < 0.01). However, maximal activity occurred in the 1.0 ppm group, rather than in the 2.0 ppm group as occurred after 2 weeks. Activity in the 1.0 ppm group was elevated above the 0.1 ppm water control (P < 0.01), while activity in the 1.5 ppm group was significantly lower than both the control (P < 0.05) and the 1.0 ppm group (P < 0.01). GSH Pxx activity in the 2.0 ppm group did not differ from control, but was also lower than the 1.0 ppm group (P < 0.01).

Figure 12 also shows the effect of time on each level of selenium administered. Control GSH-Px activity increased from baseline to 2 weeks and from 2 weeks to 10 weeks (P < 0.01). Activity in the 1.0 ppm group
LIVER SELENIUM DEPENDENT GSH-Px ACTIVITY

![Graph showing liver GSH-Px activity](image)

FIGURE 12. All rats except the 0.1 ppm controls were made deficient for 3 weeks, and were then repleted with 1.0, 1.5 or 2.0 ppm selenium as selenite for 10 weeks. Liver GSH-Px activity was measured at 0, 2 and 10 weeks following repletion by the method of Paglia and Valentine as described in methods. Values represent the mean ± S.E.M. Significant differences between groups are noted (*) following the time point at which they occurred, P<0.01.
increased dramatically (P<0.01) from 2 weeks to 10 weeks, while in the 1.5 ppm group it decreased (P<0.01). Although GSH-Px activity also appeared to decrease from 2 to 10 weeks in the 2.0 ppm group, this change was not statistically significant.

Prior to repletion, kidney GSH-Px activity in deficient animals was lower (P<0.01) than the control (Figure 13). At both 2 and 10 weeks of repletion, there were no significant differences between any levels of repletion (0.1 ppm control, 1.0, 1.5, and 2.0 ppm). No trend of an increase in activity at either 2 or 10 weeks corresponding to increasing levels of selenium was observed, as was for liver GSH-Px after 2 weeks of repletion. However, the 10 week kidney activities tended to be elevated above their respective 2 week values (1.5 ppm group, P<0.05), and the 2 and 10 week control values were elevated above the baseline value (P<0.01).

Plasma GSH-Px activity was also significantly depressed (P<0.01) in deficient animals compared to the control (Figure 14). There was a significant dose effect upon GSH-Px activity after 2 weeks of repletion (P<0.025). The enzymatic activity of the 2.0 ppm group was elevated above the control and the 1.0 ppm groups (P<0.01), and the 1.5 ppm group (P<0.05). After 10 weeks of repletion, a dose effect also occurred (P<0.025) having the pattern of increasing enzyme activity corresponding to increasing levels of selenium repletion. The plasma GSH-Px activities of both the 1.0 ppm and 2.0 ppm groups were elevated above control (P<0.05 and P<0.01, respectively). The activity of the 2.0 ppm group was also elevated above the 1.0 ppm group (P<0.05). As occurred in kidney, 10 week plasma GSH-Px activities tended to be elevated above
FIGURE 13. Rats were treated as described in Figure 12, and GSH-Px activity was measured in kidney at 0, 2 and 10 weeks following repletion. Values represent the mean ± S.E.M. for three rats.
FIGURE 14. Rats were treated as described in Figure 12, and GSH-Px activity was measured in plasma at 0, 2 and 10 weeks following repletion. Values represent the mean ± S.E.M. for three rats. Significant differences between groups are noted (*) following the time points at which they occurred, P<0.025.
their respective 2 week values (in 1.0 ppm group, P <0.01), and the 2 and 10 week control values were higher compared to the baseline control value (P <0.01).

A comparison of 10 week liver, kidney, and plasma GSH-Px between the 1.0 ppm group, and the 2.0 ppm group is shown in figure 15. There was no change in the activity of the kidney enzyme and activity in the plasma increased in a dose-related manner. However, GSH-Px activity in the liver decreased significantly in the high selenium group, indicating that liver is a target organ for inorganic selenium toxicity and that the kidney and plasma are not. A decrease in GSH-Px in liver activity has been shown to occur in livers exhibiting gross pathological changes following the chronic administration of 5.0 ppm selenium (Hafeman, 1974).

3. Specific Activity Determinations

The values for specific activity, expressed as nCi $^{75}$Se/ug total Se, for liver, kidney and plasma are shown in Table 3.

At baseline, specific activity in liver was elevated in the deficient group as compared to the 0.1 ppm control, although not significantly. This elevation in the deficient group resulted from a lower uptake of radioactivity and a lower selenium content in liver. After 2 weeks of repletion, specific activities tended to decrease in a concentration-dependent manner, resulting from both a slight decrease in uptake of $^{75}$Se and an increase in selenium content with increasing levels of selenium repletion. Ten weeks following repletion, specific activities in the 1.0, 1.5 and 2.0 ppm groups were lower than the 0.1 ppm control, but did not differ from each other (P <0.01). Uptake of $^{75}$Se tended to be lower
FIGURE 15. GSH-Px activities in liver, kidney and plasma were compared to each other 10 weeks following repletion with 1.0 and 2.0 ppm as selenite. Values represent the mean ± S.E.M for three rats; significant at P<0.01 by Student's t-test (*).
### TABLE 3

Specific Activities in Liver, Kidney and Plasma 24 Hours Following A Single Dose of $^{75}\text{Se-} \text{Na}_2\text{O}_3$

<table>
<thead>
<tr>
<th>Selenite (ppm)</th>
<th>LIVER $^{75}\text{Se/µg Se}$</th>
<th>KIDNEY $^{75}\text{Se/µg Se}$</th>
<th>PLASMA $^{75}\text{Se/µg Se}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 (Control)</td>
<td>58.4 ± 10.6$^a$</td>
<td>57.2 ± 10.0</td>
<td>85.8 ± 7.7</td>
</tr>
<tr>
<td>Deficient</td>
<td>89.6 ± 5.0</td>
<td>102.3 ± 9.2</td>
<td>188.1 ± 19.4$^b$</td>
</tr>
<tr>
<td><strong>Two Weeks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>27.3 ± 3.6</td>
<td>45.5 ± 6.6</td>
<td>62.9 ± 4.3</td>
</tr>
<tr>
<td>1.0</td>
<td>21.2 ± 2.8</td>
<td>39.5 ± 2.3</td>
<td>57.9 ± 7.9</td>
</tr>
<tr>
<td>1.5</td>
<td>19.0 ± 2.2</td>
<td>24.3 ± 2.5$^b,c$</td>
<td>38.1 ± 7.1$^b,c$</td>
</tr>
<tr>
<td>2.0</td>
<td>14.4 ± 2.8</td>
<td>18.9 ± 1.2$^b,c$</td>
<td>22.5 ± 1.4$^b,c$</td>
</tr>
<tr>
<td><strong>Ten Weeks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>39.3 ± 2.5</td>
<td>53.0 ± 4.7</td>
<td>62.3 ± 5.0</td>
</tr>
<tr>
<td>1.0</td>
<td>20.3 ± 0.6</td>
<td>24.9 ± 2.6$^b$</td>
<td>47.0 ± 5.2$^b$</td>
</tr>
<tr>
<td>1.5</td>
<td>21.4 ± 1.1$^b$</td>
<td>33.9 ± 4.0$^b$</td>
<td>45.1 ± 1.9$^b$</td>
</tr>
<tr>
<td>2.0</td>
<td>21.2 ± 0.5$^b$</td>
<td>39.9 ± 4.8$^b$</td>
<td>49.8 ± 3.6</td>
</tr>
</tbody>
</table>

$^a$ Values are means ± S.E.M. for three rats. At each time point in each tissue, values were tested by analysis of variance to determine whether significant differences existed between groups. Duncan's multiple range was used as a post-test to determine between which groups these differences existed. The level of significance was at least $P < 0.05$.

$^b$ Statistically different from 0.1 ppm group by Duncan's multiple range.

$^c$ Statistically different from 1.0 ppm group by Duncan's multiple range.
and selenium content higher in these groups than the control, with only small differences between groups.

In kidney at baseline, specific activity was also elevated in deficient rats compared to control, mainly as a result of a decreased selenium content. As in liver, this increase was not statistically significant. As occurred in liver, specific activities in kidney decreased in a dose-related fashion after 2 weeks of repletion (P < 0.01). Specific activities in both the 1.5 ppm and 2.0 ppm groups were significantly lower than the values of the 0.1 and 1.0 ppm groups. This decrease was largely due to increases in selenium content with increasing levels of repletion. After 10 weeks of repletion, specific activities at all levels of repletion were lower than the 0.1 ppm control (P < 0.025).

In plasma at baseline, the specific activity in deficient animals was elevated above the control (P < 0.01), again mainly due to a decrease in selenium content. Two weeks following repletion, the dose-dependent decrease in specific activity with increasing levels of selenium repletion was again evident (P < 0.01), resulting from a decrease in radioactivity uptake of $^{75}$Se and an increase in selenium content with increasing levels of repletion. After 10 weeks of repletion, the specific activity in the 1.0 and 1.5 ppm groups was lower than the 0.1 ppm control (P < 0.025). The activity in the 2.0 ppm group was also lower than the control, although the difference was not statistically significant.

The results show that specific activities in liver, kidney and plasma are elevated in deficiency and that they decline following repletion with various amounts of selenite. However, there were no differences between any levels of repletion at 10 weeks (1.0, 1.5 or 2.0 ppm) which could be used to define toxic changes.
4. Excretion of Selenium

The $^{75}$Se excreted in the expired air was collected in charcoal filters and counted. Two weeks following repletion, the amount of radioactivity excreted in the expired air increased with increasing levels of repletion. At 10 weeks, the group repleted with 1.0 ppm selenium as selenite excreted a greater amount of radioactivity than the control, while the amount of $^{75}$Se excreted by the lungs in the 1.5 and 2.0 ppm groups did not differ from control. The total amount in all groups, however, was less than 0.1% of the administered dose. In all groups except the control, the amount of radioactivity excreted by the lung decreased from the 2 week value to the 10 week value. There were no differences in the amount of radioactivity excreted in the feces (0.35% of the administered dose) at any level of selenite repletion at 10 weeks.

Two weeks following repletion, the amount of radioactivity excreted in 24 hours in the urine increased above the 0.1 ppm control in a dose-related manner ($P<0.01$; Table 4). After 10 weeks of repletion, the amount of radioactivity in the 24 hour urine also increased in a dose-related manner ($P<0.05$). The absolute amount of $^{75}$Se excreted at both time points per level of selenite was comparable. However, if expressed as a percentage of the original dose, the percentage excreted in the 1.0, 1.5 and 2.0 ppm groups was lower at the 10 week time point as compared to each of these groups at 2 weeks, since each rat received 20 μCi/kg body weight. The total amount of selenium in urine excreted in the 24 hour period at both 2 and 10 weeks increased with increasing levels of repletion. However, if the 2 week data is compared to the 10 week data, approximately 50% less total selenium was excreted at each level of repletion.
### TABLE 4

**Urinary Excretion of Selenium**

<table>
<thead>
<tr>
<th>Selenite (ppm)</th>
<th>Total Se Output 24 hrs (µg)</th>
<th>$^{75}$Se Output 24 hrs (nCi)</th>
<th>% Mean $^{75}$Se-Dose Excreted 24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 Weeks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>$1.15 \pm 0.07^b$</td>
<td>$502.2 \pm 114.4^c$</td>
<td>9.3</td>
</tr>
<tr>
<td>1.0</td>
<td>$8.35 \pm 1.79^c$</td>
<td>$1241.1 \pm 133.8^c$</td>
<td>23.0</td>
</tr>
<tr>
<td>1.5</td>
<td>$15.45 \pm 3.08^c,d$</td>
<td>$1446.6 \pm 2.8^c,d$</td>
<td>25.2</td>
</tr>
<tr>
<td>2.0</td>
<td>$24.26 \pm 2.93^c,d$</td>
<td>$1694.3 \pm 101.3^c,d$</td>
<td>29.5</td>
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<tr>
<td><strong>10 Weeks</strong></td>
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<tr>
<td>0.1</td>
<td>$3.41 \pm 0.35$</td>
<td>$895.4 \pm 125.4$</td>
<td>10.5</td>
</tr>
<tr>
<td>1.0</td>
<td>$4.63 \pm 0.21$</td>
<td>$1234.3 \pm 109.8$</td>
<td>14.5</td>
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<tr>
<td>1.5</td>
<td>$7.05 \pm 0.42^c,d$</td>
<td>$1903.9 \pm 158.7^c$</td>
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</tr>
<tr>
<td>2.0</td>
<td>$11.09 \pm 2.25^c,d$</td>
<td>$1592.8 \pm 45.8^c$</td>
<td>18.4</td>
</tr>
</tbody>
</table>

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*a* At 2 and 10 weeks after repletion with the concentrations of selenium listed in the table, rats were given a single dose of $^{75}$Se-Na$_2$SeO$_3$ by intubation, placed in glass metabolic chambers, and the urine collected for 24 hours.

*b* The values are means ± S.E.M. for three rats. At each time point, values were tested by analysis of variance to determine whether significant differences existed between groups. Duncan's multiple range was used as a post-test to determine between which groups these differences existed. The level of significance was at least P< 0.05.

*c* Significantly different from the 0.1 ppm group by Duncan's multiple range.

*d* Significantly different from the 1.0 ppm group by Duncan's multiple range.
(1.0, 1.5 and 2.0 ppm) at 10 weeks than at 2 weeks. Total selenium excretion at 10 weeks in the 0.1 ppm control was greater than the excretion at 2 weeks.

C. Biochemical Effects of Prolonged Exposure to Selenite and Selenomethionine

Experiments combining exposure to an inorganic form of selenium, selenite, administered in water, and an organic, selenomethionine administered in food, were conducted. In phases 1, 2 and 3, the amounts of selenium as selenomethionine used were 0.12, 0.6 and 1.2 ppm, respectively. In each phase, varying amounts of selenite were administered in combination with the above levels of selenomethionine.

1. Body Weights and Liver to Body Weight Ratios

Body weights were recorded weekly and at the time of sacrifice. In phase 1 (0.12 ppm as selenomethionine), there was a trend toward a decrease in body weight in the 1.0 and 1.5 ppm groups, which began after approximately 5 weeks of repletion. There were, however, no significant differences in body weights between groups at each time point. In phase 2 (0.6 ppm as selenomethionine), there were no apparent differences in body weights between groups throughout the 11 weeks of the experiment, and there were no differences in body weight between groups at each time point. In phase 3 (1.2 ppm as selenomethionine), the mean body weight in the group also receiving 2.0 ppm as selenite tended to be lower than the other groups, beginning after approximately 3 weeks of repletion. The only significant differences in body weights between groups occurred at the 8 week time point. Body weights in both the 1.0 and 2.0 ppm groups
(473 ± 13 and 490 ± 26, respectively) were significantly lower than the 0.1 ppm selenite control and the 0.1 ppm (+SM) groups (575 ± 15 and 558 ± 19, respectively; P<0.01).

Liver to body weight ratios were calculated. As occurred previously in the selenite study, ratios in the rats repleted with the selenite-selenomethionine combination decreased with age. However, there were no significant differences in liver to body weight ratios between any levels of selenite repletion at each time point in either phase 1, 2 or 3.

2. Blood Cell Counts

Both red and white blood cells were counted at all time points at all levels of selenite-selenomethionine repletion. All values were within the normal limits which have been established for rats.

3. Food and Water Consumption

Food and water consumption were measured at weekly intervals. No significant differences in food consumption were observed between groups at any time points. Also, the addition of increasing amounts of selenomethionine to the diet did not seem to affect the food intake which ranged from 20-24 g/day, depending upon the age of the rat. In all phases, water consumption decreased somewhat in groups with higher amounts of selenite administered in water. In phase 1, the 1.5 ppm group consumed approximately 5% less water than the other groups. In phase 2, in those rats repleted with 4.0 ppm as selenite, water consumption decreased approximately 21% compared to other groups. In phase 3, the highest amount of selenite administered in water was decreased from 4.0 ppm to 2.0 ppm.
in an attempt to restore water consumption to that of control. Even so, the 2.0 ppm group consumed approximately 20% less water than the other groups.

4. Gamma-Glutamyl Transpeptidase

Gamma-glutamyl transpeptidase (γGTP) activity, an enzyme which is elevated in serum following liver damage, was determined at all levels of repletion at all time points. The normal range for males is from 4.5-24.8 mU/ml (Szasz, 1969; Kachmar and Moss, 1976).

In both phase 1 and 2 of the combined inorganic-organic selenium experiment γGTP activity was within normal limits in all cases, thus indicating that liver cell membrane damage had not occurred to any appreciable degree. (Huseby, 1979). In phase 3, γGTP for all groups except the 2.0 ppm group after 8 weeks was within the normal range of serum enzyme activity (range: 10.3-25.1 mU/ml). The two and four week values for the 2.0 ppm group were normal, 23.3 ± 4.2 and 12.8 ± 3.9, respectively. However, after eight weeks of repletion, γGTP activity in the 2.0 ppm group was elevated above the normal range (34.5 ± 4.5 mU/ml) indicating that some degree of liver damage had occurred (Wu et al., 1976; Huseby, 1979).

5. GSH-Px Determinations

a. Phase 1

In phase 1 of the combined inorganic-organic selenium experiment, four levels of selenium, as sodium selenite, 0.1, 0.5, 1.0 and 1.5 ppm, were administered in water in combination with 0.12 ppm
selenium as selenomethionine in food to rats previously made deficient for 3 weeks. Total and selenium-dependent (SD) GSH-Px activities were measured in liver, kidney and testes at 0, 2, 4, and 8 weeks following repletion.

In baseline deficient rats (0 weeks), liver SD GSH-Px activity was significantly depressed compared to all repleted values (Figure 16). Two weeks following repletion, the activity in all groups had increased markedly from deficient values. Although there were no significant differences between any groups, there was a slight increase in mean activity with increasing levels of selenium repletion as occurred previously after 2 weeks with repletion of increasing amounts of selenite alone. Four weeks following repletion with 0.12 ppm selenium as selenomethionine and varying amounts of selenium as selenite, there were again no significant differences between any groups. However, the mean SD activity in the 1.0 ppm and 1.5 ppm groups was somewhat lower than in the 0.1 ppm and 0.5 ppm groups. After 8 weeks of repletion, a significant treatment effect occurred (P < 0.05), which was determined by analysis of variance. Duncan's multiple range was used to determine between which groups the differences existed. The liver SD GSH-Px activity in the groups receiving 0.1 ppm and 1.5 ppm as selenite (+ 0.12 ppm as selenomethionine) was significantly lower than the 0.5 ppm group (+ selenomethionine), in which maximal enzyme activity occurred. The activity in the 1.0 ppm group was also below that in the 0.5 ppm group, although the difference was not statistically significant. The effect of time at each level of selenium administration can also be seen in figure 16.
All animals were given 0.12 ppm Selenium as Selenomethionine in food.

* p<0.05

FIGURE 16. (PHASE 1) All rats were made deficient for 3 weeks, and then repleted with 0.1, 0.5, 1.0 or 2.0 ppm selenium as selenite in combination with 0.12 ppm as selenomethionine. Liver GSH-Px activity (SD) was measured in deficient rats at 0 weeks, and in repleted rats at 2, 4 and 8 weeks following repletion. Values represent the mean ± S.E.M. for 4 rats. Significant differences between groups are noted (*) after the time point at which they occurred, P<0.05.
GSH-Px activity tended to increase from 2 to 4 to 8 weeks in the 0.5 ppm group (where maximal 8 week activity occurred), and to decrease from 2 to 4 to 8 weeks in the 1.5 ppm group.

Total liver GSH-Px activities for phase 1 are shown in Table 5. A similar pattern of changes in activity with time and dose occurred with total activity as occurred with SD GSH-Px activity. Activity was depressed in deficient animals. There was an apparent increase in total activity with increasing amounts of selenium after 2 weeks of repletion, this increase was no longer evident after 4 weeks of repletion, and by 8 weeks following repletion there was a significant decrease in the 1.0 and 1.5 ppm groups (P < 0.05) below the 0.5 ppm group, where maximal activity occurred. In addition, total 8 week GSH-Px activity in the 0.5 ppm group was significantly elevated above both its 2 and 4 week values, and total activity in the 1.5 ppm group tended to decrease from 2 to 4 to 8 weeks.

An estimate of the activity of the non-selenium dependent (NSD) form of GSH-Px, expressed as percentage of total activity, was obtained by subtracting the activity of the SD form from total enzymatic activity. In deficiency, liver NSD activity comprised 37% of the total activity, the greatest percentage of NSD activity compared to all other levels of repletion. The NSD form of the enzyme ranged from 10-34% of total activity in all levels of repletion throughout the 8 week duration, but did not follow any pattern such as either an increase or a decrease at toxic levels of repletion.

Kidney SD GSH-Px activity was also depressed in deficiency (figure 17). Two weeks following repletion, activity was maximal in the 1.0 ppm group, and it was significantly elevated above both the 0.1 ppm and 1.5
TABLE 5

Total GSH-Px Activity in Liver, Kidney and Testes
(Phase 1)

<table>
<thead>
<tr>
<th>SELENITE (ppm)</th>
<th>GSH-Px LIVER (nmoles/mg protein/min)</th>
<th>GSH-Px KIDNEY (nmoles/mg protein/min)</th>
<th>GSH-Px TESTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient (&lt;0.01)</td>
<td>47.3 ± 10.2⁴</td>
<td>39.3 ± 4.3</td>
<td>19.0 ± 0.7</td>
</tr>
<tr>
<td>2 Weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>787.1 ± 94.7</td>
<td>536.5 ± 48.1</td>
<td>37.3 ± 6.2</td>
</tr>
<tr>
<td>0.5</td>
<td>853.6 ± 43.8</td>
<td>521.2 ± 33.4</td>
<td>46.4 ± 9.2</td>
</tr>
<tr>
<td>1.0</td>
<td>1128.3 ± 130.9</td>
<td>655.5 ± 27.2⁵</td>
<td>48.9 ± 11.5</td>
</tr>
<tr>
<td>1.5</td>
<td>1166.3 ± 158.8</td>
<td>486.6 ± 33.5</td>
<td>43.0 ± 4.8</td>
</tr>
<tr>
<td>4 Weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>919.1 ± 113.6</td>
<td>530.5 ± 58.4</td>
<td>46.9 ± 1.5⁶</td>
</tr>
<tr>
<td>0.5</td>
<td>936.8 ± 62.6</td>
<td>463.7 ± 74.7</td>
<td>55.0 ± 3.5</td>
</tr>
<tr>
<td>1.0</td>
<td>787.2 ± 132.0</td>
<td>621.3 ± 48.1</td>
<td>61.2 ± 2.5</td>
</tr>
<tr>
<td>1.5</td>
<td>884.8 ± 149.8</td>
<td>711.8 ± 87.8</td>
<td>54.1 ± 2.2</td>
</tr>
<tr>
<td>8 Weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>867.8 ± 131.4</td>
<td>459.6 ± 49.9</td>
<td>46.1 ± 1.5</td>
</tr>
<tr>
<td>0.5</td>
<td>1182.5 ± 97.8</td>
<td>565.0 ± 41.4</td>
<td>43.7 ± 6.7</td>
</tr>
<tr>
<td>1.0</td>
<td>780.9 ± 53.5⁷</td>
<td>621.3 ± 128.8</td>
<td>50.1 ± 4.2</td>
</tr>
<tr>
<td>1.5</td>
<td>632.7 ± 95.0⁷</td>
<td>652.7 ± 62.8</td>
<td>51.0 ± 1.8</td>
</tr>
</tbody>
</table>

⁴Each number represents the mean ± S.E.M. (n=4). All rats except deficient received the above levels of selenium as selenite in water and 0.12 ppm as selenomethionine in food. At each time point in each tissue, values were tested by analysis of variance to determine whether significant differences existed between groups. Duncan's multiple range was used as a post-test to determine between which groups these differences existed. The level of significance was at least P < 0.05.

⁵Significantly different from 0.5 ppm group by Duncan's multiple range.

⁶Significantly different from 0.5 ppm group by Duncan's multiple range.

⁷Significantly different from 1.0 ppm group by Duncan's multiple range.

⁸Significantly different from 1.5 ppm group by Duncan's multiple range.
FIGURE 17. (PHASE 1) Rats were treated as described in Figure 16. SD GSH-Px activity was measured in kidney in deficient rats at 0 weeks, and in repleted rats at 2, 4 and 8 weeks following repletion. Values represent the mean ± S.E.M. for 4 rats. Significant differences between groups are noted (*) after the time point at which they occurred, P<0.05.
ppm groups (P < 0.05). There were no significant differences between groups at the four week time point, although the values in the 1.0 ppm and 1.5 ppm groups tended to be higher than those in the 0.1 ppm and 0.5 ppm groups. After 8 weeks of repletion, there were again no differences between groups. In addition, no significant time effects occurred at any level of selenium repletion.

Total kidney GSH-Px activity was also depressed in deficient rats, and the percentage of NSD GSH-Px was 34.4% (Table 5). As occurred in liver, total kidney GSH-Px closely followed the pattern of the SD form of the enzyme. The only significant difference between groups occurred after two weeks of repletion; activity in the 1.0 ppm group was elevated above both the 0.5 ppm and the 1.5 ppm groups. The NSD form of the enzyme ranged from 13-37%, and as in liver, it varied in a random fashion.

In testes, both SD and total activity were significantly depressed at baseline in deficient rats. Two weeks following repletion, SD GSH-Px activity increased approximately three-fold (Figure 18). There were no differences between the values at any level of repletion. A significant treatment effect occurred at the 4 week time point (P < 0.01), as activity increased in a dose-related manner. The activity in both the 1.5 ppm and 1.0 ppm groups was elevated above the 0.1 ppm group (P < 0.05 and P < 0.01, respectively). The activity in the 1.0 ppm group was also elevated above the 0.5 ppm group (P < 0.05). There was no difference in enzyme activity between the 1.0 and 1.5 ppm groups. Eight weeks following repletion, there were again no differences between the values at any level of repletion. The only significant time effect occurred in the 1.5 ppm group, in which both the 4 and 8 week activities were elevated above the 2 week values (P < 0.05).
FIGURE 18. (PHASE 1) Rats were treated as described in Figure 16. SD GSH-Px activity was measured in testes in deficient rats at 0 weeks, and in repleted rats at 2, 4 and 8 weeks following repletion. Values represent the mean ± S.E.M. for 4 rats. Significant differences between groups are noted (*) after the time point at which they occurred, P<0.01.

All Animals were given 0.12 ppm Selenium as Selenomethionine in Food.

* p<0.01
Total GSH-Px activity in testes also closely followed the SD activity. A significant treatment effect occurred only at the four week time point (Table 5). Total activity in the 1.0 ppm group was elevated above the 0.1 ppm group and activity tended to increase with increasing levels of selenium repletion. The percentage of the NSD form was 43.2% in deficiency, and ranged from 22-34% at all other points.

b. Phase 2

Since total GSH-Px activity closely followed the pattern of the SD form of the enzyme in liver, kidney and testes in phase 1, only the activity of the SD form of the enzyme is included for phases 2 and 3.

In phase 2, rats were again made deficient for 3 weeks. Five levels of selenium as selenite, 0, 0.1, 0.5, 1.0 and 4.0 ppm, were then administered in water in combination with 0.6 ppm as selenomethionine in food for an eight week period. In liver, the SD activity was significantly depressed after 3 weeks of deficiency (Figure 19). Two weeks following repletion, after supplementing with selenomethionine only (0 ppm as selenite), the SD activity was elevated approximately nine times that of the deficient state, and increased slightly at 4 and 8 weeks. Activity in the 0 ppm group did not differ from that in the 0.1 ppm group at any time point. There were no differences in liver SD activities between any groups at the 2 week time point. However, by 4 weeks, although no significant differences occurred, certain changes were evident. Activity tended to increase in the 0.1, 0.5 and 1.0 ppm (+ selenomethionine) groups and to decline in the 4.0 ppm group. Eight weeks following repletion, a significant treatment effect occurred (P<0.05). Enzyme activity increased from the 0.1 ppm group to the 0.5 ppm group, in which
FIGURE 19. (PHASE 2) All rats were made deficient for 3 weeks, and then repleted with 0.1, 0.5, 1.0 or 4.0 ppm selenium as selenite in combination with 0.6 ppm as selenomethionine. Liver SD GSH-Px activity was measured in deficient rats at 0 weeks, and in repleted rats at 2, 4 and 8 weeks following repletion. Values represent the mean ± S.E.M for 4 rats. Significant differences between groups are noted (*) after the time point at which they occurred, P<0.05.
maximal activity occurred. GSH-Px activity then declined in the 1.0 and 4.0 ppm groups. Activity in the 4.0 ppm group was significantly lower than the 0.5 ppm group (P < 0.05). No significant time effects per dose occurred.

In kidney, the SD GSH-Px activity was again depressed in deficiency (Table 6). As in liver, selenomethionine alone (0 ppm as selenite) yielded adequate enzymatic activity at all time points. After 2 weeks, significant differences between repletion groups occurred (P < 0.01). Enzyme activity in the 0.1 ppm and in the 0.5 ppm groups was elevated above both the 1.0 ppm and 4.0 ppm groups. However, at both 4 and 8 weeks following repletion, there were no differences between groups. Several time effects per dose occurred. In the 0.5 ppm group, the 4 week value was elevated above both the 2 and 8 week values (P < 0.05) and in the 1.0 ppm and 4.0 ppm groups the 4 and 8 week values were elevated above their respective 2 week values (P < 0.01).

In testes, SD GSH-Px activity was depressed in deficiency, and increased after 2 weeks of repletion and further after 4 weeks of repletion (Table 6). There were however, no differences between any groups at either 2 weeks or 4 weeks of repletion. After 8 weeks of repletion, there were again no differences between groups, however, activity in all groups declined to values near those obtained after 2 weeks. Adequate GSH-Px activity occurred at all time points in the 0 ppm group which received only selenomethionine.
<table>
<thead>
<tr>
<th>SELENITE (ppm)</th>
<th>GSH-Px KIDNEY (nmole/mg protein/min)</th>
<th>GSH-Px TESTES (nmole/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Deficient (&lt;0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>138.1 ± 11.2a</td>
<td>20.0 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>2 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>757.0 ± 119.4</td>
<td>41.3 ± 1.5</td>
</tr>
<tr>
<td>0.5</td>
<td>598.4 ± 73.1b,c</td>
<td>38.7 ± 1.4</td>
</tr>
<tr>
<td>1.0</td>
<td>332.4 ± 15.9b,c</td>
<td>37.1 ± 2.3</td>
</tr>
<tr>
<td>4.0</td>
<td>328.9 ± 31.9b,c</td>
<td>38.2 ± 0.8</td>
</tr>
<tr>
<td>4 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>1006.1 ± 37.9</td>
<td>49.2 ± 2.1</td>
</tr>
<tr>
<td>0.5</td>
<td>982.1 ± 104.1</td>
<td>52.9 ± 1.7</td>
</tr>
<tr>
<td>1.0</td>
<td>916.9 ± 131.1</td>
<td>45.3 ± 0.7</td>
</tr>
<tr>
<td>4.0</td>
<td>908.5 ± 16.3</td>
<td>52.5 ± 9.0</td>
</tr>
<tr>
<td>8 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>696.2 ± 69.6</td>
<td>35.4 ± 2.7</td>
</tr>
<tr>
<td>0.5</td>
<td>649.9 ± 64.7</td>
<td>37.9 ± 1.5</td>
</tr>
<tr>
<td>1.0</td>
<td>819.7 ± 7.0</td>
<td>36.2 ± 0.8</td>
</tr>
<tr>
<td>4.0</td>
<td>698.8 ± 93.5</td>
<td>41.9 ± 1.0</td>
</tr>
</tbody>
</table>

Each number represents the mean ± S.E.M. (n=4). All rats except deficient received the above levels of selenium as selenite in water and 0.6 ppm as selenomethionine in food. At each time point, values were tested by analysis of variance to determine whether significant differences existed between groups. Duncan's multiple range was used as a post-test to determine between which groups these differences existed. The level of significance was at least P <0.05.

bStatistically different from 0.1 ppm group by Duncan's multiple range.
cStatistically different from 0.5 ppm group by Duncan's multiple range.
c. **Phase 3**

In phase 3 of the combination inorganic-organic experiment, five levels of selenium as selenite, 0, 0.1, 0.5, 1.0 and 2.0 ppm, were administered in drinking water in combination with 1.2 ppm as selenomethionine given in food. In addition, a control group, equivalent to the control group used in the previous selenite-water study, was maintained on 0.1 ppm selenium as selenite in drinking water and a selenium deficient rat diet (<0.01 ppm). All rats were made deficient for 3 weeks prior to repletion with the above levels of selenite. Changes in SD GSH-Px activity in liver are shown in figure 20. The activity was significantly depressed in deficient (baseline or 0 time) animals compared to any level of repletion at any time point. Two weeks following repletion, after supplementing with selenomethionine only (0 ppm as selenite), the SD activity was elevated to values comparable to the 0.1 ppm control (C) and the 0.1 ppm (+ 1.2 ppm as selenomethionine) groups. Activity in the 0 ppm group increased slightly after 8 weeks of repletion. However, it did not differ from the control or the 0.1 ppm group at any time point. Significant treatment effects in liver occurred after both 2 and 8 weeks of repletion (P<0.025). After 2 weeks, GSH-Px activity in the 1.0 ppm group was elevated above all other groups, which did not differ from one another. By four weeks, there were no differences between any groups. After 8 weeks of repletion, enzyme activity increased in increasing selenium repletion in the 0.5 and 1.0 ppm groups. The activity in the 0.5 ppm group was significantly elevated above the 0.1 ppm group (P<0.05) and the activity in the 1.0 ppm group was elevated above the control (P<0.05) and the 0.1 ppm group (P<0.01). However, as occurred in both
FIGURE 20. (PHASE 3) All rats were made deficient for 3 weeks, and then repleted with 0.1, 0.5, 1.0 or 2.0 ppm selenium as selenite in combination with 1.2 ppm as selenomethionine. Control rats (C) received 0.1 ppm as selenite alone. Liver SD GSH-Px activity was measured in deficient rats at 0 weeks, and in repleted rats at 2, 4 and 8 weeks following repletion. Values represent the mean ± S.E.M for 4 rats. Significant differences between groups are noted (*) after the point at which they occurred, P<0.025.
phases 1 and 2, phase 3 SD GSH-Px activity in the group receiving the highest amount of selenite, the 2.0 ppm group, was significantly depressed compared to the group having maximal enzyme activity, the 1.0 ppm group ($P<0.05$). Significant time effects per dose occurred in both the 0.5 ppm ($P<0.025$) and the 2.0 ppm group ($P<0.01$). In both the 0.5 ppm and 2.0 ppm groups, the 4 and 8 week values were elevated above their respective 2 week values.

Kidney SD GSH-Px was depressed in deficiency (Table 7). The 0 ppm group, which received selenomethionine only, responded similarly to the 0.1 ppm group at all time points. In general, activity in all groups except the control, which received no selenomethionine, was near maximal after 2 weeks. At both 2 and 4 weeks following repletion, the control activity was lower than all other groups which were repleted with both selenite and selenomethionine. After 4 weeks, the activity in the 0.1 ppm group was also lower than the 0.5 and 2.0 ppm groups. There were no differences between any groups at the 8 week time point. There was a significant time effect only in the control group; the 8 week value was elevated above both the 2 week ($P<0.01$) and the 4 week values ($P<0.05$).

Testes SD GSH-Px activity was depressed in deficiency compared to the 0.1 ppm group at 2 weeks, but not compared to the control at 2 weeks (Table 7). The 0 ppm selenite group which received only selenomethionine did not differ from the 0.1 ppm group at any time point. Treatment effects occurred at the 2 and 8 week time points ($P<0.01$), and were the result of an increase in activity at higher levels of selenium repletion. There were significant time effects in all groups. In the control and 0.1 ppm groups, the 4 and 8 week values were elevated above their
<table>
<thead>
<tr>
<th>SELENITE (ppm)</th>
<th>GSH-Px (nmoles/mg protein/min)</th>
<th>GSH-Px (nmoles/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIDNEY</td>
<td>TESTES</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>82.4 ± 2.5^a</td>
<td>16.3 ± 0.1</td>
</tr>
<tr>
<td>(&lt;0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>267.1 ± 16.7</td>
<td>14.8 ± 1.1</td>
</tr>
<tr>
<td>0.1</td>
<td>506.8 ± 15.1^c</td>
<td>21.3 ± 0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>589.8 ± 12.7^c</td>
<td>44.8 ± 3.6^b,c</td>
</tr>
<tr>
<td>1.0</td>
<td>530.7 ± 50.3^c</td>
<td>47.0 ± 3.5^b,c</td>
</tr>
<tr>
<td>2.0</td>
<td>539.5 ± 52.9^c</td>
<td>47.0 ± 2.8^b,c</td>
</tr>
<tr>
<td>4 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>301.7 ± 23.1_b</td>
<td>29.5 ± 3.7</td>
</tr>
<tr>
<td>0.1</td>
<td>448.0 ± 39.0^c</td>
<td>31.9 ± 2.3</td>
</tr>
<tr>
<td>0.5</td>
<td>585.8 ± 53.9^b,c</td>
<td>36.7 ± 3.2</td>
</tr>
<tr>
<td>1.0</td>
<td>524.7 ± 26.8^b</td>
<td>39.9 ± 1.6</td>
</tr>
<tr>
<td>2.0</td>
<td>574.0 ± 50.9^b,c</td>
<td>38.8 ± 2.2</td>
</tr>
<tr>
<td>8 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>397.1 ± 29.0</td>
<td>25.6 ± 1.5</td>
</tr>
<tr>
<td>0.1</td>
<td>500.8 ± 22.6</td>
<td>27.5 ± 1.6</td>
</tr>
<tr>
<td>0.5</td>
<td>536.7 ± 55.1</td>
<td>28.7 ± 2.8^b,c,d</td>
</tr>
<tr>
<td>1.0</td>
<td>498.7 ± 50.6</td>
<td>36.6 ± 1.3^b,c,d</td>
</tr>
<tr>
<td>2.0</td>
<td>608.4 ± 55.1</td>
<td>35.8 ± 2.5</td>
</tr>
</tbody>
</table>

^a Each number represents the mean ± S.E.M. (n=4). All rats except deficient received the above levels of selenium as selenite in water and 1.2 ppm as selenomethionine in food. At each time point, values were tested by analysis of variance to determine whether significant differences existed between groups. Duncan's multiple range was used as a post test to determine between which groups these differences existed. The level of significance was at least P<0.05.

^b Statistically different from control by Duncan's multiple range.

^c Statistically different from 0.1 ppm group by Duncan's multiple range.

^d Statistically different from 0.5 ppm group by Duncan's multiple range.
respective 2 week values (P<0.01), while in the 0.5 and 1.0 ppm groups, the 2 week values were elevated above their respective 8 week values (P<0.01 and P<0.05, respectively). In the 2.0 ppm group, both the 4 and 8 week values were lower than the 2 week value (P<0.05).

d. Summary

Maximal SD GSH-Px activity in liver in each phase of the selenite-selenomethionine experiment compared to the highest amount of selenite administered in each phase is shown in figure 21. In all phases, enzyme activity was significantly depressed at the highest level of selenite repletion. In rats repleted with 0.12 ppm and 0.6 ppm as selenomethionine, maximal activity occurred in the group also receiving 0.5 ppm selenium as selenite. However, in rats repleted with 1.2 ppm as selenomethionine, the highest amount of organic selenium administered, the group in which maximal GSH-Px activity occurred was shifted to the group which also received 1.0 ppm selenium as selenite. In addition, the greater the amount of selenomethionine in the diet, the less the inhibitory effect of selenite. Figure 21 also shows kidney GSH-Px activities at the above levels of selenite-selenomethionine repletion. There were no differences in kidney GSH-Px activities between groups in any of the three phases of selenite-selenomethionine repletion. There were also no differences in testes SD GSH-Px activities in the above groups.
GSH-Px ACTIVITY FOLLOWING ADMINISTRATION OF INORGANIC AND ORGANIC SELENIUM COMPOUNDS FOR 8 WEEKS.

**LIVER**

**KIDNEY**

0.12 ppm as Selenomethionine

0.60 ppm as Selenomethionine

1.20 ppm as Selenomethionine

![Graph](image)

FIGURE 21. Liver SD GSH-Px activities (the level of selenium at which maximal activity occurred vs the highest amount given) were compared at each level of selenomethionine given in rats receiving both forms of selenium. Kidney SD GSH-Px activities in the same groups were also compared. Values represent the mean ± S.E.M for 4 rats. Values were compared using Student's t-test; P<0.05 (*), P<0.02 (**).
6. **Enzymatic Determinations**

a. **Protein Methylase I**

Protein methylase I (PM) activity was measured in liver and testes eight weeks following repletion with selenite and selenomethionine. There were no significant differences in liver PM activities between any groups repleted with 0.12 ppm selenium as selenomethionine and 0.1, 0.5, 1.0 or 1.5 ppm as selenite (phase 1), although activity decreased somewhat in the 1.0 ppm and 1.5 ppm groups (Table 8). In phase 2 (0.6 ppm as selenomethionine + selenite), significant differences in liver PM activity between groups occurred (P<0.025). Activity in the 1.0 ppm group was below that in both the 0.1 and 0.5 ppm groups. Activity in the 4.0 ppm group was also lower than the activity in the 0.1 and 0.5 ppm groups, although not significantly. In rats repleted with 1.2 ppm selenium as selenomethionine and selenite (phase 3), liver PM activity did not decline at higher selenite doses, but rather it increased in the 2.0 ppm group above all other groups (P<0.05).

Significant differences between groups in testes PM I activity occurred in each phase of selenite-selenomethionine repletion (P<0.01; Table 8). In phase 1, PM activity in the 1.0 ppm and 1.5 ppm groups decreased below the activity in the 0.1 and 0.5 ppm groups. In phase 2, testes PM activity in the two highest selenite groups, the 1.0 and 4.0 ppm groups, was also lower than the 0.1 ppm and 0.5 ppm groups. In phase 3, however, activity in testes did not follow the pattern of a decrease in product formed with increasing amounts of selenite repletion as occurred in phases 1 and 2. An increase in PM activity in the 0.5, 1.0 and 2.0 ppm groups above the 0.1 ppm group occurred, rather than the previously
<table>
<thead>
<tr>
<th>SELENITE (ppm)</th>
<th>LIVER (DPM $^{14}$C Product Formed/Hr)</th>
<th>TESTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>855 ± 67$^a$</td>
<td>849 ± 245</td>
</tr>
<tr>
<td></td>
<td>481 ± 82</td>
<td>736 ± 83</td>
</tr>
<tr>
<td></td>
<td>6140 ± 162</td>
<td>6172 ± 50$^b,c$</td>
</tr>
<tr>
<td></td>
<td>4259 ± 308$^b,c$</td>
<td>4708 ± 68</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>708 ± 40</td>
<td>649 ± 45</td>
</tr>
<tr>
<td></td>
<td>328 ± 131$^b,c$</td>
<td>3976 ± 67</td>
</tr>
<tr>
<td></td>
<td>4120 ± 112</td>
<td>3976 ± 67</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1315 ± 72</td>
<td>1342 ± 92</td>
</tr>
<tr>
<td></td>
<td>1350 ± 77$^b,c,d$</td>
<td>1736 ± 136$^b,c,d$</td>
</tr>
<tr>
<td></td>
<td>6653 ± 116$^b$</td>
<td>7122 ± 111$^b$</td>
</tr>
<tr>
<td></td>
<td>7504 ± 172$^b$</td>
<td>7305 ± 165$^b$</td>
</tr>
</tbody>
</table>

$^a$ Each number represents the mean ± S.E.M. (n=4). Rats were repleted with the above levels of selenite in water in combination with 0.12 ppm (phase 1), 0.6 ppm (phase 2), or 1.2 ppm (phase 3) as selenomethionine in food. At each time point, values were tested by analysis of variance to determine whether significant differences existed between groups. Duncan's multiple range was used as a post-test to determine between which groups these differences existed. The level of significance was at least P<0.05.

$^b$ Statistically different from 0.1 ppm group by Duncan's multiple range.

$^c$ Statistically different from 0.5 ppm group by Duncan's multiple range.

$^d$ Statistically different from 1.0 ppm group by Duncan's multiple range.
observed decrease.

b. **Imidazole-N-Methyl Transferase**

Imidazole-N-Methyl transferase (IMT) activity was also measured in liver eight weeks following repletion in phases 2 and 3. There were no significant differences between groups at any level of selenite-selenomethionine repletion, as individual variation was large (range 10,000 - 30,000 DPM product formed/100μl/hr.). In phase 2, there was a slight decrease in IMT activity and in phase 3 a slight increase in activity at higher levels of selenite repletion, similar to the changes which occurred in PM I activity.
D. Chronic Toxicity

Rats were maintained on 0, 0.1, 0.5, 1.0 or 2.0 ppm selenium as sodium selenite in combination with 1.2 ppm selenium as selenomethionine for 24 weeks. Those rats receiving only selenomethionine in food and no selenite in water (0 ppm group) served as the control for the groups receiving both selenite and selenomethionine.

An additional group of rats received 2.0 ppm selenium as selenite in water and a selenium deficient diet for 24 weeks. The control group for the 2.0 ppm selenite group received 0.1 ppm selenium as selenite and a selenium deficient diet.

Rats were evaluated for toxicity histologically and by GSH-Px activity, and adenosyl derivatives were measured to determine the effects of chronic toxicity on methyl metabolism.

1. GSH-Px Determinations

The selenium-dependent form of GSH-Px was measured in liver in an attempt to establish the presence of toxicity using enzyme activity as a biochemical index. Previous results have demonstrated a decline in GSH-Px activity following a 10 week exposure to >1.0 ppm selenium as selenite administered in drinking water (Results, B.2.) rather than an increase in enzyme activity due to the availability of increased dietary selenium as occurs in non-toxic states (Hafeman, 1974). This decline has been defined as indicative of a biochemical toxicity which precedes gross pathological changes, including weight loss, liver disease and death.

There were no significant differences between the control group which received selenomethionine alone and any of the groups receiving selenomethionine and varying amounts of selenite, or between any of the
### TABLE 9

**SELENIUM DEPENDENT GLUTATHIONE PEROXIDASE ACTIVITY IN RAT LIVER.**

<table>
<thead>
<tr>
<th>SELENITE (ppm)</th>
<th>GSH-Px nmoles/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 (Control)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>975.8 ± 55.5&lt;sup&gt;b&lt;/sup&gt; (3)</td>
</tr>
<tr>
<td>0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1046.0 ± (10.4) (2)</td>
</tr>
<tr>
<td>0.5</td>
<td>872.1 ± (96.9) (2)</td>
</tr>
<tr>
<td>1.0</td>
<td>761.6 ± 94.2 (3)</td>
</tr>
<tr>
<td>2.0</td>
<td>980.2 ± 41.4 (4)</td>
</tr>
</tbody>
</table>

<sup>a</sup>All animals received 1.2 ppm Se as Selenomethionine in food in combination with the above levels of Se as Selenite in water for 24 weeks.

<sup>b</sup>Each number represents the Mean ± SEM for (N) rats where N is greater than or equal to 3 and the mean (range) for N = 2.

<sup>c</sup>Another control group of 3 rats received 0.1 ppm Se as selenite in water and no selenomethionine in food for 24 weeks. The liver GSH-Px value was 581.3 ± 65.7 units.
groups receiving the two chemical forms of selenium in combination (Table 9). There appeared to be a slight decline in enzyme activity in the groups receiving 0.5 and 1.0 ppm as selenite and the selenomethionine, but it was not statistically significant. This decline did not occur in the group receiving 2.0 ppm selenium as selenite and 1.2 ppm as selenomethionine. In addition, in the group which received 0.1 ppm as selenite and 1.2 ppm as selenomethionine, GSH-Px activity was approximately doubled as compared to the group receiving only 0.1 ppm as selenite and no selenomethionine (1046 vs. 581 units, see Table 9,C). These results indicate that selenomethionine contributes more to GSH-Px activity than does selenite, and that the presence of relatively high amounts of selenomethionine seems to protect against the decline in GSH-Px activity occurring after the administration of >1.0 ppm selenium as selenite for 10 weeks.

Liver GSH-Px was also measured in one rat receiving 2.0 ppm selenium as sodium selenite for 24 weeks (no selenomethionine). The value of GSH-Px was 393.7 nanomoles/mg protein/min. This value followed the trend of a decline in activity (0.1 ppm control GSH-Px = 581 ± 65.7) following prolonged exposure to high levels of selenite, although a greater number of animals would have to be included in order to confirm this observation. This value was approximately 40% of the group receiving 2.0 ppm as selenite and 1.2 ppm as selenomethionine, again indicating the contribution of selenomethionine toward GSH-Px activity.

2. Pathology

Organs were examined histologically using light microscopy. A control liver is shown in figure 22. Pathological changes occurred in liver in the groups receiving 0.5, 1.0 and 2.0 ppm selenium as selenite
and 1.2 ppm as selenomethionine. The livers of the rats from these groups exhibited sinusoidal congestion, especially in the 2.0 ppm group. In addition, in the 0.5 ppm and 1.0 ppm groups (Figure 23), focal areas of hydropic or vacuolar degeneration of the liver parenchyma occurred, and in the 2.0 ppm group (Figure 22) hyperplasia with dark staining chromatin was present. In kidney in these rats, interstitial and glomerular congestion also occurred.

In rats repleted with 2.0 ppm as selenite and no selenomethionine for 24 weeks, fatty degeneration of the liver parenchyma occurred. Glycogen was also present in many nuclei (Figure 23).

3. Body Weights and Liver to Body Weight Ratios

Body weights were taken and liver to body weight ratios were calculated in the groups of rats receiving the selenite-selenomethionine combination for 24 weeks. The mean body weight of control rats was 817 ± 30 g. There was a significant decrease (P<0.01) in the mean body weight to 622 ± 12 g in the group of rats receiving the highest level of selenite, 2.0 ppm, in combination with 1.2 ppm as selenomethionine. There was also a significant decrease (P<0.05) in the liver to body weight ratio in the group receiving 2.0 ppm as selenite and 1.2 ppm as selenomethionine (0.0259 ± .0007) as compared to the control (0.0287 ± .0002). There were no other differences between the ratios of the control and any other level of repletion.

4. Effects on Adenosyl Derivatives

a. S-Adenosylhomocysteine and S-Adenosylmethionine

Tissues were prepared for HPLC analysis of the adenosyl
Photomicrographs from livers of rats receiving the following amounts of selenium for 24 weeks:

A. Control (1.2 ppm selenium as selenomethionine), 80 X magnification.
B. Control (1.2 ppm selenium as selenomethionine), 320 X magnification.
C. Combination of 2.0 ppm selenium as selenite and 1.2 ppm as selenomethionine, 80 X magnification.
D. Combination of 2.0 ppm selenium as selenite and 1.2 ppm as selenomethionine, 320 X magnification.
FIGURE 23

Photomicrographs from livers of rats receiving the following amounts of selenium for 24 weeks:

A. Combination of 0.5 ppm selenium as selenite and 1.2 ppm as selenomethionine, 80 X magnification.
B. Combination of 1.0 ppm selenium as selenite and 1.2 ppm as selenomethionine, 320 X magnification.
C. 2.0 ppm as selenite, 80 X magnification.
D. 2.0 ppm as selenite, 320 X magnification.
derivatives as described in methods. In rats receiving both selenite and selenomethionine, there was a dose-dependent decrease in liver SAH concentrations, expressed as nanomoles/gram original tissue, with increasing amounts of selenite (Table 10). This treatment effect was significant at the P<0.01 level of significance by analysis of variance (only those values where N>2 were analyzed statistically). Liver SAH concentrations in the 1.0 and 2.0 ppm selenite and selenomethionine groups were significantly lower than the control which received only selenomethionine (Duncan's multiple range test, P>0.05 and P>0.01, respectively). Pathological changes were evident in livers from rats from both of the above groups. There were no significant changes in SAM concentrations at any level of selenite-selenomethionine administration. A significant decrease in testes SAH also occurred in the group receiving 2.0 ppm selenite - 1.2 ppm selenomethionine as compared to control (P>0.01).

The pattern of a dose-related decline in SAH concentration with increasing amounts of selenium as selenite was present (Table 11). There was no difference in the testes SAM concentration between the 2.0 ppm selenite-1.2 ppm selenomethionine group and the control group.

In the group receiving 2.0 ppm selenium as selenite for 24 weeks without additional selenium as selenomethionine, liver SAH was again depressed by approximately 50%, as compared to the control which received 0.1 ppm selenium as selenite (P>0.01), while SAM concentrations remained constant (Table 12). In testes, there was also an apparent depression in SAH concentration in the 2.0 ppm selenite group as compared to the 0.1 ppm selenite control (approximately 63%), and no apparent change in SAM concentration (Table 13).
TABLE 10

S-ADENOSYLHOMOCYSTEINE AND S-ADENOSYL METHIONINE CONCENTRATIONS IN RAT LIVER FOLLOWING ADMINISTRATION OF VARIOUS LEVELS OF SELENIUM.

<table>
<thead>
<tr>
<th>Selenite (ppm)</th>
<th>S-Adenosylhomocysteine (nmoles/gram)</th>
<th>S-Adenosylmethionine (nmoles/gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0(^a) (Control)</td>
<td>28.2 ± 2.8(^{b,c})</td>
<td>48.5 ± 5.0(^{(3)})</td>
</tr>
<tr>
<td>0.1</td>
<td>23.1 (5.6)</td>
<td>40.6 (4.0)(^{(2)})</td>
</tr>
<tr>
<td>0.5</td>
<td>17.4 (11.2)</td>
<td>49.4 (2.4)(^{(2)})</td>
</tr>
<tr>
<td>1.0</td>
<td>16.7 ± 1.2(^d)</td>
<td>43.6 ± 3.2(^{(3)})</td>
</tr>
<tr>
<td>2.0</td>
<td>13.4 ± 2.5(^d)</td>
<td>55.3 ± 4.9(^{(3)})</td>
</tr>
</tbody>
</table>

\(^a\) All Animals received 1.2 ppm Se as Selenomethionine in Food in Combination with the above Levels of Se as Selenite in Water for 24 weeks.

\(^b\) Each Number represents the Mean ± S.E.M. for (N) Rats where N = 3 and the Mean (Range) for N = 2.

\(^c\) Statistically Significant Difference between Treatment Groups by Analysis of Variance, p < 0.01.

\(^d\) Statistically Significant from Control by Duncan's Multiple Range.
### TABLE 11

**S-ADENOSYLHOMOCysteine and S-ADENOSYLmethionine Concentrations in Rat Testes Following Administration of Various Levels of Selenium.**

<table>
<thead>
<tr>
<th>Selenite (ppm)</th>
<th>S-Adenosylhomocysteine (nmoles/gram)</th>
<th>S-Adenosylmethionine (nmoles/gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0° (Control)</td>
<td>21.3 ± 1.6b</td>
<td>9.2 ± 1.5(3)</td>
</tr>
<tr>
<td>0.1</td>
<td>16.0 (0.4)</td>
<td>7.5 (1.4)(2)</td>
</tr>
<tr>
<td>2.0</td>
<td>10.5 ± 0.3c</td>
<td>8.7 ± 1.6(3)</td>
</tr>
</tbody>
</table>

- All Animals received 1.2 ppm Se as Selenomethionine in Food in Combination with the above Levels of Se as Selenite in Water for 24 weeks.
- Each Number represents the Mean ± S.E.M. for (N) Rats where N=3 and the Mean (Range) for N=2.
- Statistically Significant from Control by Student T-Test, p<0.01
### TABLE 12

**S-ADENOSYLHOMOCYSTEINE AND S-ADENOSYLMETHIONINE CONCENTRATIONS IN RAT LIVER FOLLOWING SELENITE ADMINISTRATION.**

<table>
<thead>
<tr>
<th>Selenite (ppm)</th>
<th>S-Adenosylhomocysteine (nmoles/gram)</th>
<th>S-Adenosylmethionine (nmoles/gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1&lt;sup&gt;a&lt;/sup&gt; (Control)</td>
<td>28.1 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.7 ± 3.7&lt;sup&gt;(3)&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.0</td>
<td>14.4 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>57.1 ± 0.7&lt;sup&gt;(3)&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rats were maintained on the above Levels of Se as Selenite in Water for 24 weeks.

<sup>b</sup> Each Number represents the Mean ± S.E.M. for (N) Rats.

<sup>c</sup> Statistically Significant from the 0.1 ppm Control by Student's T-Test, p<0.01
<table>
<thead>
<tr>
<th>Selenite (ppm)</th>
<th>S-Adenosylhomocysteine (nmoles/gram)</th>
<th>S-Adenosylmethionine (nmoles/gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1&lt;sup&gt;a&lt;/sup&gt; (Control)</td>
<td>26.4 (12.4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.7 (1.0)</td>
</tr>
<tr>
<td>2.0</td>
<td>16.6 (1.2)</td>
<td>10.2 (2.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rats were maintained on the above Levels of Se as Selenite in Water for 24 weeks.

<sup>b</sup> Each Number represents the Mean (Range) for (2) Rats.
b. Adenosine and 5-Methylthioadenosine

In order to better understand the dynamics of the changes in SAH concentrations and the stability of the SAM concentrations, the concentrations of adenosine, formed from the hydrolysis of SAH by SAH hydrolase, and the concentrations of MTA, formed from SAM during polyamine synthesis (Figure 2), were also measured in the same tissues as were SAH and SAM.

The values of adenosine and MTA in liver and testes from rats receiving the selenite-selenomethionine combination are shown in Table 14. There were no changes in adenosine concentrations in either liver or testes at any level of selenite-selenomethionine administration, and values were comparable in both tissues. Control MTA concentrations were approximately two fold higher in testes than in liver. Both liver and testes MTA values appeared to decrease in a dose-related manner.

Adenosine and MTA concentrations were also measured in liver and testes in animals receiving selenite alone, with no additional selenium in the form of selenomethionine. Compared to animals receiving the combination of selenite-selenomethionine, animals receiving only selenite had higher adenosine concentrations, which were comparable in both liver and testes (Tables 14 and 15). Again, there was no change in the adenosine concentration between the control (0.1 ppm as selenite) and the toxic (2.0 ppm as selenite) group in either testes or liver (Table 15). Liver MTA concentrations did not vary between the selenomethionine control (0 selenite and 1.2 ppm selenomethionine) and the selenite control (0.1 ppm selenite), while testes MTA levels were lower in the selenite control group as compared to the selenomethionine
TABLE 14

ADENOSINE AND 5-METHYLTHIOADENOSINE CONCENTRATIONS IN RAT LIVER AND TESTES FOLLOWING ADMINISTRATION OF VARIOUS LEVELS OF Selenium.

<table>
<thead>
<tr>
<th>Selenite (ppm)</th>
<th>LIVER</th>
<th>TESTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine (nmoles/gram)</td>
<td>MTA (nmoles/gram)</td>
</tr>
<tr>
<td>0.0&lt;sup&gt;a&lt;/sup&gt; (Control)</td>
<td>24.5±1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103.5±17.9</td>
</tr>
<tr>
<td>0.1</td>
<td>28.3 (5.9)</td>
<td>114.5 (38.5)</td>
</tr>
<tr>
<td>0.5</td>
<td>25.4 (3.3)</td>
<td>75.0 (22.2)</td>
</tr>
<tr>
<td>1.0</td>
<td>26.6±3.5</td>
<td>69.0±5.8</td>
</tr>
<tr>
<td>2.0</td>
<td>25.6±2.2</td>
<td>57.2±1.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>All Animals received 1.2 ppm Se as Selenomethionine in Food in Combination with the above Levels of Se as Selenite in Water for 24 weeks.

<sup>b</sup>Each Number represents the Mean ± S.E.M. for (N) Rats where N=3 and the Mean (Range) for N=2.
### TABLE 15

**ADENOSINE AND 5-METHYLTIOADENOSINE CONCENTRATIONS IN RAT LIVER AND TESTES FOLLOWING SELENITE ADMINISTRATION.**

<table>
<thead>
<tr>
<th>Selenite (ppm)</th>
<th>LIVER</th>
<th>TESTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenosine</td>
<td>MTA</td>
</tr>
<tr>
<td>0.1° (Control)</td>
<td>39.5 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98.2 ± 15.9 (3)</td>
</tr>
<tr>
<td>2.0</td>
<td>41.2 ± 5.9</td>
<td>49.4 ± 9.8 (3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rats were maintained on the above levels of Se as Selenite in Water for 24 weeks.

<sup>b</sup> Each Number represents the Mean ± S.E.M. for (N) Rats where N=3 and the Mean (Range) for N=2.
control. There was also an apparent decrease in liver MTA concentration in the 2.0 ppm selenite group compared to the 0.1 ppm control.

5. **Enzymatic Determinations**

Imidazole-N-methyl transferase (IMT) and protein methylase I were measured in vitro from liver, and liver and testes, respectively, from the animals receiving the selenite-selenomethionine combination.

Total IMT activity, measured with substrate added varied but in a random fashion (activities ranged from 2400-15,000 DPM product formed/100 µl/hour). Endogenous activity, measured in the absence of substrate, remained near the control value in all groups except the 2.0 ppm group, where it decreased slightly (400-250 DPM product formed/100 µl/hour).

Protein methylase I activity did not differ from control in any group in either liver or testes (1600 and 6880 DPM product formed/hour).
A. Biochemical Effects of Prolonged Exposure to Selenite

Following the discovery by Rotruck et al. (1973) that selenium was an integral portion of the enzyme glutathione peroxidase, several studies were conducted to determine the effect of dietary levels of selenium, especially deficient levels, on the activity of this selenoenzyme. Omaye and Tappel (1974) found that at non-toxic intakes, GSH-Px activity in the tissues of chicks was related to the dietary selenium level (as selenite), the specific activity increasing with the available selenium. In deficient states, GSH-Px activity decreased dramatically. Chow and Tappel (1974) also found that GSH-Px activity in rat tissues declined in deficiency; the relative decrease in activity after 17 days on a selenium deficient diet was plasma > kidney, heart, liver and lung > erythrocytes > testes. Following supplementation with 2.0 ppm selenium as selenomethionine for 11 days, activity in all tissues except testes increased linearly above the non-supplemented controls. The relative increase was plasma > kidney > heart, erythrocytes, lung and liver > testes. Hafeman et al. (1974) reported that liver GSH-Px activity in unsupplemented rats fed a Torula yeast diet (<0.01 ppm) fell to less than 1% of that found in weanling rats fed a control diet (0.5 ppm selenium as
sodium selenite) in 24 days. As in the chick, erythrocyte GSH-Px activities increased as the amount of selenium in the diet increased from 0.05 ppm to 5.0 ppm. Also, the activity at any given dose did not plateau, but increased with time over the 134 day period. Liver GSH-Px activity in the rats supplemented with 0.05-1.0 ppm selenium also increased in a dose-related manner, and maximal activity occurred in the group receiving 1.0 ppm. However, the activity in the group fed 5.0 ppm, a chronically toxic amount, declined below that in the 1.0 ppm group after 10 days of supplementation and remained depressed throughout the 134 day experiment. This decline was attributed to readily observable cellular damage, i.e., irregularly mottled and pitted lobes with accompanying fibrosis. It was concluded that at non-toxic states the liver was a sensitive index of selenium adequacy. The effects of the decline in GSH-Px in the 5.0 ppm group were not discussed.

The purpose of the experiments in this thesis effort involving exposure to adequate and low toxic levels of selenite administered in drinking water (Results, B.) was to determine at what levels of selenium supplementation biochemical alterations occurred prior to the onset of more severe symptoms of toxicity including weight loss, liver degeneration, and mortality.

The upper limit of selenium supplementation of 2.0 ppm was chosen based on the findings of Palmer and Olson (1974), in an attempt to produce a minimal yet toxic response. They reported that levels of 2-3 ppm of either selenite or selenate produced a small decrease in body weight in 4-6 weeks, but no mortality. Although no decreases in body weight occurred between any groups (0.1-2.0 ppm) at any time point in the
present study, this may be attributable to either differences in strains of rats used and/or variations in the diets. Also, the inherent variations in body weight of the small number of animals used could mask experimental changes.

Glutathione peroxidase activity in rats made deficient for 3 weeks (at baseline or 0 weeks) was 27% of the 0.1 ppm water control in plasma, 20% in kidney and 4% in liver (Figures 12-14). This data is in agreement with the studies of Hafeman (1974) with respect to declines in liver GSH-Px activity in deficient states. The GSH-Px activities from the 0.1 ppm water control and from the 0.5 ppm food control were compared at the baseline time point, as they had received these amounts of selenium during the 3 week period during which the remaining rats were being made deficient. There were no differences in GSH-Px activities in either tissues or plasma between these two control groups. It was thus established that the 0.1 ppm water control used for the remainder of the experiment was a comparable control to the feeding studies of others (Hafeman, 1974).

After 2 weeks of repletion with 1.0, 1.5 and 2.0 ppm selenium in water, GSH-Px activity increased in both liver and plasma in a concentration-related manner, as would be expected. Although in kidney, GSH-Px activity increased in response to supplementation, there were no differences between groups at any level of repletion, indicating that although the kidney contained significant amounts of activity, it was not sensitive to differences in repletion when greater than 0.1 ppm.
Ten weeks following repletion, maximal enzyme activity in liver
corecurred in the group receiving 1.0 ppm selenium in water, rather than
in the 2.0 ppm group as occurred after 2 weeks of repletion. Hafeman
also observed maximal liver GSH-Px activity after supplementing with
1.0 ppm selenium as selenite in food. The decline in activity that
occurred in liver was significant; the value in the 2.0 ppm group was
only approximately half that of the 1.0 ppm group, and the value in the
1.5 ppm group was slightly lower than the 2.0 ppm group. This decline in
GSH-Px activity occurred prior to any observable changes in the liver.
Since this decline occurred in the presence of seemingly normal liver, it
seems to reflect a biochemical change which occurs prior to gross degener­
erative changes rather than because of them. It can be used, therefore,
as an index of toxic changes existing at the cellular level preceeding
observable pathological alterations, when compared to a range of liver
GSH-Px activities from nutritionally sufficient animals.

Whether excess selenite was the cause of the decline in GSH-Px
activity, either by an inactivation of enzyme, a decrease in synthesis
of enzyme, or an increase in degradation; or whether the decline in
activity resulted from other detrimental metabolic changes in the cell
due to the presence of excess selenite, remains to be determined.
Selenious acid (H₂SeO₃) due to its oxidation state and known reactivity
with thiols (Painter, 1941), especially with GSH in its reduction to
selenide, may form selenotrisulfide bridges with thiol containing com­
pounds within the cell when present in excess amounts and thereby disturb
normal processes within the cell.
It should also be recognized that the observed decrease in GSH-Px activity may be detrimental to the liver, for although it was only slightly below that of the 0.1 ppm value, excess amounts of selenite have been shown to produce oxidative stress (Levander and Morris, 1970; Beutler et al., 1975).

In plasma, GSH-Px activity continued to increase with increased amounts of selenite available in the drinking water. Because plasma has no way of maintaining an adequate supply of reducing equivalents, this apparent increase in enzyme activity may reflect tissue leakage, probably from either liver or erythrocytes. Erythrocytes, may in fact, be the source of the plasma activity, since it has been shown that in the RBC, GSH-Px activity continues to increase, even in the presence of chronically toxic intakes (Hafeman, 1974). This is supported by the low absolute activity in plasma as compared to GSH-Px activity present either in liver or kidney.

Specific activities, which represented the $^{75}$Se uptake relative to total selenium content, were elevated in liver, kidney and plasma in deficiency as compared to the 0.1 ppm control, and declined after 2 weeks of repletion with various amounts of selenite (Table 3). There were no differences, however, between any levels of repletion at 10 weeks (1.0, 1.5, or 2.0 ppm) which could be used to determine the existence of toxic changes. In addition, at all levels of repletion, which were 10-fold or greater than the control, tissue selenium content varied only slightly (data not shown) after 10 weeks of repletion. Selenium content in liver and kidney was approximately 1.7 ppm and 2.7 ppm, respectively. In the control group, the liver had a selenium content of 1.1 ppm and
the kidney a content of 1.2 ppm. Although there was no apparent
difference in specific activities between sufficient and toxic condi­
tions, specific activities were dramatically increased in deficient
animals, especially in plasma. This measurement would therefore, be
useful in determining the existence of selenium deficiency.

The amount of radioactivity excreted in both the expired air and
the feces over a 24 hour period at all levels of repletion at both
2 and 10 weeks was less than 0.5% of the administered dose, and was thus
considered minor. These routes of excretion have previously been shown
to be relatively nonimportant pathways in chronic selenium ingestion
(Smith, 1938; Burk et al., 1972). Urinary excretion of $^{75}\text{Se}$ was signif­
icant, accounting for between 9-29% of the original dose of $^{75}\text{Se}-
\text{Na}_2\text{SeO}_3$ (Table 4). The amount of $^{75}\text{Se}$ excreted as a percentage of the
original dose tended to increase with increasing levels of selenium
repletion, however, the percentage excreted in the 1.0, 1.5 and 2.0 ppm
groups was lower at 10 weeks than in each of these respective groups at
2 weeks. The total excretion ($\mu$g) of selenium also declined at 10 weeks
from the corresponding amount at 2 weeks, except in the 0.1 ppm control
rats. Approximately 50% less selenium was excreted at each level of
repletion at 10 weeks. The relative decrease in percentage of $^{75}\text{Se}$
excreted at 10 weeks was less than the relative decrease in total selenium
output at 10 weeks, suggesting the existence of more than one pool of
selenium within the body. Although the total selenium output decreased
at 10 weeks relative to 2 weeks, a greater amount was excreted as a
function of level of repletion at both 2 weeks and at 10 weeks. A
portion of this decrease in excretion of Se with time can be accounted
for on the basis of increasing liver weight with age. Although selenium content in liver (as μg/g tissue, ppm) increased somewhat from 2 to 10 weeks (range 1.3-1.8 μg/g, data not shown), liver weight increased from 10-20% during this period. Therefore, the selenium content in the total liver (organ basis) was greater at 10 weeks than at 2 weeks by at least a factor of 10-20%. This partially accounts for the apparent decrease in excretion after 10 weeks, as accumulation occurred largely as a result of increased liver mass. Further experiments studying the distribution and excretion of selenium at various times after repletion are necessary to explain the apparent decrease in excretion which was observed.

The data obtained in this study suggest that nutritionally sufficient but not toxic amounts of selenium can be achieved in rats drinking both 0.1 and 1.0 ppm selenium as sodium selenite. The decline in GSH-Px activity in liver at levels above 1.0 ppm after 10 weeks of repletion suggests the occurrence of a biochemical hepatotoxicity, prior to the onset of gross pathological changes, including weight loss, liver degeneration and death. This fairly early decline in activity can be used as a biochemical index of selenite toxicity, when compared to selenium sufficient animals. GSH-Px activities in plasma and kidney did not exhibit this decline. This coincides with the fact that the liver is the major target organ in chronic selenium toxicity in the rat.
B. Biochemical Effects of Prolonged Exposure to Selenite and Selenomethionine

The purpose of the experiments combining exposure to selenite and selenomethionine was to test exposure to both inorganic and organic forms of selenium, as would occur environmentally, and to determine the potency of the inorganic versus the organic forms. In addition, this appears to be the first report of controlled exposure to inorganic and organic selenium compounds within the same animal. Usually, in comparative studies, various forms are given individually to several groups of rats, which are then compared. As in the previous experiment, fairly low levels of both selenite and selenomethionine were used to avoid the production of marked toxic changes in the rats.

The only significant decreases in body weight occurred in phase 3 of this study, in which the highest amount of selenomethionine, 1.2 ppm, was given. Body weights in the groups receiving 1.0 ppm and 2.0 ppm as selenite and 1.2 ppm as selenomethionine were lower than in the groups which received either 0.1 ppm as selenite and 1.2 ppm as selenomethionine or 0.1 ppm as selenite alone. There were however, no significant changes in liver to body weight ratios in any groups. In addition, both red and white blood cell counts from all rats were within normal limits.

Gamma-glutamyl transpeptidase (\(\gamma\)GTP) activity was determined in plasma in all rats at all time points, as this enzyme is elevated in any and all forms of liver disease (Kachmar and Moss, 1976), especially in cirrhosis (Wu et al., 1976). The only group which exhibited elevated serum \(\gamma\)GTP activity was the group which received the combination of 2 ppm
as selenite and 1.2 ppm as selenomethionine. This elevation did not occur until the 8 week time point, and indicated that some degree of liver damage had occurred.

Both total GSH-Px activity and the SD form of GSH-Px were measured at 0, 2, 4 and 8 weeks of repletion in liver, kidney and testes at all levels of repletion. In all cases, similar patterns of changes in activity with time and dose which occurred with SD activity also occurred with total activity. The NSD form (estimate) tended to be greater in deficiency than in any other selenium repleted state. However, no pattern in the ZNSD activity was evident, such as an elevation in toxic states to compensate for the decline in SD GSH-Px which occurred. GSH-Px activity was not measured in plasma, as it may have represented enzyme leakage rather than inherent activity. Instead, GSH-Px activity was measured in testes because ingestion of toxic amounts of selenomethionine, the principal form of selenium involved in the production of alkali disease, has been shown to decrease reproductive performance.

Neither testes nor kidney GSH-Px activities exhibited any distinct patterns with respect to increasing levels of repletion with the selenomethionine-selenite combination. Enzyme activity in both organs declined significantly in deficiency and responded well to repletion. Overall activity varied somewhat, but it did not decline at high selenium intakes. Absolute activity in kidney was generally lower than in liver, and absolute activity in testes was much lower than that in either kidney or liver.
In liver, however, a decline in GSH-Px activity, characteristic of the decline which occurred following exposure to high levels of selenite, occurred in all levels of selenomethionine repletion (phases 1, 2 and 3) when combined with relatively high amounts of selenite for a period of eight weeks. In rats receiving 0.12 ppm as selenomethionine and 0.1, 0.5, 1.0 or 1.5 ppm as selenite, maximal activity occurred in the 0.5 ppm group and then declined in the 1.0 and 1.5 ppm groups. In phase 2, rats received 0.6 ppm selenium as selenomethionine and from 0.1 to 4.0 ppm as selenite. Maximal SD GSH-Px also occurred in the 0.5 ppm group (+ selenomethionine, SM) and declined with concentrations of selenite greater than 0.5 ppm. In phase 3 (1.2 ppm as selenomethionine), there was an increase in the amount of selenium as selenite which yielded maximal enzyme activity. Rather than maximal activity occurring at either 0.5 ppm as selenite or lower, since a greater amount of selenium was present due to the high level of selenomethionine repletion, maximal activity occurred in the group also receiving 1.0 ppm as selenite. Also, the greater the amount of selenomethionine present in the diet, the smaller the magnitude of the decline in GSH-Px activity in liver (Figure 21). The magnitude of the decline between maximal activity and the activity in the group receiving the highest amount of selenite in that phase of the experiment was 48%, 28% and 22% for repletion with 0.12, 0.6 and 1.2 ppm as selenomethionine. Although the decline in the group receiving 1.2 ppm as selenomethionine and 2.0 ppm as selenite was only 22%, animals in this group exhibited a decrease in body weight and an increase in the activity of γGTP in plasma. Both of these indicate that some degree of toxicity existed in this group.
Although food and water consumption were not equal (water consumed ranged from 22-30 ml/day; food from 20-24 g/day), an approximation of total selenium intake can be obtained by summing the two. The group which exhibited maximal GSH-Px activity in liver at the highest level of selenomethionine (1.2 ppm as SM + 1.0 ppm as selenite) received approximately 2.2 ppm total selenium, an amount which when given as selenite resulted in a marked decrease in GSH-Px activity. The decline in activity occurred in the 2.0 ppm group (+ 1.2 ppm as SM), which received about 3.2 ppm total selenium. These results suggest that selenomethionine, when administered concurrently with selenite, has a protective effect on the activity of the selenoenzyme, GSH-Px.

Selenomethionine alone (0 ppm as selenite), at levels of both 0.6 and 1.2 ppm, also produced adequate GSH-Px activity at 2, 4, and 8 weeks following repletion, indicating that both selenite and selenomethionine are ultimately metabolized to in vivo to selenocysteine, the form of selenium which is present in GSH-Px.

Liver GSH-Px activities were also determined in rats which received either 0.1 ppm as selenite, or 1.2 ppm selenium as selenomethionine in combination with 0, 0.1, 0.5, 1.0 or 2.0 ppm as selenite for 24 weeks (Table 9). Activity in the 0.1 ppm selenite group (no additional selenium in the form of SM) was lower than all other groups; the activity in the group which received 0.1 ppm as selenite and 1.2 ppm as selenomethionine was twice that of the 0.1 ppm selenite group. However, there were no significant differences between the GSH-Px values in any groups which received both selenite and selenomethionine or selenomethione alone. This data suggests that in the presence of both selenite and selenomethionine,
selenomethionine contributes more to liver SD GSH-Px activity than does selenite. The protective influence of selenomethionine on the maintenance of GSH-Px activity in the presence of excess selenite was again evident. The characteristic decline in activity did not occur even at the highest level of selenite given (2.0 ppm), after repleting for a 24 week period. Pathological changes had occurred in liver by 24 weeks in groups receiving 0.5, 1.0 and 2.0 ppm as selenite combined with 1.2 ppm as selenomethionine (Figures 22, 23), suggesting that even in the presence of relatively high GSH-Px activity, toxic histological changes could occur. However, the degenerative changes appeared to be more advanced in the livers of animals receiving 2.0 ppm selenium as selenite alone (Figure 23, C and D) compared to the other groups which received selenomethionine and selenite in combination. In addition, liver GSH-Px activity in one rat which received 2.0 ppm as selenite was 394 nanomoles/min/mg protein, which was approximately half the lowest value in the selenite-selenomethionine groups. Therefore, the "normal" GSH-Px activities in the selenite-selenomethionine rats seemed to either slow the progression or decrease the severity of the pathological changes, but it did not prevent their development.

Selenomethionine may exert this apparent protective effect in several ways.

1) Cary et al. (1973) reported that body retention was greater in rats fed a diet containing 0.12 ppm selenium as selenomethionine than in rats fed a diet containing 0.146 ppm as selenite, and that at levels of supplementation less than 0.1 ppm selenium no differences in retention existed. At much higher intakes (20-30 ppm), Martin and Hurlbut (1976)
found that selenomethionine produced a delayed response to selenium toxicity, a higher body retention, and a slower recovery from toxicity than equal amounts of selenite. In addition, the incorporation of $^{75}\text{Se-}\text{Na}_2\text{SeO}_3$ and $^{75}\text{Se}$-selenomethionine into proteins of several tissues has been observed, and the incorporation of selenomethionine occurred to a much greater extent than did the incorporation of selenite (Fuss and Godwin, 1975). Collectively, these results indicate that selenomethionine is incorporated into proteins producing a higher tissue content than equivalent doses of selenite, and that in this sequestered form, it is relatively non-toxic. Therefore, part of the protection afforded by selenomethionine may be a decrease in the total, freely available selenium pool by its incorporation into proteins, presumably by substituting for methionine.

2) Levander and Morris (1970) noted that in rats fed 10 ppm selenium as selenate, the addition of 0.5% methionine protected against the toxic liver damage produced by the selenium, reducing it from moderate to either grossly normal or mild. Selenium concentrations in liver and kidney were also significantly reduced in the methionine supplemented rats. In addition, the presence of 0.4% guanidoacetic acid to the diet inhibited the protective effect of methionine. They suggested that methionine exerted its protective effect by increasing the amount of methyl groups available for the detoxification and metabolism of selenium, presumably by increasing S-adenosylmethionine concentrations.

The similarities (and differences) between selenium and sulfur were discussed in the introduction (Section B). It appears that in the form of amino acids, similar metabolic pathways exist for sulfur and selenium
(Pan et al., 1964). It is possible, therefore, that selenomethionine increases the formation of methylated metabolites as does methionine, by increasing the available methyl group pool via the formation of a seleno-analog of SAM (Seleno-adenosylmethionine, SeAM). This possibility is supported by the work of Mudd and Cantoni (1957) who demonstrated the activation of DL-selenomethionine by a yeast transferase to seleno-adenosylmethionine, and the subsequent transfer of its methyl group to guanidoacetic acid. SeAM was also found to be a better methyl donor than SAM for choline biosynthesis in an in vitro rat preparation (Bremer and Natori, 1960). However, selenomethionine reacted more slowly than methionine with rat methionine adenosyltransferase. It should be noted that upon donation of its methyl group, selenoadenosylhomocysteine would be formed (SeAH, Figure 2) as the seleno-analog of SAH. Accumulation of SeAH would probably inhibit methyltransferases, as does SAH, should it occur.

3) The protective effect of selenomethionine on the activity of GSH-Px may occur as the result of different metabolic pathways in the metabolism of selenomethionine and selenite to selenocysteine. As previously mentioned, selenocysteine (Se-Cys) has been identified as the catalytic site of rat liver GSH-Px, and has been found to be located within the polypeptide chain of the native enzyme. Two possible mechanisms for the incorporation of Se-Cys into GSH-Px seem feasible.

a. Se-Cys may be formed after the synthesis of the protein by a post-transcriptional modification of an amino acid such as serine or cysteine. This could occur by a cysteine synthase-like reaction, which produces cysteine from serine and sodium sulfide (Na$_2$S; Olson and Palmer 1976). Cysteine synthase activity has been identified in rat liver.
preparations (Huovinen, 1968) and the production of \( \text{H}_2\text{Se} \) (Figure 1) has been shown to occur. \( \text{Se-Cys} \) could therefore, be formed by the substitution of \( \text{H}_2\text{Se} \) for \( \text{Na}_2\text{S} \). This experiment has not been done. Also, the configuration of GSH-Px would have to be such that only one serine residue per monomer would be converted to \( \text{Se-Cys} \).

b. Alternatively, \( \text{Se-Cys} \) may be incorporated during translation. Tappel's group (Zakowski et al., 1978) has postulated the existence of a specific \( \text{Se-Cys} \) tRNA, and that the specificity of incorporation of \( \text{Se-Cys} \) may be obtained from the sequence of nucleotides surrounding the insertion site. This has been described for other amino acid derivatives (Uy and Wold, 1977).

Assuming that selenomethionine is metabolized like methionine, \( \text{Se-Cys} \) could be readily formed by the cleavage of \( \text{Se-cystathionine} \) (Figure 24), or \( \text{Se-Cys} \) could be formed from \( \text{H}_2\text{Se} \) (formed from reduction of selenite) and serine via cysteine synthase, prior to GSH-Px synthesis rather than post-translationally.

The protective effect observed in the combination selenite-selenomethionine experiments, especially at 24 weeks, favors the translation hypothesis (3b). Enzymatic activity declined in the presence of 2.0 ppm selenium as selenite after 10 weeks. If the first hypothesis were correct, it would be necessary for selenomethionine to be converted oxidatively to selenite, and then reduced to \( \text{H}_2\text{Se} \) prior to the modification of GSH-Px. This would in effect, result in the production of additional selenite which would be expected to decrease GSH-Px activity rather than to maintain it. Since selenomethionine tended to maintain GSH-Px levels in the presence of excess selenite, it appears that selenomethionine was converted to
Proposed Metabolism of Selenomethionine

SELENOMETHIONINE → \text{SE-ADENOSYL METHIONINE} → \text{CH}_3\text{-ACCEPTOR}

Serine

SELENOHOMOCYSTEINE

SELENOCYSTATHIONINE

\text{H}_2\text{O}

\text{Selenocysteine} → \text{GSH-PX}

\text{HOMOSERINE} → \alpha\text{-KETO BUTYRIC ACID}

\text{CO}_2

\text{PROPIONYL-CoA}

NADH + H^+

\text{NAD}^+

FIGURE 23. Proposed metabolism of selenomethionine.
Se-Cys and incorporated into GSH-Px during synthesis as the amino acid and that this conversion was greater than the conversion of selenite to selenocysteine.

In summary, a combination of all three ways, incorporation into proteins, increased availability of methyl donors, and maintenance of GSH-Px activity, may contribute to the protective effects of selenomethionine in the presence of excess selenite.

As was mentioned in the introduction, Hoffman recently reported that a decrease in methylating capacity in liver (a decrease in SAM and an increase in SAH concentrations) resulted following acute exposure to selenite. It was of interest to determine if a decrease in methylating capacity also occurred during chronic exposure to selenium, especially since formation of trimethylselenonium ion is the major excretory product in states of chronic exposure.

Two in vitro transmethylating enzymes, PM I and IMT, were measured in liver and testes, and in liver, respectively, 8 weeks after exposure to the various combinations of selenite and selenomethionine. In animals repleted with both 0.12 or 0.6 ppm as selenomethionine and with selenite, activity in both liver and testes tended to decrease at the two highest levels of selenite repletion, indicating a decrease in the methylating capacity of protein methylase I. However, in rats repleted with 1.2 ppm as selenomethionine, an increase in activity occurred in the group also receiving 2.0 ppm as selenite (in liver) and in the groups also receiving 0.5, 1.0 and 2.0 ppm as selenite (in testes). Again selenomethionine may have served a protective role when present in amounts of 1.2 ppm by increasing the amount of methyl donor present. However, this was an in
vitro assay which had exogenously added $^{14}$CH$_3$-SAM present, and may therefore reflect only an increase in the total amount of enzyme present, rather than an increase in in vivo methylating capacity. Also, due to the dilution effects present in this assay, any endogenous inhibitor (SAH or SeAH, for example) present in vivo may have been undetectable. IMT activity was measured because it is a soluble enzyme which transmethylates a small substrate, and was a model of sorts for the methylation of selenium. However, individual variation among all animals was great, and no conclusions could be drawn.

C. Chronic Toxicity: Effects on Adenosyl Derivatives

The purpose of this experiment was to measure the concentrations of SAM and SAH directly after 24 weeks of exposure to determine whether the changes accompanying acute selenium toxicity (a decrease in SAM and an increase in SAH) also occurred during chronic toxicity. Rats were given 1.2 ppm selenium as selenomethionine in combination with 0, 0.1, 0.5, 1.0 or 2.0 ppm selenium as selenite, or 0.1 or 2.0 ppm selenium as selenite alone.

GSH-Px activities in liver were discussed previously. Pathological changes occurred in the livers of animals which received 0.5, 1.0 and 2.0 ppm as selenite and 1.2 ppm as selenomethionine and also in the livers of animals which received 2.0 ppm selenium as selenite (Figures 22, 23), indicating the presence of toxicity.

In both liver and testes, SAH concentrations decreased in a dose-dependent manner with increasing amounts of selenite repletion in addition to the constant amount of selenomethionine present in the diet.
SAH concentrations also decreased in liver and testes in rats which received 2.0 ppm as selenite and no selenomethionine, and the magnitude of the decrease was comparable to that which occurred in the group that received 2.0 ppm as selenite and the selenomethionine. There were no changes in SAM concentrations in any of the above groups in either liver or testes. The decrease in SAH and the stability of SAM levels did not seem to depend on the presence of selenomethionine in the diet, since equivalent decreases occurred both in its presence and in its absence. Rather, the effect seemed to depend upon the presence of relatively high amounts of selenite or some metabolite of selenite not readily formed in vivo from selenomethionine.

Adenosine and 5-methylthioadenosine (MTA) concentrations were also measured in the above animals. Since MTA is formed from decarboxylated SAM after it donates its propylamino group to putrescine (Figure 2) an increase in MTA levels could suggest an increased utilization of SAM for polyamine synthesis (Ferro, 1979), and thus explain the constant amount of SAM and the decreased amount of SAH. Also, changes in adenosine concentrations might reflect changes in the activity of SAH hydrolase. No changes in adenosine concentrations occurred at any level of repletion. MTA concentrations appeared to decrease in both liver and testes with increasing amounts of selenite repletion. This decrease occurred both in the presence and absence of selenomethionine. It suggests a decrease in the synthesis of polyamines. However, they would have to be measured directly in order to prove this. The decrease in MTA concentrations and the decrease in SAH concentrations together suggest that there may have been an increase in SAH turnover.
The results of this experiment indicate that chronic toxicity and acute toxicity differ in their effects on both S-adenosylmethionine and S-adenosylhomocysteine levels, and therefore, that the mechanisms by which chronic and acute toxicity occur may differ. In addition, a decrease in the metabolism of selenium and other important physiological methylations do not appear to occur with chronic toxicity, either by a depletion of SAM as a methyl donor or by an elevation of SAH as a physiological inhibitor.
SUMMARY

Studies were conducted in an attempt to detect biochemical changes occurring in selenium toxicity prior to weight loss, liver disease and death, as compared to both deficient and nutritionally adequate animals. Since selenium occurs in both inorganic (selenite and selenate) and organic (selenomethionine, selenocystathionine, etc.) forms, studies were designed to: 1) test exposure to an inorganic form, selenite alone, which would occur primarily in drinking water; 2) test exposure to selenite administered in drinking water in combination with an organic form, selenomethionine, administered in food, as would occur environmentally, and 3) determine the potency of the inorganic versus the organic forms.

In order to determine the effects of selenite alone, rats, previously maintained on selenium deficient diets, received in drinking water various levels of selenium as Na$_2$SeO$_3$ (1.0, 1.5, and 2.0 ppm) for 10 weeks. Controls received 0.1 ppm selenium as selenite. Changes in selenium dependent glutathione peroxidase (GSH-Px) activities and specific activities ($\text{nCi}^{75}\text{Se}/\mu\text{g Se}$) were determined in liver, kidney and plasma at baseline and at two and ten weeks after repletion, and the excretion of $^{75}\text{Se}$ and total selenium were determined in urine for a 24 hour period. In selenium deficient rats, GSH-Px activities were markedly depressed and specific activities elevated as compared to 0.1 ppm controls. After two weeks, liver and plasma GSH-Px activities increased, and plasma, 141
liver and kidney specific activities decreased in a concentration dependent manner. In kidney, there were no differences in enzyme activity at either two or ten weeks, and in plasma activity increased with increasing levels of selenite repletion at ten weeks. At ten weeks, liver GSH-Px activities continued to increase in the 1.0 ppm group, but were depressed at both the 1.5 and 2.0 ppm levels. Specific activities also remained low in liver, kidney and plasma, and did not differ from one another at any level of repletion (1.0, 1.5 or 2.0 ppm). Total excretion of selenium increased with increasing levels of selenite repletion at both the two and ten week time points. However, there was an apparent decrease in excretion at ten weeks at each dose (1.0, 1.5 and 2.0 ppm) compared to its respective two week value. The decline in liver GSH-Px at high levels of selenite repletion suggests the presence of a biochemical toxicity in liver at levels above 1.0 ppm Se after ten weeks, prior to the onset of gross pathological changes, and can be used as an index of selenium toxicity due to the presence of excess selenite.

In the experiments using combined forms of selenium, rats received 0.1, 0.5, 1.0, and 1.5, 2.0 or 4.0 ppm selenium as Na$_2$SeO$_3$ in combination with 0.12, 0.60, and 1.20 ppm selenium as selenomethionine. GSH-Px activities were determined in liver, kidney and testes at zero, two, four and eight weeks following repletion with the above combinations. In animals receiving 0.12 ppm as selenomethionine, a decline in liver GSH-Px activity (indicating biochemical toxicity) occurred in animals also receiving 1.0
and 1.5 ppm as selenite. Animals receiving 0.1 ppm and 0.12 ppm, appeared to have adequate enzyme activity, while animals receiving 0.5 ppm as selenite and 0.12 ppm as selenomethionine, showed maximal activity after eight weeks. At the intermediate level of selenomethionine, 0.60 ppm, a similar trend occurred after eight weeks of repletion. Animals receiving 0.1 ppm selenium as selenite in addition to the selenomethionine showed sufficient GSH-Px activity, while those receiving 0.5 ppm again had maximal enzyme activity. At levels above 0.5 ppm, 1.0 and 4.0 ppm as selenite, the GSH-Px levels were depressed. In rats receiving the highest amount of selenomethionine, 1.2 ppm, in combination with selenite in water, the significant decline in enzyme activity, indicating toxic biochemical changes, also occurred at eight weeks following repletion in the group receiving the highest amount of selenite, 2.0 ppm. However, maximal activity occurred at 1.0 ppm selenium as selenite, rather than at 0.5 ppm as in the two lower selenomethionine treatments. Also, the magnitude of the decline decreased as selenomethionine concentrations were increased. Therefore, selenomethionine, when given in conjunction with selenite, appeared to have a protective effect on GSH-Px with respect to total amount of selenium given. Also pathological changes which occurred in liver after 24 weeks of selenite-selenomethionine exposure were less severe than those resulting from exposure to selenite alone.

Neither testes nor kidney GSH-Px showed any distinct patterns with respect to levels of repletion with the combination of selenomethionine and selenite, indicating they were not target organs of toxicity, at
least in reference to GSH-Px as the index. Although two transmethylating enzymes were measured in vitro in liver and testes after 8 weeks, the significance of the changes which occurred were difficult to interpret.

Metabolism of selenium and thus detoxification occur primarily by the formation of methylated derivatives, dimethyl selenide and trimethylselenonium ion which are excreted by the lung and the kidney respectively, with S-adenosylmethionine (SAM) as the methyl donor. Acute toxic doses of selenite have been shown previously to result in a depletion of liver SAM, an elevation of S-adenosylhomocysteine (SAH), and an inactivation of methionine adenosyltransferase. Experiments were performed to determine whether changes in methylation capacity accompanying acute toxicity also occurred chronically, resulting in accumulation of selenium and alterations in normal methyl metabolism. Rats were maintained on 0, 0.1, 0.5, 1.0, and 2.0 ppm selenium as selenite in combination with 1.2 ppm selenium as selenomethionine or 0.1 and 2.0 ppm selenium as selenite alone, for a period of 24 weeks. Toxicity was defined histologically. SAM, SAH, MTA and adenosine concentrations were measured in liver and testes. A dose-dependent decrease in liver SAH concentrations occurred with increasing levels of selenium administered (combination of both forms). There were no significant changes in SAM concentrations at any level of combined selenium administration. A decrease in testes SAH concentration and no change in SAM was also observed in the group of animals receiving the highest level of selenium (2.0 ppm as selenite for 24 weeks and 1.2 ppm as selenomethionine). In animals given only 2.0 ppm as selenite for 24
weeks, liver and testes SAH was also depressed while SAM levels remained constant. MTA levels also appeared to decrease in liver and testes with increasing concentrations of selenite in both the presence and absence of selenomethionine. Adenosine concentrations did not change in any group, regardless of treatment.


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