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Title: Effect of Dietary Fluoride on Selenite Toxicity in the Rat

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Two factorial experiments were conducted to determine if high dietary fluoride would inhibit selenite toxicity in rats. In each study, two levels of selenite (0.05 and 5 mg/kg diet) were matched against two levels of fluoride (1 and 150 mg/kg diet) for either 6 or 8 weeks. Fluoride failed to prevent the depressive effect of selenite on food intake and body weight gain in either study. Although liver selenium concentration was slightly (15%) but significantly ($P < 0.005$) reduced when the highest fluoride and selenium level were combined in the first study, this effect could not be repeated. These three measures therefore failed to provide evidence for a fluoride and selenium interaction. Fluoride, however, prevented hepatic necrosis seen in most of the selenite-toxic rats. Hepatic lesions seen histologically in selenite-toxic rats were not observed for either kidney or

heart. With regard to a possible mechanism for the fluoride effect upon selenite liver pathology, fluoride partially (26%) but significantly ($P < 0.025$) reduced thiobarbituric-reactive substances (an indicator of peroxidative cell membrane damage) in selenite-toxic rats, but there was no fluoride effect on an enzyme system (liver xanthine oxidase) that potentially could generate an initiator of lipid peroxidation. In agreement with results of others, fluoride deposition into bone was inconsistently affected by selenite. Overall, the protective effect of fluoride on selenite toxicity appears to be confined to liver pathology. The exact mechanism for this effect, however, remains unclear.

Effect of Dietary Fluoride on Selenite Toxicity in the Rat

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INTRODUCTION

Selenium is both an essential and toxic trace element (Oldfield, 1987). Selenium, for example, is a necessary constituent at the active site of the selenoenzyme glutathione peroxidase (Rotruck et al., 1973). This selenoenzyme functions as part of a multicomponent antioxidant defense system within the cytosol and mitochondrial matrix space. Glutathione peroxidase protects against peroxidative damage of cell membranes by reducing organic and inorganic hydroperoxides, prooxidant compounds, to a non-toxic form. On the other hand, selenium has also been shown to be toxic to humans as the result of excessive dietary intake, inappropriate use of oral supplements, and by way of occupational exposure (Olson, 1986; Combs and Combs 1986b). Recently, for example, a chronic selenosis in humans, which had peak prevalence during 1961 to 1964 in Enshi county, Hubei Province of the People's Republic of China, was documented (Yang et al., 1983). The 248 residents within this region suffered a morbidity of almost 50%. The typical symptoms of selenosis, which are characterized by hair and nail loss, nausea, vomiting, gastrointestinal

disturbances, skin lesions, peripheral neuropathy, fatigue and irritability, were found in the residents. The source of selenium was a stony coal (Se $\geq$ 300 μg/g). The local use of this coal as fuel in the home exposed people to large amounts of selenium by inhalation. Selenium from the coal also got into the soil by weathering and was available for uptake by crops. Through these foods, considerable amounts of selenium entered the human body and caused intoxication. Besides selenium, other elements such as fluoride and sulfur were also found at high levels in coal, corn and vegetables within the Enshi area (Whanger, 1989; Liu and Li, 1987).

At the Third International Symposium on Selenium Metabolism, Liu and Li (1987) pointed out that cases of endemic selenosis and fluorosis occurred simulataneously in humans and animals within Enshi county. They found some fluorosis patients in areas of endemic selenosis. Furthermore, the residents within the high fluorine areas did not have any symptoms of endemic selenosis despite their high level of blood and hair selenium. Thus, Liu and Li suggested that high fluoride might inhibit selenium intoxication by accelerating the excretion of selenium through hair and urine.

Although Chinese scientists suggested that they had uncovered a selenium and fluoride interaction, previous results by others offer little support for a selenium and fluoride interaction. Moxon and DuBois (1939), for example, reported that mortality caused by selenite in the diet (11

mg/kg) of rats was exaggerated by also providing fluoride (5 mg/kg diet). Hadjimarkos (1969), however, was unable to duplicate this result when selenium (3 mg/kg diet) was combined with 50 mg/kg fluoride. Shearer and Ridlington (1976) also failed to find any evidence for a fluoride and selenium interaction in either hard (femur and molar enamel) or soft tissue (liver and kidney) of rats.

A study by Cerklewski and Whanger (unpublished data), however, provided support for the suggestion by Chinese scientists that a selenium and fluoride interaction might occur. Cerklewski and Whanger suggested that high dietary fluoride (150 mg/kg) would prevent cellular damage to liver caused by selenite (5 mg/kg). Problems with fixing liver tissue in their study however did not allow for a complete histological examination of the liver. Therefore, the present study (Experiment I), a 2 x 2 factorial experiment, was designed to test the hypothesis that a nutritionally high but non-toxic level of fluoride (150 mg/kg diet) will reduce liver damage of selenite toxicity (5 mg/kg diet) when compared to control levels of selenium (0.05 mg/kg diet) and fluoride (1 mg/kg diet). A 2 x 2 factorial experiment design was selected because it will not only define each individual factor effect but it will also define whether there is an interaction between the two factors (selenium and fluoride). In addition, Experiment I will present a histological description of rat liver.

The present study (Experiment II) was also designed to

determine how high dietary fluoride could prevent selenite damage to the liver. One hypothesis to be tested in this regard is that fluoride may inhibit lipid peroxidative damage caused by selenite. A recent study (Shayiq et al., 1986), for example, found that a significant inhibition of lipid peroxidation in liver and intestine could be demonstrated at 10 mM NaF in vitro. A further increase in NaF to 50 mM resulted in a spontaneous decrease in malondialdehyde formation. This finding provided a possible explanation of how fluoride prevents selenite toxicity because selenite has been reported to have a pro-oxidant effect both in vivo and in vitro (Bunyan et al., 1960; Dougherty and Hoekstra, 1982). The lipid peroxidation effect of selenite toxicity, for example, was observed by accumulation of lipofusion pigments in the liver of mice given additional sodium selenite (0.1 µg/ml) in the drinking water for 9 months (Csallany et al., 1984). Earlier, it had also been suggested that inhibition of lipid peroxidation by fluoride could occur by stimulation of the reducing capacity of tissues (Bus et al., 1976). However, the preliminary study by Cerklewski and Whanger (unpublished data) failed to support this possibility. Recently much interest has also focused on xanthine oxidase as a potential source of oxygen-derived free radicals in tissues (McCord, 1985). The oxygen-derived free radicals have been shown to play a role in the pathogenesis of ischemic tissue injury (eg, caused by lipid peroxidation) in the heart, kidney and brain. During ischemia, decreased

oxygen supply promotes the dephosphoryation of adenosine triphosphate (ATP). A consequence of decreased ATP level is that the cell can not maintain a proper ion gradient across the cell membrane resulting in an influx of calcium into the cell. This increase in intracellular calcium is believed to activate the Ca-dependent proteolytic enzyme calpain, which is responsible for the enzymatic conversion of xanthine dehydrogenase to xanthine oxidase. Increased xanthine oxidase activity has been proposed to cause a burst of free radical production. The inability to counteract the deleterious effects of these free radicals would then lead a loss of cell function and increased membrane permeability. The present study (Experiment II), which is a 2 x 2 factorial experiment (same as Experiment I), was designed to determine whether high dietary fluoride combines with calcium to form an insoluble complex thereby preventing the conversion of xanthine dehydrogenase to xanthine oxidase which would in turn prevent oxygen-derived free radical generation. Specifically, Experiment II will determine if liver damage caused by selenite in rats can be correlated with an increase in xanthine oxidase activity. Furthermore, it examines whether fluoride can inhibit xanthine oxidase, thus explaining why fluoride is protective against selenite toxicity in the liver. The next section will review selenium bioavailability, selenium toxicity and prior evidence for a selenium-fluoride interaction to develop the rationale for the studies to be described.

LITERATURE REVIEW

I. Selenium Sources and Requirements

A. Sources of dietary selenium

The most important sources of human dietary selenium are fish, meat and cereals (National Research Council, 1989). The selenium content of human foods is related to its protein content and geographical origin. Seafoods (shell fish & fish), organ meats (liver & kidney), and to a lesser extent muscle meats (beef, lamb & pork), which are high in protein, are generally good sources of selenium. Combs and Combs (1986a), for example, summarized data from several countries and found that meats and fish contribute around 40%-50% of the total selenium intake per person per day. Grains and cereal products contain variable amounts of selenium depending on the selenium content of the soils where they are grown. They contribute 1/4 to 2/3 of total selenium consumption in countries with selenium intake greater than 40 µg/person/day (South Dakota, Colorado, parts of Iowa in the US; Eastern, Western and Northern part of Canada) and 1/10 to 1/4 of total selenium consumption in countries with a selenium intake less than 40 µg/person/day (Finland, New Zealand, Italy). Foods low in protein, such as vegetables and fruits, have been shown to contain very little selenium. Drinking water usually contributes a small amount of selenium

intake for humans.

B. Selenium requirements

There are many problems associated with quantitatively defining the human requirement for selenium because the nutritional bioavailability of selenium in different foods is not the same. One way to estimate human selenium requirements is by examining dietary selenium intake in both selenium deficient and non-deficient regions. Data from China, for example, showed that the minimum selenium requirements were 19 µg/day for men and 13 µg/day for women (Yang et al., 1988). The physiological human selenium requirement, which is related to the body weight and individual variations, was estimated according to the level of glutathionine peroxidase activities in the plasma. Using this procedure, Yang et al.(1987) suggested approximately 40 µg Se/day was required to meet the requirement for Chinese adult males (60 kg). Extrapolation of this data from China to the U.S. population, with consideration of body weight and individual factors, led the Food and Nutrition Board of the National Research Council (1989) to recommend a dietary selenium allowance of 0.87 µg/kg or around 70 µg/day for adult American men (79 kg). Similar calculations yielded a selenium requirement of 55 µg/day for an American adult female (63 kg).

II. Selenium Bioavailability

A. Selenium absorption

a. Chemical forms of selenium affect absorption

The sites of selenium absorption in humans are the gastrointestinal tract, the respiratory tract and the skin. The biochemical mechanism of selenium absorption, which has not been entirely elucidated, depends upon the chemical form of selenium in the diet (Combs and Combs, 1984; Levander, 1986; Favier, 1988).

Selenium appears in the periodic table as a group VI element and exhibits valences of -2, 0, +4, and +6 (Table 1). Although many chemical forms of selenium have been identified in humans diets, selenium occurs naturally in foods almost exclusively as the organic compounds selenocystine, selenocysteine (from meat source), and selenomethionine (from cereal source, such as wheat). At physiologic levels, the intestinal absorption of selenium by mammals in the form of soluble organic compounds (selenomethionine & selenocysteine) and soluble inorganic compounds (selenite & selenate) is rapid and efficient (70%-100%), but insoluble inorganic compounds such as metal selenides and elemental selenium are poorly absorbed. Organic forms of selenium such as selenomethionine are absorbed by active transport (McConnell and Cho, 1965) mainly in the duodenum (Whanger et al, 1976) and absorption is generally higher than inorganic forms of selenium in human. Absorption of inorganic selenium such as selenite is by non-active transfer and is generally much lower and more variable (44%-70%) than selenomethionine (95%-97%). Selenate is absorbed in the ileum by carrier-mediated transfer and is better absorbed than selenite which

Table 1

Chemical forms of selenium

Name	Valence	Formula
Selenate	+6	SeO_4^{2-}
Selenite	+4	SeO_3^{2-}
Elemental Selenium	0	Se^o
Selenodiglutathionine	0	G-S-Se-S-G
Selenotrisulfide	0	R-S-Se-S-R
Selenocysteine	-2	$HSe-CH_2-CH(NH_2)-COOH$
Selenomethionine	-2	$CH_3-Se-CH_2-CH_2-CH-(NH_2)-COOH$
Trimethylselenonium	-2	$(CH_3)_3Se^+$
Dimethylselenide	-2	$CH_3-Se-CH_3$
Selenide	-2	H_2Se

absorption may be by simple diffusion (Thomson and Robinson, 1986).

Quantitative human data on the pulmonary absorption of selenium (WHO, 1987) is unavailable. The forms of inhaled selenium are usually selenium dioxide and hydrogen selenide. Pulmonary absorption of selenium has been indicated by high urinary selenium excretion in workers exposed to selenium in the air. Limited data from animal studies showed that inhaled selenium deposited in the pulmonary region was absorbed into blood and then transported to liver and kidney. At selenium levels normally present in the atmosphere, inhalation of selenium compounds do not contribute significantly to the body burden of selenium, but at higher levels ($\geq$ 0.2 mg Se/m^3), tissue selenium concentration becomes 3 to 4 times higher in occupationally-exposed workers compared to that observed from oral ingestion (Medinsky et al., 1981).

b. Dietary factors affecting selenium absorption

Protein: The absorption of selenium can be increased by feeding a high protein diet. It has been reported, for example, that apparent absorption of selenium was 63% to 90% when 8 adult male subjects were fed a high protein diet (24.1 nitrogen/day) and 9% to 72% when subjects were fed a low protein diet (8.1 nitrogen/day) for 51-day (Greger and Marcus, 1981). Because L-selenomethionine is transported across the intestinal membrane by active transport similar to L-methionine, it may explain why selenomethionine absorption

has been reported to be inhibited by methionine (McConnell and Cho, 1965).

Vitamin C: Data from animals, humans and in vitro studies have suggested that an interaction between selenium and ascorbic acid can occur. Selenite and ascorbic acid have been shown to be incompatible in a total parenteral nutrition solution because the reducing power of ascorbic acid makes selenium less soluble in solution. Increasing the concentration of ascorbic acid from 100 mg/L to 2000 mg/L, for example, induced selenium precipitation from 6% to 37% respectively (Shils and Levander, 1982). Selenite absorption was also shown to be reduced in humans administrated an intravenous solution containing a high selenium dose (1 mg) plus 1 g of ascorbic acid (Robinson et al.,1985a).

In animal studies, the information regarding the effect of ascorbic acid on selenium absorption appears contradictory. Studies by Combs and Pesti (1976) indicated that ascorbic acid increased the absorption of radioselenite from duodenal loops of chicks. They therefore suggested that reducing agents such as ascorbic acid can actually enhance the absorption of inorganic forms of dietary selenium. However, another study (Mykkanen and Mutanen, 1983) with chicks, conducted to determine the effects of ascorbic acid on the absorption of orally or introduodenally administrated sodium selenite, showed that moderate doses of ascorbic acid (10 mM) could inhibit the intestinal absorption of sodium selenite.

In humans, a single dose of selenium given as $^{74}SeO_3^{2-}$ with orange juice was just as available for absorption as $^{74}SeO_3^{2-}$ given with water (Mutanen & Mykkanen, 1985). But in another study, administration of 1 mg selenium as selenite with 200 mL orange juice (about 60 mg ascorbic acid) to young women increased selenium absorption (Robinson et al., 1985a). Martin et al., (1989) gave an oral dose of ascorbic acid (1 g/day) and selenium (82 µg of selenium as $Na_2{}^{74}SeO_3$; total selenium intake was 300 µg/day) to eight healthy male college students. They found that both the absorption and the retention of this stable isotope of selenium were significantly greater among the subjects given ascorbic acid supplements (1 g/day for 25 days) than those in the control group (20 mg/day). They also found that the selenium-exchangeable metabolic pool of ascorbic acid restricted subjects was only about one-third that found in the ascorbic acid supplemented subjects. These results therefore suggest that ascorbic acid may favorably affect selenium homeostasis in humans at least when selenium is the form of the selenite ion.(Anonymous, 1990).

c. Physiological factors affect selenium absorption

Species: On the basis of limited available data, it appears likely that selenium absorption is less efficient in man than in the rat. Selenomethionine, for example, has been reported to be 91% in rats (Thomson and Stewart, 1973) compared to 75% in humans ((Robinson et al., 1978). The absorption of selenium as selenite has been reported to be as low as 45% in

humans (Robinson et al., 1978) and as high as 93% in rats (Thomson and Stewart, 1973). Applying results from rat studies to humans should therefore consider this absorption difference.

B. Selenium transport

After selenium is absorbed, it is rapidly taken up and metabolized to reduced form such as selenoglutathione or selenide by erythrocytes using reduced glutathione (Lee et al., 1969; Gasiewicz and Smith, 1978). The subsequent release of the reduced forms of selenium is in a protein-bound form which accounts for almost all of the selenium in plasma. The selenoprotein glutathione peroxidase represents only a small part of the plasma selenium level (about 1.5%) (Behne and Wolters, 1979) so it doesn't play a role in transfer of selenium. Many authors (Favier, 1988) have observed a variation of selenium bound with plasma protein and a majority have reported that selenium is associated with an alpha-2-globulin. In humans selenium is mainly carried by very low-density lipoprotein (VLDL). Montzenbocker and Tappel (1982) identified a selenocysteine-containing plasma protein synthesized in the rat liver, called selenoprotein P, which they believe may serve as a selenium transfer protein to redistribute selenium from liver to extrahepatic tissues. So far, no selenium transport protein has been found in humans.

C. Cellular uptake and distribution

Cellular uptake of selenium varies with the tissue and

is influenced by the level and form of selenium in the diet. When selenium is ingested by animals at the nutritional requirement level (0.05-0.2 mg/kg), selenium is at high concentration (0.2-2 μg/g) in kidney and liver. These organs attract selenium very fast but have a first rate of elimination. Cardiac muscle (0.15-0.20 μg/g) is consistently higher in selenium than skeletal muscle (0.05-0.20 μg/g). Brain and nervous tissue (< 0.1 μg/g) tend to be low and adipose tissue is very low in selenium. With low selenium intake (< 0.05 mg/kg), blood and liver selenium markedly decrease but kidney is less affected. At a high intake of selenium (> 1 mg/kg), tissue selenium concentrations rise steadily until levels as high as several μg/g in liver and kidneys are reached. Beyond these levels, excretion keeps pace with absorption. Among different forms of selenium, organic forms of selenium produce greater level of tissue selenium concentration than inorganic selenium. Although the selenium concentration of muscle is low, it accounts for 40% of total body selenium because of its large mass. Liver accounts for about 30% of total body selenium.

D. Selenium biotransformation

The assimilation of different chemical forms of selenium by tissues undergoes complex metabolic transformation to be fully utilized as a biocatalyst (Young et al., 1982; Levander, 1986). In animals, selenium compounds tend to be metabolized to more reduced states. Selenoamino acids, such as selenomethionine, are first converted to selenocysteine

and released as selenite by postabsorptive catabolism. Selenate is also reduced to selenite which in turn is further reduced to selenide (H_2Se) by way of enzymatic and non enzymatic steps. Selenide with an oxidation state of -2 is a very important metabolite because in this form selenium may be incorporated into proteins. Selenide, for example, can be incorporated into selenocysteine which is the form of selenium at the active site of the best known selenoenzyme, glutathione peroxidase. Selenide may also be either oxidized to elemental selenium, interact with plasma or cellular proteins or be methylated for elimination as the trimethylselenonium ion. Selenium biotransformation in humans has not been well studied, but it is hypothesized that selenium in humans has a similar biotransformation as those for animals.

E. Function of selenium

Selenium is one of the essential trace elements for normal performance, growth and fertility in mammals including humans. Evidence for the essentiality of selenium was first provided by Schwarz and Foltz (1957) who demonstrated that liver necrosis in vitamin E-deficient rats could be prevented by selenium. Evidence for the essentiality of selenium for humans was lacking until 1973 when Rotruck and coworkers (Rotruck et al., 1973) demonstration that selenium is part of the metalloenzyme glutathionine peroxidase. More recently, Chinese scientists have reported an association between low selenium status and Keshan disease. Keshan

disease is an cardiac myopathy that affects children and women who live in Keshan County, Heilongjiang Province of China. This apparent selenium responsive disorder was virtually eliminated after selenium supplementation with either table salt supplemented with 15 mg/kg as Na_2SeO_3 or orally administered tablets of 0.5 or 1.0 mg Se as Na_2SeO_3 given once per week to each child (Keshan Disease Research Group, 1979).

The requirement of selenium as an essential constituent at the active site of glutathione peroxidase is the best known biochemical function of selenium in mammals. This selenoenzyme was described by Rotruck et al. (1973). It is present in the cytosol and mitochondrial matrix space with a molecular weight of 80,000. Glutathione peroxidase is composed of four identical subunits with a molecule of selenosysteine incorporated with the peptide chains of each subunit. The selenoprotein glutathione peroxidase functions as part of a multicomponent antioxidant defense system within the cell (cytosol and mitochondrial). This system protects unsaturated phospholipids and critical proteins from adverse effects of reaction oxygen and free radical interactions of oxygen. Various prooxidant compounds which promote formation of hydrogen peroxide and free radicals can occur in cells as a result of normal cellular functions (e.g., superoxides are formed during the reduction of oxygen) or from the metabolism of toxic substances. Depending on the origin and target of these prooxides, various antioxidants are needed to protect

the cells. Glutathione peroxidase detoxifies both organic and inorganic peroxides in the cytosol.

Selenium may also play a metabolic role in the detoxification of heavy metals such as mercury (Ganther et al., 1972), cadmium (Chen et al., 1974; Magos and Webb, 1980) and lead (Cerklewski and Forbes, 1976). Evidence has been cited that links certain types of cancers and cardiovascular diseases with low selenium status (Schrauzer et al., 1977; Keshan Disease Research Group, 1979). Selenium compounds have also been suggested for cancer chemotherapy because selenium status and cancer mortality appear to be inversely correlated (Griffin, 1979).

F. Selenium excretion

In humans with a normal level of dietary selenium intake (50-200 μg/day), selenium can be excreted from the body through two major routes: the urinary tract and intestinal tract. Urinary selenium excretion is markedly higher than fecal excretion and major urinary metabolite form is trimethylselenonium (Levander et al., 1981). The amount of selenium excreted in the urine is directly related to the dietary intake in man so that the proportion of urinary selenium in terms of the total selenium output remains relatively constant (50%-60%) (Levander et al., 1981). It has been suggested that urinary excretion is the most important mechanism by which selenium homeostasis is achieved (Robinson et al., 1985b). Fecal excretion accounts for a relatively constant fraction of total output over a wide

range of dietary selenium intake (35%-45%) (Wei et al., 1984). Very high intake of selenium is associated with the production of the volatile compound dimethylselenide which is eliminated by pulmonary excretion. In rats, dimethylselenide excretion greatly increased from lung only when the dose of selenium exceeded 200 µg (1.4 mg/kg weight). There is only a few percentage of selenium excretion via pulmonary, dermal or saliva routes under normal selenium intake (Favier, 1988).

III. Selenium Toxicity

A. Human selenosis

The possible toxic effects of dietary selenium overexposure to people was of little concern until the discovery in the early 1930s by Frank that selenium in grains, grasses and weeds causes alkali disease in livestock. Several surveys were done in seleniferous areas in Northern Great Plains of the USA , part of Venezuela, and Columbia. Correlation of high urinary selenium with some symptoms of selenium toxicity such as hair and nail loss, garlic odor of breath, nausea, gastrointestinal disturbances, dermatitis, and fatigue was found.

The disease syndromes produced in man by excessive intake of selenium have been documented for many centuries and are well reviewed by Olson (1986) and Combs and Combs (1986b). Chronic selenosis has also been reported in human as the result of the use of various oral supplements which provided selenium. Recently, the US Food and Drug Administration (FDA) and the Center for Disease Control (CDC)

in 1984 reported that thirteen people developed selenium intoxication after taking an improperly manufactured dietary supplement. Analysis of the supplement showed that it contained 27.3 to 31 mg of selenium which was about 182 times higher than that declared on the label. The total number of tablets consumed by the thirteen individuals ranged from one tablet taken once to seventy-seven tablets over a two and half month period. Eleven additional cases have been reported subsequently. The most common symptoms reported have been nausea and vomiting, abdominal pain, diarrhea, nail and hair changes, peripheral neuropathy, fatigue and irritability (Helzlsouer et al., 1985).

Although water have not generally been considered to be a potential source of selenium toxicity even in seleniferous areas, it was reported that an Indian family from Wyoming suffered from selenium poisoning by consuming well-water containing 9 μg/g Se. Members of the family experienced hair loss, weakened nails, and listlessness (Anonymous, 1962).

B. Mechanism of selenium toxicity

The mechanism by which selenium toxicity interferes with tissue structure and function remains to be established. It was first pointed out by Dougherty and Hoekstra (1982) that a toxic level of selenite has a pro-oxidant effect in vivo. They estimated lipid peroxidation in vivo by monitoring the production of ethane, a volatile product formed by peroxidation of omega-3-unsaturated fatty acids. Using this technique, they found a 15-fold increase over controls of

ethane exhalation in rats fed a vitamin E and selenium deficient diet after they were injected with 2mg/kg sodium selenite. The pro-oxidant effect of selenite is consistent with the observations by others that selenite stimulated lipid peroxidation in vitro (Bunyan et al., 1960) and chronic intoxication could be induced by relatively low levels of selenite (1.25 μg/g) in rats severely deficient in vitamin E (Wright and Bell, 1964). Morever, it was reported that mice given a modest amount of sodium selenite (0.1 μg/ml) in the drinking water and fed a normal dietary levels of vitamin E and selenium (0.05 μg/g), developed increased hepatic concentrations of lipofuscin pigments by 9 months (Csallany et al., 1984). The accumulation of lipofuscin pigments would indicate lipid peroxidation having occurred as a result of the selenite supplement.

C. Dietary factors modify selenium toxicity

The toxicity of selenium can be greatly modified by several dietary factors. These factors include level and type of protein, antioxidants (vitamin E), inorganic sulfur, level of heavy metals and many other factors (Combs and Combs, 1986b).

a. Dietary protein and methionine

A high protein diet can significantly alter the toxicity of selenium. Gortner (1940) first showed that growth depression in rats fed a diet containing 35 μg/g selenium could be alleviated by feeding a high level (30% of the diet) of protein as casein. Casein was the most effective type of

dietary protein in this regard. Similar but less effective protection was found for lactalbumin.

The sulfur-containing amino acid methionine has a beneficial effect against selenite toxicity in rats that may depend on vitamin E or another fat soluble antioxidant in the diet. It has been demonstrated that methionine and vitamin E have a combined ability to reduce the liver and kidney level of selenium. It was suggested that methionine provided labile methyl groups for production of easily excreted methylated metabolites and that vitamin E served as a lipid-soluble antioxidant to increase the availability of the methionine-methyl group in the process of detoxification of selenium.

b. Inorganic sulfur

Supplemental sulfur can reduce toxicity signs of selenium. It was found by Ganther and Baumann (1962), for example, that growth depression in rats fed excess sodium selenate (5 mg/kg) were prevented by feeding sulfate (1%). A similar finding with respect to growth depression and liver damage in rats was found by Halverson and Monty (1960). Ganther and Baumann (1962) suggested that sulfate reduced selenium toxicity by increasing urinary excretion of selenium.

c. Heavy metals

Dietary arsenic (As), silver (Ag) and copper (Cu) have been shown to have possible potential in alleviating selenium toxicity in experimental animals with each element apparently

exerting its protecting action by its own mechanism. Unfortunately, most of what is known about these interactions is ill-defined.

Moxon (1938) discovered that the growth-depressing effects of feeding seleniferous grains (15 µg/g selenium) to rats could be protected by adding sodium arsenite (5 mg/l) to their drinking water. This finding was confirmed by numerous workers in the later years and reviewed by Diplock (1976). Either arsenate or arsenite was equally effective and selenium could by presented as either seleniferous grain , selenite or selenocysteine. Levander and Baumann (1966) demonstrated that arsenic was protective against selenium toxicity because arsenic had the ability to increase the biliary elimination of selenium in rats. It has been observed in chicks, that dietary silver (1000 mg/kg) and copper (1000 mg/kg) can prevent the mortality of selenium toxicity (5 mg/kg and 10 mg/kg) and reduce the growth depression (Jensen, 1975). Stowe (1980) showed that ponies given 6 and 8 mg Se/kg without copper pretreatment developed selenium toxicity syndromes and all ponies given 8 mg Se/kg died within 36 hours of selenium administration. Ponies pretreated with either 20 or 40 mg Cu/kg given the same amount of the selenium did not show any toxicity signs. It has been suggested that copper may interfere with selenite absorption by formation of insoluble complexes. The ability of silver to decrease selenium toxicity may be by interfering with selenium absorption and by increasing the nontoxic

selenium compounds in tissues.

IV. Selenium and Fluoride Interactions

A. Evidence for a fluoride and selenium interaction

At the third international symposium on selenium metabolism, Liu and Li (1987) pointed out that cases of endemic selenosis and fluorosis occurred simulataneously in human and animals within Enshi county, Hubei province of People's Republic of China. Their investigation indicated that both selenium and fluorine were rich in this area and that the source of the two elements was coal in the environment and corn from diet. The concentration of fluorine of coal and corn were higher in the fluorosis area (F in coal range from 433-1128 mg/kg; content of F in corn was 40.94 $\pm$ 8.03 mg/kg) than in the selenosis area (fluoride in coal range from 252-657 mg/kg; content of fluoride in corn was 15.20 $\pm$ 4.56 mg/kg). The level of selenium in coal was higher in the selenosis area but the selenium level of corn in the selenosis area was similar to that found in the fluorosis area. They found some fluorosis patients in areas of selenosis but in the high fluorosis areas symptoms of endemic selenosis were not found in all residents despite the presence of high level of blood and hair selenium. Thus, they suggested that high fluorine may have inhibited selenium intoxication by accelerating excretion of selenium through hair and urine. In another study they also found that the monocellular organism tetrahymena pyriformis would not grow in a medium containing 5 μg Se/ml and 2 μg F/ml. However,

when fluoride in the medium was increased to 5 μg/ml, with the same amount of selenium, growth rate were similar to control (Whanger, 1989).

B. Interaction mechanism of fluoride and selenium toxicity

It is necessary to review the bioavailability of dietary fluoride in order to understand the the interaction of fluoride and selenium. The bioavailability of dietary fluoride depends on the ratio of free and bound forms of fluoride in food. In general, the higher the free fluoride the greater the fluoride bioavailability. The bioavailabliity of NaF intake in water or tablets by human is 100%. Fluoride in foods and beverages, which are either protein bound or in organic forms, appears to be less bioavailabile (50%-80%)). This result is in agreement with the studies with rat (Rao, 1984). Aluminum, calcium and magnesium form insoluble complexes with fluoride thus reducing the absorbability of fluoride. Selenium, however, does not form an insoluble complex with fluoride. Ingested fluoride rapidly enters the circulating blood and is distributed into all organs and tissues but the skeleton is the major deposition site (98%) of fluoride .

Although the data from China suggest a protective effect of high fluoride on selenosis, not all investigators agree. Moxon and DuBois (1939), for example, reported that mortality caused by selenite in the diet (11 mg/kg) of rats was exaggerated by also providing fluoride (5 mg/kg diet). Hadjimarkos (1969), however, was unable to duplicate this

result when selenium (3 mg/kg diet) was combined with 50 mg/kg fluoride. Shearer and Ridlington (1976) also failed to find any evidence for a fluoride and selenium interaction in either the hard (femur and molar enamel) or soft tissues (liver and kidney) of rats. Shearer and Ridlington (1976) indicated that the failure to see an interaction may be due to the fact that fluoride and selenium are located in two distinctly different compartments of the body. Fluoride as fluorapatite is located in the mineralized region of hard tissue whereas selenium as selenoamino acids and selenotrisulfides are found in the protein fraction of soft tissues.

Current studies (Shayiq et al., 1986) indicated that a significant inhibition of lipid peroxidation in liver and intestine was noticed at 10 mM NaF and a further increase in NaF concentration resulted in a spontaneous decrease in malondialdehyde (MDA) formation. Lipid peroxidation is the oxidative degradation of polyunsaturated lipids. During the reduction of molecular oxygen in the cell, reactive free radicals such as the superoxide radical (O_2^-) are readily formed leading to an attack on the polyunsaturated fatty acid portion of biological membranes thus initiating the process of lipid peroxidation. Active oxygen species mediating lipid peroxidation is believed to be one of the important causes of cell membrane destruction and cell damage.

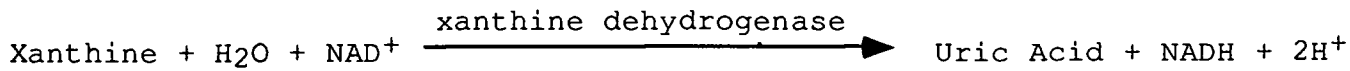
Earlier, it had been suggested that inhibition of lipid peroxidation formation by fluoride could be due to

stimulation of reducing capacity of tissues (Bus et al., 1976), but preliminary results by Cerklewski and Whanger (unpublished observation) failed to support this possibility.

Recently, much interest has been focused on xanthine oxidase as a major source of oxygen-derived free radicals in tissues. These radicals, including the superoxide anion and hydroxyl radical, have been implicated in mediating ischemic tissue damage (Granger et al., 1981). These oxygen-derived free radicals have been shown to play a role in the pathogenesis of ischemic tissue injury in the heart, kidney and brain. According to McCord (1985), xanthine dehydrogenase, the naturally occurring form of the enzyme, which uses NAD as an electron acceptor, does not produce free radicals when xanthine is metabolized to uric acid. However, under certain conditions, including that catalyzed by prooxiants, the dehydrogenase can be converted to the oxidase (Figure 1). The danger here is that xanthine oxidase can transfer electrons directly to molecular oxygen generating the superoxide anion O_2^- and H_2O_2. These two chemicals interact to form the highly reactive hydroxyl molecule, which can initiate cell damage such as lipid peroxidation. Figure 2 summarizes these events using reperfusion injury as an example.

Preliminary studies at Oregon State University, (Cerklewski and Whanger, unpublished observations) suggested that high dietary fluoride (150 mg/kg) would prevent cellular damage to liver caused by selenite (5 mg/kg) but problems

with fixing the tissue did not allow for a histological description of the liver. The present study (Experiment I) will test the hypothesis that a nutritional high, but non-toxic, level of fluoride will reduce liver damage of selenium toxicity. Liver damage will be assessed histologically. Experiment II will test the hypothesis that a high, but non-toxic, level of fluoride will reduce the toxic effect of selenite by inhibiting free-radical initiated lipid peroxidation. Experiment II will also determine if tissue damage caused by selenite (pro-oxident) in rats can be correlated with an increase in xanthine oxidase activity. Furthermore it will test whether fluoride can inhibit xanthine oxidase thus explaining why fluoride is protective against selenite toxicity in liver.



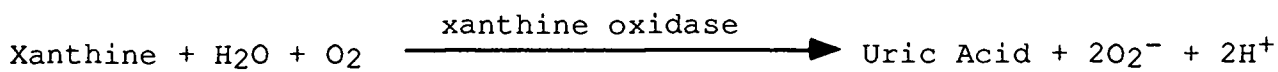


Figure 1. Reaction of xanthine (McCord, 1985)

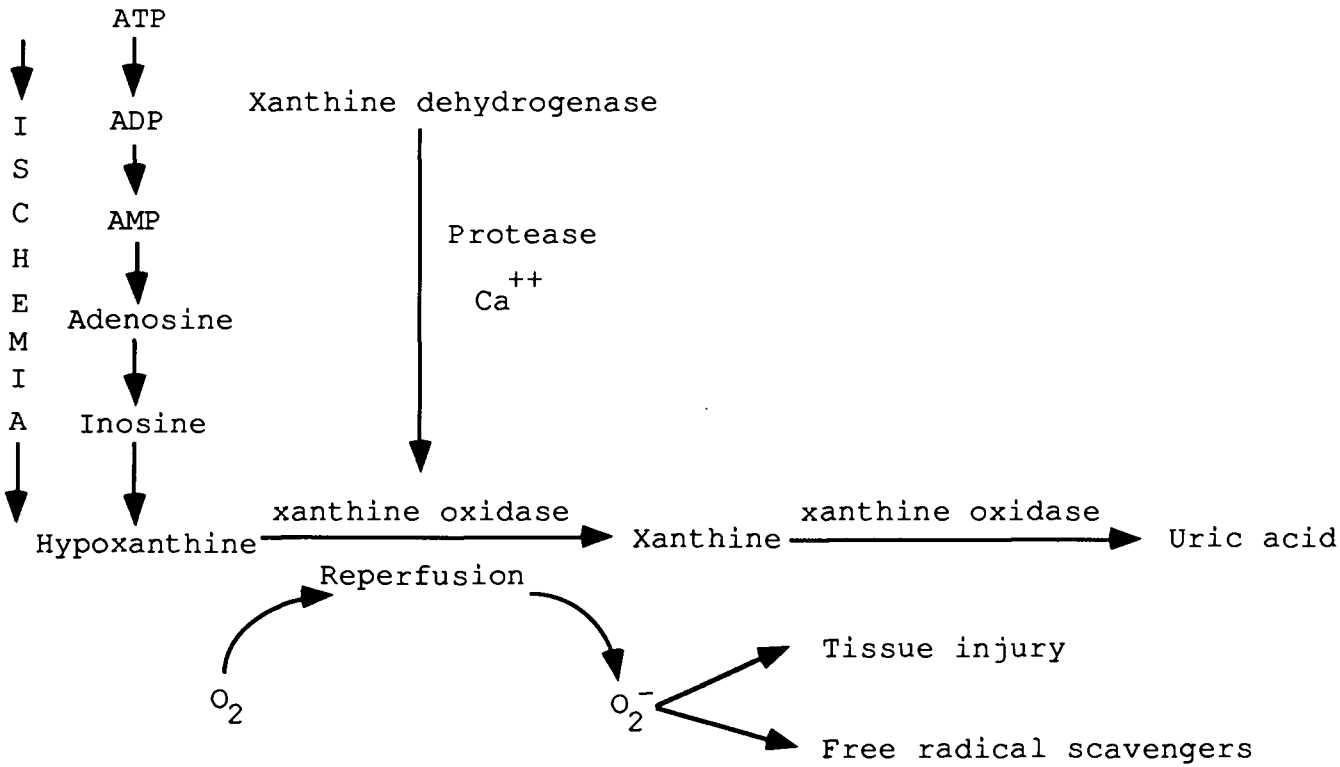


Figure 2 Mechanism responsible for production of superoxide radical by xanthine oxidase (McCord, 1985)

MATERIALS AND METHODS

Experiment I

The purpose of this study was to determine if a nutritionally high but non-toxic level of fluoride (150 mg/kg diet) could reduce liver damage of selenite toxicity (5 mg/kg diet). The experimental design was based upon results obtained from a pilot study (Cerklewski, F. L. and Whanger, P. D., unpublished observations).

Animals/Conduct of the study

Twenty five, male Sprague-Dawley rats, initially weighing 70-85 g (Batin-Kingman, Freemont, California), were individually housed in suspended stainless steel cages in a temperature- and humidity-controlled animal room with a 12 hour light-dark cycle. All rats were fed the control diet (low Se, low F^-) for a three-day quarantine. Rats (5/group) were then fed diets containing either 0.05 or 5 mg/kg Se as the selenite and either 1 or 150 mg/kg F^- as sodium fluoride in a factorial arrangement of treatments for eight weeks. A pair-fed control, where food intake of the low Se, low F^- group was restricted to that of the high Se, low F^- group, was also included because the pilot study indicated that the high selenium level would depress food intake. Rats were given free access to the powder-type diet which was provided

every other day in glass jars. Distilled-deionized water was provided in a glass bottle fitted with a stainless steel sipper and silicone rubber stopper. All unconsumed food was recorded and discarded. Body weight was recorded weekly.

Diets

Composition of the basal diet is shown in Table 2. Composition of the vitamin and mineral mixtures is shown in Tables 3 and 4 respectively. The basal diet, which provided selenium at 0.05 mg/kg and F^- at 1 mg/kg (control diet), was partitioned into four diets (Table 5). The higher levels of selenium and fluoride concentration in the individual diets was made by adding $Na_2SeO_3 \cdot 5H_2O$ and NaF using dextrose as a dispersing agent. $NaHCO_3$ was added to balance the sodium ion when more NaF was added. Sodium fluoride at 1 mg/kg is within the range of the suggested requirement of this nutrient for the rat (Subcommittee on Laboratory Animal Nutrition, 1978). The high fluoride level in this diet represents a nutritional high but nontoxic amount of fluoride. The diets were mixed and stored in plastic bags at $-4^{o}C$.

Sample collection and preparation for assay

Rats were anesthetized with sodium pentobarbital (3 mg/100 g body weight) following a 24 hour fast. Blood was removed from the abdominal aorta for analyses not reported in this thesis. This procedure also served to drain blood from the liver prior to preservation of this tissue for histological examination. For this purpose, a sample of

Table 2

Composition of the basal diet

Component	g/kg
Casein-vitafree[1]	150
DL-methionine[2]	3
Cellulose powder[3]	40
Vitamin mix[4]	50
Mineral mix[5]	30
Corn oil (0.01% BHT)[6]	50
Cornstarch[7]	150
Dextrose[8]	527

[1]U.S. Biochemical Corp., Cleveland, OH.

[2]Sigma Chemical Co., St. Louis, MO.

[3]Alphacel, ICN Nutritional Biochemicals, Cleveland, OH.

[4]See Table 3.

[5]See Table 4.

[6]A.E. Staley Co. Gregg Food Division, Portland, OR.

[7]CPC International, Summit-Argo, IL.

[8]Cerulose, CPC International, Summit-Argo, IL.

Table 3

Vitamin mixture[1]

Component	grams
thiamin-HCl	0.20
riboflavin	0.12
pyridoxine-HCl	0.08
calcium pantothenate	0.32
biotin	0.04
niacin	0.50
folic acid	0.02
vitamin B_{12} (0.1% trituration in mannitol)	1.00
menadione	0.01
ergocalciferol (500,000 IU/g)	0.08
retinyl palmitate (250,000 IU/g)	0.50
d-alpha-tocopheryl succinate (1210 IU/g)	0.80
choline chloride	30.0

[1]Made to 1 kg with dextrose.

Table 4

Mineral mixture

Component	g/kg
$CaHPO_4$	556.48
$CaCO_3$	50.00
NaCl	77.85
K_2CO_3	45.96
K_2SO_4	60.60
$K_3C_6H_5O_7 \cdot H_2O$	145.51
$MgCO_3 \cdot nH_2O$ (26% Mg)	51.28
$ZnCO_3$	0.8950
$MnCO_3$	4.0650
$CuCO_3$	0.3044
$FeC_6H_5O_7 \cdot nH_2O$ (18.42% Fe)	6.3355
KIO_3	0.0112
$Na_2SeO_3 \cdot 5H_2O$	0.0036
$CrK(SO_4)_2 \cdot 12H_2O$	0.6403
NaF	0.0626

Table 5

Composition of the individual diets

Factor	A = Selenite (mg/kg)	
	Level	a_1 =0.05 a_2 = 5
B = Fluoride (mg/kg)	b_1 = 1	a_1b_1 a_2b_1
	b_2 = 150	a_1b_2 a_2b_2

liver, approximately 2 mm in thickness, was immediately plunged into 10% neutral buffered formalin for routine histological examination. For histological examination of liver, tissue slices of preserved liver were embedded in paraffin, dehydrated in alcohol, cut into 5-6 μm sections with a Reichert-Jung microtome, and stained with hematoxylin and eosin prior to light microscopy examination by Dr. Olaf Hedstrom, College of Veterinary Medicine, Oregon State University, Corvallis, OR. Liver was also saved for selenium analysis and femur was saved for fluoride analysis. Tissues for mineral analysis were stored frozen (-20^{o}C) in plastic Ziploc bags.

Femurs were steam autoclaved for 8 minutes at 15 psi to allow defleshing. Each femur was broken in half, wrapped in cheesecloth identified with pencil, and extracted for successive 24 hours periods in a Soxhlet apparatus with 95% ethyl alcohol followed by petroleum ether. Femurs were then ashed at 590^{o}C in a muffle furnace (Thermolyne Corp., Dubuque, IA) for 24 hours, weighed and stored in a desiccator prior to analysis.

For liver selenium assay, samples were weighed and placed in a tared 50 mL acid-washed Erhlemeyer flask labelled on 2 sides with a water soluble marker. Samples were dried overnight in an oven and a dry weight was recorded. A set of standards which would bracket the unknown was also included. Both samples and standards were predigested with 10 mL concentrated nitric acid (HNO_3) and 3 mL concentrated

perchloric acid ($HClO_4$) for at least 4 hours at room temperature. After predigested samples were digested on a hot plate to a "white fume", digestion was continued for an additional 15 minutes. Samples were then removed from heat, and allowed to cool after which 1 mL concentrated hydrochloric acid was added and digestion continued to a "white fume" for an additional 15 minutes. This digestion allowed selenium to be converted to the +4 oxidation state. After cooling, 15 mL of 0.027 M EDTA and 2 drops of the combination indicator (cresol red and bromocresol green) were added to each sample. The pH of the samples were adjusted to 2-3 (yellow color) before assay.

Analytical methods

A. Femur fluoride determination

a. Reagents

3 N HCl: A solution was prepared by adding 250 mL of concentrated HCl in about 500 mL reagent water and diluted to 1000 mL.

5 M NaOH: A solution was prepared by dissolving 20 g NaOH in about 80 reagent water and diluted to 100 mL.

Fluoride standard stock solution (100 µg/mL): Purchased from Orion Research Incorporated, Cambridge Massachussetts.

Fluoride standard working solution: The fluoride standard solution was diluted 1/100 and 1/10 to give 1 µg/mL and 10 µg/mL working solution, respectively, and stored in labeled plastic bottles.

Total ionic strength adjustment buffer (TISAB II, pH=5.5):

This buffer was prepared by dissolving 57 mL glacial acetic acid, 58 g of sodium chloride and 4 g of disodium 1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid (DCTA) in 500 mL reagent water. The pH of this solution was adjusted to 5.5 by addition of 5 M NaOH. The solution was cooled to room temperature and diluted with distilled water to 1000 mL. This buffer was described by Sekerka and Lechner (1973).

b. Assay technique

Femur fluoride was analyzed using the method of Singer and Armstrong (1968). Ashed femur samples were dissolved in 3 N HCl in 20 mL polystyrene beaker cups (VWR Scientific, Seattle, WA) and transferred to a 50 mL polymethylpentene flask (Nalge Co., Rochester, NY) which contained 25 mL of total ionic strength adjustment buffer (TISAB II, pH=5.5). Final pH was adjusted to 5.2 with 1 mL of 5 M sodium hydroxide (NaOH). The contents were then made to volume with redistilled water. Femur fluoride concentration was determined with a fluoride combination electrode (Model 96-09 and Model 901 Ionalyzer, Orion Research Inc., Cambridge, MA) against similarly prepared fluoride standards (1 µg/mL and 10 µg/mL).

To minimize contamination of fluoride all laboratory ware was made of plastic because fluoride can be adsorbed onto standard laboratory glassware. Plastic bottles were used to keep fluoride solutions. Because tap water in Corvallis contains supplemental fluoride, it was necessary to use redistilled water to prepare all reagents related to the

fluoride assay.

B. Liver selenium determination

a. Reagents

5 N NH_4OH: This solution was prepared by adding 83 mL of concentrated NH_4OH to a flask and diluted to 250 mL with deionized water.

0.1 N HCl: This solution was prepared by adding 8.3 mL of concentrated HCl to a flask with about 800 mL of deionized water and diluted to 1000 mL.

0.1% diaminonaphthalene (DAN) reagent: This solution was prepared by mixing 0.05 g DAN and 50 mL of 0.1 N HCl in a shaking water bath at 40-50°C for about 20-30 minutes. After mixing, the solution was extracted 3 times with spectrograde cyclohexane and filtered into a brown container containing 25 mL of cyclohexane.

0.8 M NH_2OH-HCl: This solution was prepared by dissolving approximately 55 g of NH_2OH HCl in deionized water and diluted to 1000 mL.

Spectrograde cyclohexane: This was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ.

0.09 M ethylenediamine tetraacetic acid (EDTA): This solution was prepared by adding 2.63 g EDTA to 50 mL deionized water and slowly adding 5 N NH_4OH until the EDTA dissolved. The solution was then diluted to 1000 mL with deionized water.

Selenium standards (stock) 1000 ng/mL: To prepare this stock solution, 2.19 g dried Na_2SeO_3 (45.65% Se) was dissolved in

water and made to 1000 mL (HNO_3 added to acidify) to give 1 mg/mL. Further dilution, 1:1000 with reagent water, gave 1 µg/mL or 1000 ng/mL.

Selenium working standards: Selenium working standards of 100, 200, 400, 600, and 800 ng/ml were prepared by appropriate dilution of the stock standard.

b. Assay technique

Rat liver selenium concentration was determined by a semiautomated fluorometric method (Brown and Watkinson, 1977) using an autoanalyzer II (Alpkem Corp, Clackamas, OR) (Beilstein and Whanger, 1986). The prepared samples were complexed with 2,3-diaminonaphthalene (DAN) to form 4,5-benzopiazselenol which was extracted with cyclohexane. The fluorescence of 4,5-benzopiazselenol was measured with a 325 nm filter for excitation and a 556 nm filter for emission. Peak heights of each samples were measured in mm and a standard curve was plotted. Selenium content of each sample was calculated from the standard curve in both µg/g wet and µg/g dry liver weight.

Experiment II

The purpose of this study was to determine if the cellular damage to liver by selenite toxicity could be associated with free radical-mediated lipid peroxidation and to determine if the source of free radicals in selenite-toxic rats was due to a stimulation of the enzyme xanthine oxidase. Furthermore, the intent was to determine if fluoride could

inhibit xanthine oxidase thus explaining why fluoride was protective against selenite toxicity in rat liver.

Animals/Conduct of study

Thirty-two, male Sprague-Dawley rats, initially weighing 74-88 g, were purchased from Charles River Laboratories, Wilmington, Mass. The experimental design was similar to Experiment I except that there were seven rats in each of the two high selenium treatment groups and six rats in the other three treatment groups. The experimental period was 6 weeks. Because of the complex procedures associated with one of the assays (xanthine oxidase), it was necessary to use a staggered start of 16 rats each, separated by one week (all treatments represented by each start). By this procedure, all 32 rats were on diet simultaneously for most of the time.

All rats were fed an adjustment diet (as described in Experiment I) for a three-day quarantine and continuously fed the experimental diet for 6 weeks. The diets were replaced every other day and all unconsumed material was recorded before it was discarded. Dietary intake was recorded every day and body weights were recorded weekly.

Diets

The composition of the basal diet and individual diets described earlier for Experiment I was repeated in Experiment II.

Sample collection and preparation for assay

At the end of the experimental period, all rats were anesthetized with sodium pentobarbital (3 mg/100 g body

weight) following a 24 hour fast. Liver was dissected free of any attached muscle, peritoneum, or fibrous tissue and 2 gram of liver sample from each rat was weighed and placed in ice-cold buffered sucrose at pH=7.8. Liver samples were homogenized in four volumes of ice-cold buffered sucrose at pH=7.8 for 45 seconds in a Potter-Elvehjem homogenizer fitted with a motor-driven Teflon pestle. The homogenate was centrifuged at 30,000 x g for 30 minutes (Beckman model J-2K centrifuge) and at 100,000 g for 1 hour (Beckman model L5-75 Ultracentrifuge). The supernanate was dialyzed against the homogenizing buffer for 24 hours with two changes of buffer, then centrifuged at 30,000 x g for 5 minutes before assay. One gram of another piece of the same liver was saved for lipid peroxidation assay. The liver sample for this purpose was cut into pieces in 9 mL 1.15% KCl iced-cold buffer and homogenized with a Potter-Elvehjem homogenizer. A fraction of liver was also saved for histological examination as described in Experiment 1. The remaining liver sample was weighed and saved for selenium assay. Femur was saved for fluoride assay. Samples for mineral analyses were placed in plastic Ziploc bags and stored frozen at -20°C.

Analytical methods

A. Xanthine oxidase activity

a. Reagents

2.5 M phosphate buffer: A 2.5 M stock solution was prepared by dissolving 30.9 g of KH_2PO_4 (anhydrous) and 396 g of K_2HPO_4 (anhydrous) in about 800 mL reagent water and diluted

to a final volume of 1000 mL with reagent water. The final pH was 7.8 (Rajagopalan, 1985).

Buffered sucrose: This buffer was prepared by dissolving 77.1 mg dithioerythritol, 85.6 g sucrose and 29.2 mg ethylenediamine tetraacetic acid (EDTA) in 20 mL 2.5 M phosphate buffer and diluted to 1000 mL with reagent water. The buffer was stored in a refrigerator.

60 mM phosphate buffer: This buffer was prepared by dissolving 35 mg EDTA in 24 mL 2.5 M phosphate buffer and diluted to 1000 mL with reagent water.

Xanthine solution: A 5 mM stock solution was prepared by dissolving 38 mg xanthine in 10 mL 50 mM NaOH and diluted to 50 mL with reagent water. This solution was stored at -4°C and rewarmed to room temperature before use. A working stock of this solution (500 µM) was prepared on the day of assay by diluting the stock solution 1:10 with reagent water. Any unused working stock was discarded (Owen, 1971).

b. Assay technique

Xanthine oxidase was measured based on the reaction of the enzyme with xanthine (Fridovich, 1985). Dialyzed solution (0.2 mL) was added to a ultraviolet cuvette containing 0.3 mL 500 µM xanthine and 2.5 mL 60 mM potassium phosphate buffer pH=7.8 containing 0.12 mM EDTA. The subsequent production of uric acid was monitored spectrophotometrically at 295 nm and was proportional to enzyme activity. The rate of change of optical density was recorded on a Beckman DU-64 spectrophotometer and enzyme

activity was expressed as the change in optical density per minute per gram of liver protein (measured by Lowry protein assay).

B. Lowry protein assay

a. reagents

Reagent A: This solution was prepared by dissolving 20 g Na_2CO_3 in 100 mL of 1 M NaOH and diluted to 1000 mL with reagent water.

Reagent B: This solution was prepared by separately dissolving 0.5 g $CuSO_4.5H_2O$ in about 40 mL reagent water and 1.19 g sodium tartrate dihydrate (MW = 230.1) in about 40 mL reagent water. The two solutions were combined and diluted to 100 mL with reagent water (0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate).

Reagent C: A alkaline copper solution was prepared by mixing 50 mL of reagent A with 1 mL of reagent B. Any unused reagent was discarded after one day.

Reagent E: This reagent was prepared by diluting 2 N Folin and Ciocalteu's phenol reagent (Sigma Chemical Co., Louis, MO) 1 to 1 with reagent water.

b. Assay technique

Liver sample protein was measured by adding 1 mL of diluted liver sample from the xanthine oxidase assay to a test tube containing 5 mL of reagent C, mixed well and allowed to stand for 10 minutes at room temperature. Reagent E (0.5 mL)was added and mixed within a second or two. After 30 minutes sample color was read at 660 nm with a Beckman

DU-40 spectrophotometer.

C. Lipid peroxides

a. Reagents

1.15% KCl: This buffer was prepared by dissolving 11.5 g KCl in the reagent water and made to 1000 mL with reagent water.

8.1% sodium dodecyl sulfate (SDS): This solution was prepared by dissolving 8.1 g SDS in reagent water and made to 100 mL with reagent water.

Acetate buffer (pH=3.5): This buffer was prepared by mixing 200 mL glacial acetic acid with 500 mL reagent water and adjusted pH to 3.5 with 5 M NaOH and finally diluted to 1000 mL with reagent water.

0.8% thiobarbituric acid (TBA): This solution was prepared by dissolving 0.8 g TBA in 100 mL of 50 mM NaOH solution. Any unused reagent was discarded after use.

Butanol/pyridine (15/1 volume/volume): This reagent was prepared by adding 10 mL pyridine to 150 mL n-butanol in a glass-stoppered 250 mL graduate cylinder and mixed well.

Standard stock 10 mM 1,1,3,3-tetramethoxypropane (TMP) solution: A standard stock solution was prepared by dissolving 164.2 mg TMP in 40% ethanol and diluted to 100 mL with 40% ethanol. This solution served as an external standard because the absorption spectrum of the product obtained with liver homogenate has been shown to produce a similar absorption spectrum as that with TMP (Ohkawa, et al., 1979).

Standard working stock 100 µM TMP solution: A working stock solution was prepared by diluting the initially prepared stock solution 0.1 mL to 10 mL with 40% ethanol.

b. Assay technique

Lipid peroxides were estimated by the thiobarbituric acid (TBA) test (Ohkawa et al., 1979). In this test, one of the products of peroxidation, malondialdehyde (MDA), was measured. The quantity of this material present was used as an indication of the amount of the peroxidation that had occurred in vitro. In this study it was used to analyze the peroxidation in rat liver. The reaction of TBA with MDA in the liver was detected spectrometrically at 532 nm. 1,1,3,3-tetramethoxypropane (TMP) was used as an external standard. The results were expressed as nmols/g protein.

In this assay, 0.1 mL homogenate liver sample, 0.2 mL 8.1% SDS solution, 1.5 mL acetate buffer (pH=3.5), 1.5 mL 0.8% TBA solution and 0.7 mL reagent water were combined in a test tube. TMP working stock (0, 20, 50,100 µL), diluted with 800, 780, 750 and 700 µL reagent water, respectively, replaced the 0.1 mL homogenate solution to develop a standard curve. After mixing well, the reaction mixture was heated at 95^{o}C for 60 minutes in a dry block heater. At the end of 60 minutes, tubes were cooled to room temperature and 1 mL reagent water plus 5 mL butanol/pyridine (15:1, v/v) was added. Tubes were mixed for 30 seconds. After centrifuging the samples at 2000 x g for 10 minutes, the upper organic layer was transferred to a cuvette with a disposable glass

transfer pipette and the absorbance of organic layer was read at 532 nm with a spectrophotometer (Beckman spectrophotometer, National Technical Laboratory, South Pasadena, CA). Sample protein was analyzed following the procedure of Lowry protein assay as previously described.

D. Femur fluoride determination

See analytical methods part A of Experiment I.

E. Liver selenium determination

See analytical methods part B of Experiment I.

Statistical Analysis

The experimental design of both Experiment I and Experiment II was a 2 x 2 factorial experiment involving two factors, selenium and fluoride, with two levels of each factor. There was four treatments with five replicates per treatment in Experiment I. In Experiment II one rat was eliminated from each high selenium group, based upon poor food intake, which yielded six replicates per treatment. The purpose of including a pair-fed control in each Experiment was to determine if reduced food intake itself would adversely affect biochemical test results and histological examination. Treatment effects were partitioned into that due to selenium, fluoride and the combination of fluoride and selenium (Steel and Torrie, 1980). Individual means were compared using Fisher's least significant difference method (FLSD) only if a significant F-value was found for the treatment effect. By using this procedure, the possibility

of making a type I error (null hypothesis rejected but it is actually true) was reduced while still being able to detect real differences between means. Effects were considered to be significant at $P < 0.05$.

RESULTS

Experiment I

The purpose of this study was to determine if a nutritional high but non-toxic level of fluoride (150 mg/kg diet) could reduce liver damage of selenite toxicity (5 mg/kg diet).

Food intake and body weight

Total food intake and body weight gain of rats fed the four treatment diets for 55 days are shown in Table 6. High fluoride failed to prevent the depressive effect of selenite. In fact, body weight gain was actually lowest in the high fluoride, high selenium group. An interactive effect of selenium and fluoride was not evident for either food intake or body weight. Rats fed diets containing high selenium (5 mg/kg Se) had significantly less food intake and body weight gain than rats fed the ad libitum control diet (0.05 Se, 1 F^-). Similar effects have been reported by others (Halverson, et al., 1962; Palmer and Olson, 1974). In high selenium groups, food intake was reduced 21% in the low fluoride group and 23% in the high fluoride group when compared to the ad libitum control. Body weight was reduced 22% by selenium in the low fluoride group and by 35% in the high fluoride group compared to the ad libitum control.

Depressed food intake in the high selenium groups confirmed that a pair-fed control was necessary in this study. The

Table 6

Experiment I: Effect of dietary selenium and fluoride on food intake, body weight gain, liver selenium content and femur fluoride content[1]

Measures	Dietary treatments, mg/kg					Significance Levels		
	1 fluoride			150 fluoride				
	AL control[2] 0.05 Se	PF control[3] 0.05 Se	5.0 Se	0.05 Se	5.0 Se	F	Se	F +Se
55d food intake grams	1214[a] ±37	945[c] ±60	964[c] ±63	1094[b] ±40	934[c] ±81	<0.025	<0.001	NS
55d weight gain grams	317[a] ±24	239[c] ±21	247[b,c] ±15	274[b] ±16	206[d] ±24	<0.001	<0.001	NS
Liver selenium nmole/g wet weight	7.65[c] ±0.54	8.45[c] ±1.19	26.9[a] ±1.76	7.66[c] ±1.75	23.1[b] ±1.74	<0.025	<0.001	<0.025
Liver selenium nmole/g dry weight	23.1[c] ±3.20	26.5[c] ±3.54	84.2[a] ±6.28	23.9[c] ±5.68	71.6[b] ±7.17	<0.05	<0.001	<0.025
Femur fluoride µmole/femur	1.70[c] ±0.13	1.92[c] ±0.09	1.83[c] ±0.14	122.8[b] ±3.81	147.4[a] ±9.52	<0.001	<0.001	<0.001
Femur F µmole/g ash	4.97[c] ±0.43	6.45[c] ±0.92	6.69[c] ±0.56	401.8[b] ±35.6	512.0[a] ±40.7	<0.001	<0.001	<0.001

[1]Mean ± SD, n=5 Different row superscript letters indicates a significant difference ($P < 0.05$) of means. If any letter combination matches, the difference is not significant [2]Ad-libitum control. [3]Pair-fed control to group III (1 mg/kg F; 5 mg/kg Se).

pair-fed group consumed the control diet equal to that eaten by the high selenium, low fluoride group. The primary purpose of the pair-fed group was to provide an alternate control for the histological examination to be reported in the next section. For statistical comparisons a 2 x 2 factorial ANOVA was done without considering the pair-fed group. The pair-fed control was successful because there was no significant difference between pair-fed control and high selenium, low fluoride group with regard to food intake and body weight.

Although high fluoride diets were not intended to decrease food intake and body weight, high fluoride (150 mg/kg F^-) did decrease food intake and body weight gain compared to the ad libitum control in this experiment. However, total food intake in the high fluoride, low selenium group was better than all groups except the ad libitum group. Although body weight gain in high fluoride, low selenium group was not significantly difference from the high selenium, low fluoride group, body weight gain was better than high fluoride, high selenium group and pair-fed group.

Histological examination of liver

As shown in Figure 3, livers from 4 of 5 rats consuming the high selenium, low fluoride diet had massive irregular focal areas of hepatic necrosis and hemorrhage that spanned multiple lobules. Hyaline thrombi, partially occluding portal arteries or central and portal veins either within or adjacent to affected regions, were routinely seen.

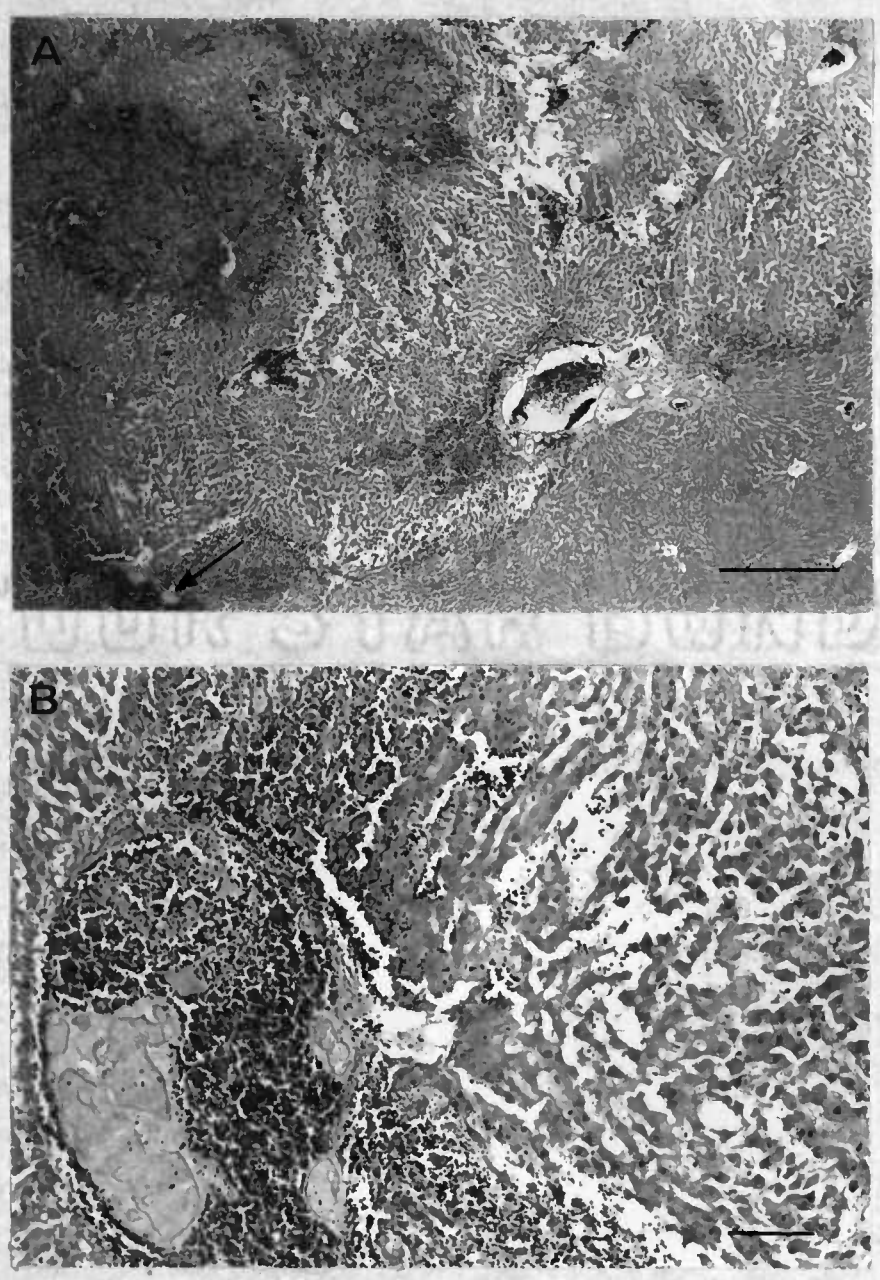


Figure 3 Liver histopathology from a rat fed a diet containing high selenite (5 mg/kg) and low fluoride (1 mg/kg). **A**. Extensive infarction and necrosis with hemorrhage extending over multiple lobules is shown. A blood vessel containing a hyaline thrombus (arrow) is associated with the necrosis. Normal liver structure is present in the right hand corner of the figure. Bar = 500 µm. **B**. Higher magnification of Figure 1A from left corner region (arrow) showing necrotic hepatocytes, hemorrhage and thrombosed blood vessel is shown in detail. Bar = 100 µm.

Sinusoidal spaces surrounding areas of necrosis were dilated and filled with red blood cells and serum. Significant numbers of inflammatory leukocytes were not associated with the necrosis. In the remaining rat liver without histological evidence of necrosis, the capsular surface was irregular and convoluted, Rats fed diets containing high selenium plus high fluoride, on the other hand, had no histological lesions in liver (Figure 4). Histological lesions of liver were likewise absent in the ad libitum control, the pair-fed control and in the high fluoride, low selenium group. Unlike liver, histological effects of selenite were not seen in either kidney or heart.

Liver selenium status

As shown in Table 6, high dietary fluoride significantly decreased selenium concentration of rat liver in the high selenium group for either on a nmole/g wet weight basis or on a nmole/g dry weight basis. This effect, however, was of small magnitude (15%). Liver selenium was presented on both a wet and dry weight basis to offer a comparison with different reports in the literature. Normally, liver selenium presented on dry weight basis is more appropriate than a wet weight basis because of difference in water content of tissue samples. An interaction effect of selenium and fluoride on selenium content in rat liver was observed because the fluoride effect was dependent upon the level of selenium (no fluoride effect for the low selenium group evidenced by similar liver selenium value for the ad libitum

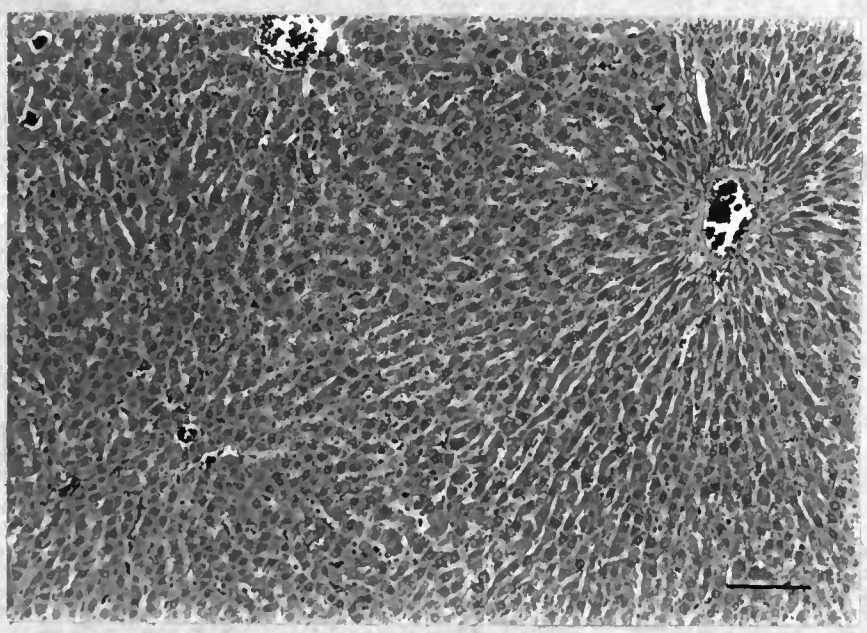


Figure 4 Liver histopathology from a rat fed a diet containing high selenite (5 mg/kg) and high fluoride (150 mg/kg). Normal hepatic lobular architecture is shown. Bar = 100 μm.

control, the pair-fed control and the high fluoride, low selenium group). As expected, liver selenium content was significantly higher in high selenium diet groups (5 mg/kg Se) than those in the low selenium groups (0.05 mg/kg Se) for either a nmoles/g wet weight basis or on a nmoles/g dry weight basis.

Femur fluoride status

Rat femur fluoride content was measured to test whether selenium level affected deposition of fluoride into bone (the reverse of the selenium and fluoride interaction) and also to indicate that the diet was really high in fluoride in high fluoride groups. As shown in Table 6, high dietary selenium significantly increased femur fluoride content at the high fluoride level. There was a fluoride and selenium interaction on femur fluoride content because the selenium effect was dependent upon the level of fluoride (there was no selenium effect at the low fluoride level). As expected, rat femur fluoride content was significantly higher in high dietary fluoride groups (150 mg/kg F^-) than those in low dietary fluoride groups (1 mg/kg F^-) when expressed as either a μmole/femur basis or as μmole/g ash. Results for femur fluoride were expressed both on a dry and wet weight basis because of differences in ash weight of bones.

Experiment II

The purpose of this study was to determine if the cellular damage to liver by selenite toxicity could be

associated with free radical-mediated lipid peroxidation and to determine if the source of free radicals in selenite-toxic rats was due to a stimulation of the enzyme xanthine oxidase. It was also the purpose of this study to determine if fluoride could inhibit xanthine oxidase thus explaining why fluoride was protective against selenite toxicity in rat liver as demonstrated in Experiment I.

Food intake and body weight

Total food intake and body weight gain of rats fed the four treatment diets for 40 days are shown in Table 7. Similar to Experiment I, high fluoride failed to prevent the depressive effect of selenite. Unlike Experiment I, body weight gain was not the lowest in the high fluoride, high selenium group. High selenite depressed both food intake and body weight gain regardless of the fluoride level (NS fluoride effect). An interactive effect of selenium and fluoride was not evident for either food intake or body weight gain. Rats fed diets containing high selenium (5 mg/kg Se) had significantly less food intake and body weight gain than rats fed the ad libitum control diet (0.05 Se, 1 F^-). Similar effects have been reported by others (Halverson, et al., 1962; Palmer and Olson, 1974). In high selenium groups, food intake was reduced 29% in the low fluoride group and 28% in the high fluoride group compared to the ad libitum control. Body weight was reduced 50% by selenium in the low fluoride group and by 52% in the high fluoride group compared to the ad libitum control.

Table 7

Experiment II: Effect of dietary selenium and fluoride on food intake, body weight gain, liver selenium content and femur fluoride content[1]

Measures	Dietary treatments, mg/kg					Significance Levels		
	1 fluoride			150 fluoride				
	AL control[2] 0.05 Se	PF control[3] 0.05 Se	5.0 Se	0.05 Se	5.0 Se	F	Se	F +Se
40d food intake grams	803^{a} ±35	554^{b} ±52	570^{b} ±87	734^{a} ±23	580^{b} ±102	NS	<0.001	NS
40d weight gain grams	280^{a} ±27	174^{c} ±30	$141^{c,d}$ ±42	236^{b} ±31	134^{d} ±30	NS	<0.001	NS
Liver selenium nmole/g wet weight	6.25^{b} ±0.86	6.61^{b} ±0.71	39.2^{a} ±7.38	6.39^{b} ±1.05	41.4^{a} ±11.0	NS	<0.001	NS
Liver selenium nmole/g dry weight	20.1^{b} ±2.72	21.5^{b} ±2.42	131.2^{a} ±23.2	20.8^{b} ±3.64	140.5^{a} ±33.2	NS	<0.001	NS
Femur F µmole/femur	0.98^{c} ±0.05	0.77^{c} ±0.06	0.68^{c} ±0.05	84.3^{a} ±5.13	60.6^{b} ±8.12	<0.001	<0.001	<0.001
Femur F µmole/g ash	3.96^{b} ±0.33	4.04^{b} ±0.13	4.27^{b} ±0.27	349.1^{a} ±49.1	321.9^{a} ±19.9	<0.001	NS	NS

[1]Mean ± SD, n=6 Different row superscript letters indicates a significant difference ($P < 0.05$) of means. If any letter combination matches, the difference is not significant

[2]Ad-libitum control. [3]Pair-fed control to group III (1 mg/kg F; 5 mg/kg Se).

Depressed food intake in the high selenium groups confirmed that a pair-fed control was necessary in this study. The pair-fed control group consumed the control diet equal to that eaten by the high selenium, low fluoride group. As in Experiment I, the primary purpose of the pair-fed group was to provide an alternate control for the histological examination to be reported in the next section. For statistical comparison a 2 x 2 factorial ANOVA was done without considering the pair-fed control. The purpose of the pair-fed control was also expanded in this experiment to include malondialdehyde and xanthine oxidase measurements. The results of these two biological assays were expressed per gram of protein because liver sample protein might be affected by decreased food intake. The pair-fed control was successful because there was no significant difference for these analyses between the pair-fed control and the high selenium, low fluoride group with regard to food intake and body weight.

Unlike Experiment I, high fluoride did not significantly depress food intake, but it did depress body weight gain compared to the ad libitum control.

Histological examination of liver

All rats consuming the high selenium, low fluoride diet had dilated sinusoids, cell necrosis and general collapse of liver architecture similar to that described in the previous study. Hemorrhage and infarction of liver seen in Experiment I, however, was not observed. The effects of selenite on

liver pathology were absent in kidney tissue slices and were of irregular occurrence in heart.

Fluoride was protective against selenite toxicity of liver for 50% of the rats in the high selenium, high fluoride group in Experiment II in contrast to 100% protection in the previous study. As in the previous study, histological lesions of liver were absent in the ad libitum control, the pair-fed control and in the high fluoride, low selenium group.

Liver selenium status

As shown in Table 7, liver selenium concentration was unaffected by dietary fluoride level for either a nmole/g wet weight basis or on a nmole/g dry weight basis. Thus, the effect seen in Experiment I was not replicated in this study. Therefore, there was no interactive effect of selenium and fluoride on selenium content of rat liver. Liver selenium was presented on both a wet and dry weight basis to offer a comparsion with different reports in the literature. Normally, liver selenium presented on dry weight basis is more appropriate than a wet weight basis because of differences in water content of tissue samples. As expected, liver selenium content was significantly higher in high selenium diet groups (5 mg/kg Se) than those in the low selenium groups (0.05 mg/kg Se) for either a nmole/g wet weight basis or on a nmole/g dry weight basis.

Femur fluoride status

Rat femur fluoride content was measured to test whether

selenium level affected deposition of fluoride into bone and also to indicate that the diet was really high in fluoride in the high fluoride groups. As shown in Table 7, high dietary selenium level significantly decreased femur fluoride content at the high fluoride level when expressed on a µmole/femur basis. When femur fluoride was expressed as a µmole/g ash basis, femur fluoride content at the high fluoride level was unaffected by dietary selenium level. Thus, the effect seen in Experiment I was replicated when femur fluoride was expressed as a µmole/femur basis but not when expressed as a µmole/g ash basis. As expected, rat femur fluoride content was significantly higher in high dietary fluoride groups (150 mg/kg F^-) than those in the low dietary fluoride groups (1 mg/kg F^-) when expressed as either a µmole/femur basis or as µmole/g ash. Results for femur fluoride were expressed both ways because of differences in ash weight of bones.

Level of lipid peroxides

The effect of dietary selenium and fluoride on liver level of malondialdehyde is shown in Table 8. This in vitro test actually estimates susceptibility of liver lipid to peroxidation. One of the products formed exhibits an absorbance spectrum similar to malondialdehyde (MDA). Therefore, results are expressed as nmole MDA/g protein. High dietary selenium significantly increased the level of malondialdehyde of rat liver in the low fluoride group (47%) whereas in the high fluoride, low selenium group the increase in malondialdehyde was only 9% compared to the ad libitum

Table 8

Experiment II: Effect of dietary selenium and fluoride on liver malondialdehyde level and liver xanthine oxidase activity[1]

Measures	Dietary treatments, mg/kg					Significance Levels		
	1 fluoride			150 fluoride				
	AL control[2] 0.05 Se	PF control[3] 0.05 Se	5.0 Se	0.05 Se	5.0 Se	F	Se	F +Se
Malondialdehyde nmole/g protein	$559^{b,c}$ ±66	633^{b} ±96	821^{a} ±98	494^{c} ±104	$609^{b,c}$ ±184	<0.025	<0.005	NS
Xanthine oxidase ΔOD/min/g protein	6.6 ±1.4	5.2 ±1.9	5.6 ±1.8	7.5 ±1.7	6.3 ±1.3	NS	NS	NS

[1]Mean ± SD, n=6. Different row superscript letters indicates a significant difference ($P < 0.05$) of means. If any letter combination matches, the difference is not significant.

[2]Ad-libitum control.

[3]Pair-fed control to group III (1 mg/kg F; 5 mg/kg Se).

control. High fluoride at the low selenium level tended to decrease malondialdehyde level (12%) compared to the ad libitum control but the effect was not significant. Therefore, there was no interaction effect of selenium and fluoride on malondialdehyde level in rat liver. The fact that there was no significant difference between the ad libitum control and the pair-fed control with respect to malondialdehyde level suggests that depressed food intake did not influence the level of liver lipid peroxidation in vitro.

Xanthine oxidase activity

The effect of dietary selenium and fluoride level on rat liver xanthine oxidase activity is shown in Table 8. Liver xanthine oxidase activity was unaffected by both the dietary level of selenium and fluoride. Therefore, no correlation could be made between the effect of selenium and fluoride on malondialdehyde level and xanthine oxidase activity.

DISCUSSION

At the Third International Symposium on Selenium Metabolism, Chinese scientists (Liu & Li, 1987) suggested that high fluoride might inhibit human selenium intoxication by accelerating the excretion of selenium through hair and urine. Therefore a study was designed to test the hypothesis that a nutritionally high but non-toxic level of fluoride (150 mg/kg diet) would reduce selenium toxicity (5 mg/kg diet) when compared to control levels of selenium (0.05 mg/kg diet) and fluoride (1 mg/kg diet) using the rat as an animal model. The results of this study (Experiment I) provided little support for an interaction between fluoride and selenite except for an apparent protective effect of fluoride on selenite liver pathology. The mechanism by which fluoride prevented selenite liver pathology, however, was not obvious from the available data. Fluoride, for example, failed to prevent the depressive effect of selenium toxicity on both food intake and body weight gain. Furthermore, liver selenium concentration was slightly lower in rats fed diets containing high selenium and high fluoride versus high selenium alone, but the magnitude of the effect was small. This latter result was in agreement with unpublished observations by Cerklewski, F. L. and Whanger, P. D. who found that dietary fluoride had little or no effect upon

tissue selenium concentration, intracellular selenium distribution in liver, plasma selenium, or on enzymatic activity of glutathione peroxidase in red blood cells. Because of these observations, no attempt was made to estimate the effect of fluoride on either urinary or fecal selenium excretion in the present studies.

Therefore a second study (Experiment II) was designed to search for other ways in which high dietary fluoride could prevent selenite damage to the liver. The hypothesis tested was that a nutritionally high but non-toxic level of fluoride may inhibit free-radical initiated lipid peroxidative damage caused by selenite. Experiment II also tested whether tissue damage caused by selenite toxicity could be correlated with an increase in xanthine oxidase activity because this enzyme has been proposed as a potential source of free radicals (McCord, 1985). Evidence was found for a pro-oxidant effect of excess selenite as suggested by others (Dougherty and Hoekstra, 1982) in terms of increased (47%) liver malondialdehyde, an indicator of peroxidative tissue damage, and the fact that fluoride could at least partially prevent this effect (26% reduction) was consistent with the observation that fluoride can inhibit lipid peroxidation (Shayiq et al., 1986). This effect of selenium and fluoride, however, was of small magnitude relative to observed liver pathology. Furthermore, the magnitude of the selenium and fluoride effect on lipid peroxidation was consistent with the observation by others (Cerklewski, F. L. and Whanger, P. D.,

unpublished data) that selenium and fluoride had no effect on liver glutathione, the most abundant non-protein thiol offering protection to cells (Meister, 1984). Similarly, neither fluoride nor selenium had any effect on the activity of the free-radical generating enzyme xanthine oxidase.

Overall, consideration of past and present studies allows several other conclusions to be made with regard to a mechanism for the protective effect of fluoride on selenite liver pathology. It is unlikely, for example, that the protective effect of fluoride on selenite liver pathology was simply due to increased urinary excretion of selenium as suggested by Liu and Li (1987) because fluoride had little or no effect upon commonly accepted indicators of selenium status. Similarly, it is unlikely that the mechanism involved increased biliary excretion of selenium as was demonstrated for an arsenic-selenium interaction (Levander and Baumann, 1966) or decreased selenium absorption as was suggested for the protective effect of silver (Jensen, 1975) and copper (Stowe, 1980) on selenium toxicity. Arsenic, for example, increased biliary elimination of selenium and decreased selenium retention in the liver (Levander and Bauman, 1966) whereas fluoride had an inconsistent effect on liver selenium in the present studies. With regard to the effect of silver on selenite toxicity, Jensen (1975) found that all tissues, except the duodenum and the lower intestine of chicks given an oral dose of radioactive selenium with silver, had significantly less ^{75}Se than that observed in

chicks intramuscularly injected. Arsenic, silver and copper also individually prevented growth depression due to selenium which was not the case for fluoride. Growth-depressing effects of selenium from seleniferous grains (15 ug/g selenium), for example, was prevented by adding sodium arsenite (5 mg/L) to the drinking water of rats. Dietary silver (1000 mg/kg) and copper (1000 mg/kg) prevented mortality and reduced growth depression of selenium toxicity (5 mg/kg and 10 mg/kg) in chicks (Jensen, 1975).

It is also important to point-out that the human data on which the present selenium and fluoride studies were based involved both oral and inhalation exposure to fluoride and selenium, whereas the route of exposure in rats was oral only. Therefore, an interaction between fluoride and selenium at the pulmonary level remains a possibility. Furthermore, the human subjects exposed to fluoride and selenium from coal used as fuel most likely were also exposed to high levels of sulfur. The significance of this observation is that sulfate, a metabolite of sulfur, has been shown to offer protection against selenium toxicity (Ganther and Baumann, 1962). Inclusion of 1% sulfate in the diet of rats, for example, increased urinary excretion of selenium, decreased selenium concentration in liver and kidney and prevented growth depressive effects of selenate (5 mg/kg).

The possibility that high dietary selenite would influence fluoride bioavailability was also determined by estimating deposition of fluoride into femur. Inconsistent

evidence for a fluoride and selenium interaction was found in this regard because selenite increased femur fluoride in Experiment I, but was without effect in Experiment II. The results in the present studies are therefore consistent with the observation that fluoride deposition into femur is unaffected by selenite (Hajimarkos, 1967). Hajimarkos found, for example, that the ingestion of water containing 50 mg/L of fluoride and 3 mg/L of selenium by weaning rats for 28 days did not reduce the amount of fluoride retained in the femurs of rats compared to the control group receiving water with only 50 mg/L fluoride.

A likely mechanism for the protective effect of fluoride on selenite liver pathology may follow the scheme outlined by Anders (1988). In this case, selenium would increase intracellular calcium concentration which would in turn disrupt normal cell membrane integrity by activating phospholipases and proteases. Fluoride could inhibit this action because of insoluble complex formation with calcium (Cerklewski and Ridlington, 1987). Estimation of xanthine oxidase activity, as a potential source of free radicals, was a step in this direction although unsuccessful, because the predominant form of this enzyme in liver (xanthine dehydrogenase) is converted to the oxidase by a calcium-dependent protease (McCord, 1985). This proposed mechanism involving some as yet unidentified phospholipase or protease would also be consistent with the observation of liver infarction and thrombosis seen in selenite-toxic rats

(Popper, 1988)

If further research is attempted to define which specific phospholipase or protease might participate in a selenium and fluoride interaction, a better approach than a whole animal study might be to use a cell culture system. Liu and Li (personal communication with P. D. Whanger), for example, found that the monocellular organism, *Tetrahymena pyriformis*, will not grow in a medium containing 0.06 mM selenium and 0.105 mM fluoride. However, when the fluoride concentration in the medium was increased two and half times, with the same selenium concentration as before, growth rates were similar to controls (Whanger, 1989). Similarly, the bactericidal effect of selenite (0.1 mM) on Streptococcus mutans GS-5 was completely inhibited by adding 1 mM fluoride to the culture medium (Eisenberg et al., 1990).

SUMMARY

The studies described in this report were designed to define the possible protective effect of excess oral fluoride on selenite toxicity as suggested by recent observation from China. An apparent protective effect of fluoride on selenite liver pathology was found. Fluoride, however, failed to prevent the depressive effect of selenite on food intake and body weight gain unlike studies by others involving arsenic, copper, silver and sulfate. Furthermore, selenium status, represented by liver selenium concentration, was largely unaffected by fluoride suggesting that some complex mechanism is involved in the protective effect seen. It therefore is likely that some factor other than fluoride such as sulfur was mostly responsible for the prevention of selenosis recently reported in China. A possible interaction between fluoride and selenium at the pulmonary level, however, remains a possibility.

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