**AN ABSTRACT OF THE THESIS OF**

Yu Sun for the degree of Doctor of Philosophy in Toxicology present on May 27, 1998.

Title: Selenoprotein W: Distribution and Function in Rat Tissues and Cultured Cells

Abstract approved: Redacted for Privacy

Philip D. Whanger

The objective of this study was to further determine the distribution of selenoprotein W (SeW) in tissues from rats and sheep fed different selenium levels and to search for the possible functions of this protein. In the rat study a total of 28 rat tissues were examined and SeW was found in all of the tissues except for liver, thyroid, pancreas, pituitary and eyes regardless of the level of Se fed. SeW was not detected in heart, lungs, prostate, esophagus, small intestine, tongue, skin diaphragm and skeletal muscle from selenium deficient rats, but was present in these tissues when the two higher levels of selenium (0.1 and 4.0 mg/kg) were fed. SeW has the highest expression in muscle, brain, testis and spleen when selenium is adequate. Interestingly, selenium deficiency resulted in undetectable SeW levels in heart and muscle from deficient sheep and rats, but the content in brain was unaffected by selenium status. Second generation selenium depleted and repleted rats indicated that the expression of SeW in cortex and cerebellum was not significantly affected by selenium, but selenium increased its levels in thalamus. Cortex had the highest SeW expression among the three parts of the rat brain. SeW levels in muscle, spleen, skin and testis were undetectable in weanling rats, but became detectable after 6 weeks of selenium repletion. Studies with various brain cell cultures indicated that Se appears to be metabolized differently by different brain cell

types. As demonstrated in neuroblastoma and glial cells, glutathione peroxidase (GPX) activity decreased at a faster rate than SeW with neuroblastoma cells whereas SeW decreased at a faster rate than GPX activity in glial cells when selenium was removed from the media. Since other work showed that glutathione was bound to SeW, it was speculated that it has antioxidant function similar to other selenoproteins. SeW overexpressed and underexpressed cell lines were established by DNA recombinant techniques. There was a greater survival rate of overexpressed cells when incubated with 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) than control cells, suggesting SeW possibly has an antioxidant function.

# Selenoprotein W: Distribution and Function in Rat Tissues and Cultured Cells

By

Yu Sun

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed May 27, 1998

Commencement June 1999

Doctor of Philosophy thesis of Yu Sun presented on May 27, 1998

APPROVED:

Redacted for Privacy

Major Professor, representing Toxicology

Redacted for Privacy

Chairman of Toxicology Program

Redacted for Privacy

Dean of Graduate School

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Yu Sun, Author

## ACKNOWLEDGMENTS

I would like to express my sincere thanks to Dr. Philip D. Whanger, my mentor, for his dedicated teaching, guidance, encouragement, and financial support throughout my entire program. The guidance and discussion on aspects of cell culture provided by Dr. David W. Barnes are especially acknowledged. I am truly grateful to my other committee members, Dr. Olaf Hedstrom for his advice in immunocytochemistry study, Dr. Ian Tinsley, Dr. Wilbert Gamble, Dr. Douglas Barofsky and Dr. Brad Smith for their encouragement and comments on my research and graduate program.

Also, I would like to thank my lab colleagues, Ms. Judy Butler for her advice on laboratory procedures, editing my thesis and assistance in everything, Mr. Mike Beilstein for his helpful suggestions, technical discussions and editing my thesis, Ms. Azizah Mohd for her friendship and assistance in some experiments. Thanks are due to Ms. Angela Helmrich for her assistance in cell culture; to Ms. Kay Fisher for her assistance in immunocytochemistry; to Dr. Qiuping Gu, Ms. Pengcheng Ha and Dr. Jan-Ying Yeh for their friendship and assistance during my studies.

Finally, my special thanks go to my family for their love, encouragement and patience.

## CONTRIBUTION OF AUTHORS

Dr. Philip D. Whanger was involved in designing experiments, interpretation of data, writing and correcting of each manuscript. Ms. Judy A. Butler assisted in selenium analysis, caring and dissecting of animals. Dr. Qiu-Ping Gu assisted in plasmid construction. Dr. Neil E. Forsberg was involved in design of sheep experiment. Ms. Peng-Cheng Ha assisted in rat experiment.

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## CHAPTER 1

## INTRODUCTION

Evidence that selenium (Se) may be an essential nutrient was obtained in the 1950's (Pinsent, 1954; Schwarz and Foltz, 1957 ). Dr. Pinsent demonstrated that selenium would promote the growth of bacteria. Dr. Schwarz and Foltz demonstrated that liver necrosis, distinct from fatty liver and liver cirrhosis, could be prevented by dietary selenium. It was definitely established to be essential for animals when it was shown to be an essential component of glutathione peroxidase, a widely dispersed mammalian enzyme (Rotruck et al., 1973; Smith et al., 1974). Selenium's notoriety as a toxic element emerged in the 1930's with its identification of selenium as the causative agent of blind staggers and alkali disease in livestock in the Great Plains of North America, in which two different versions of selenium toxicity were shown due to the ingestion of high-selenium-containing plants. In 1943, Nelson et al.(1943) reported the development of neoplasms in liver of rats ingesting 5 μg Se/g diet, earmarking Se as a carcinogenic agent. But this result could not be repeated later, suggesting that selenium is merely toxic (Harr et al., 1967; Tinsley et al., 1967). Nelson's results may have been complicated by the marginal protein content of the diet. Epidemiologic studies conducted in the late 1960's and 1970's began to provide solid evidence of an inverse relationship between selenium intake and cancer mortality at levels well above the dietary requirements (Combs and Combs, 1986; Ip, 1989; Blot et al., 1993; Clark et al., 1996).

During the past 60 years, the public image of Se gradually evolved from that of a highly toxic and carcinogenic element to an essential trace element with antioxidant and anticarcinogenic properties. Selenium occurs in all cells and tissues of the body in concentrations that vary with the tissue and the amount and chemical form of Se in the diet.

## SELENIUM METABOLISM IN HUMANS

Soluble selenium compounds are very efficiently absorbed from the gastrointestinal tract. Radiolabeled selenium compounds demonstrated that the absorption of selenomethionine in New Zealand women was much better than that of selenite (95-97% versus 44-70%). The duodenum is the main site of selenium absorption, but the selenium status of an individual appears to have no effect on selenium absorption (Edmonds et al., 1984). Therefore, humans appear to have little or no homeostatic control over the absorption of selenium compounds from the gastrointestinal tract.

Although the highest concentrations of Se occur in the liver and kidneys, the largest total body amount of Se is in the muscle mass (Behne and Wolters, 1983). The tissue distribution of selenium in autopsy samples from North Americans was: kidney > liver > spleen > pancreas > testes > heart > muscle > lungs > brain. Skeletal muscle mass would contain almost half of the total-body selenium content of typical North Americans (Levander, 1985). In humans the amount of selenium excreted in the urine (trimethylselenonium ion) is closely related to the dietary intake, and balance studies have shown that over a range of intakes from 9 to 226 μg/day the urine accounts for 50 - 60% of the total amount excreted (Levander and Morris, 1984). During low selenium

intake, the kidney shuts down selenium excretion. Therefore, trimethylselenonium is a minor component of urinary selenium excretion in low selenium status, whereas it is present at higher amounts from humans ingesting supranutritional levels of selenium. Fecal excretion accounts for a relatively constant fraction of the total excretory output over a wide range of dietary intakes. Both fecal losses and urinary losses of selenium play a role in the homeostasis of selenium (Levander et al., 1981). In addition, pulmonary excretion of volatile selenium compounds (dimethylselenide) and sweat losses represent a minor pathway of selenium excretion.

Metabolic transformation of selenium compounds has not been well studied in humans, but selenium metabolism appears to be similar to that of animals. Selenium as selenide can be methylated to form dimethylselenide, which is excreted via the lungs, or trimethylselenonium ion, which is excreted via the kidney. Mammals do not distinguish selenomethionine from methionine. Incorporation of selenomethionine into animal proteins is directly correlated with selenomethionine and methionine intakes (Waschulewski and Sunde, 1988) since mammals can not synthesize selenomethionine from inorganic sources. Therefore, depending on diet, most body selenium can be in the form of selenomethionine in non-selenoproteins. The selenomethionine pool may provide a continuous supply of selenium as the amino acid is catabolized.

Selenium has been shown to counteract the toxicity of heavy metals such as cadmium (Kar et al., 1960), inorganic mercury, methylmercury (Ganther and Sunde, 1974), lead (Cerklewski and Forbes, 1976), thallium (Whanger, 1981) and to a limited extent silver (Diplock, 1976). To protect against the toxicity of these heavy metals, Se must be administered either shortly before or during the exposure to obtain the maximum

protection (Whanger, 1992). The protective effects may occur through the promotion of binding to high molecular weight proteins and through the formation of insoluble heavy metal selenides.

## SELENIUM DEFICIENCY AND SELENIUM TOXICITY

Selenium is necessary for growth and fertility in animals and for the prevention of various disease conditions in which response varies with the vitamin E intake. The effect of selenium deficiency varies among species. These are liver necrosis in rats, exudative diathesis (DE) and pancreatic fibrosis in poultry, muscular dystrophy (white muscle disease, WMD) in lambs, calves, and hepatosis dietetica in pigs (Combs and Combs, 1986).

White muscle disease (WMD) is a degenerative disease of the striated muscles, without neural involvement, in a wide range of animal species. WMD rarely occurs in mature animals. In lambs, it can occur at birth, or at any age up to 12 months. It is most common between 3 and 6 weeks of age. WMD lambs show a stiff and stilted gait and an arched back. They are not inclined to move about, lose condition, become prostrate, and die. Animals with severe heart involvement may die suddenly without showing any such signs. Mildly affected animals may recover spontaneously. The lesions are usually most readily discernible in the thigh and shoulder muscles. The lesions in the cardiac muscle are commonly confined to the right ventricle but may occur in other compartments. WMD can be prevented by selenium supplementation. WMD has received most attention in lambs and calves because of its economic importance and natural occurrence. In the

state of Oregon alone, untreated selenium deficiency caused more than 10 million dollars loss each year in livestock (Combs and Combs, 1986).

In humans, the first report of selenium deficiency symptoms was made in a New Zealand patient undergoing parenteral nutrition (Van Rij et al., 1979). The patient developed bilateral muscular discomfort and muscle pain. The muscle pain was sufficient to aggravate walking, and a generalized muscular wasting occurred. After selenium infusion, muscle pain disappeared and the patient returned to full mobility.

Until the 1980's, people living in certain hilly and mountainous regions of China, where soil selenium status is low and transportation is very limited, developed Keshan disease when they consumed only crops grown in those areas. Keshan disease is an endemic cardiomyopathy that mainly affected children under age of 15 and women of childbearing age. It may cause sudden death in some patients and the muscular changes are irreversible. The main pathologic feature of Keshan disease are multiple focal myocardial necrosis and fibrous replacement scattered throughout the heart muscle (Sunde, 1997). Myocytolysis is present in most cases. Criteria for diagnosis include acute or chronic heart function insufficiency, heart enlargement, gallop rhythm or arrhythmia, ECG changes, and pulmonary edema. The incidence of Keshan disease was dramatically reduced once selenium supplementation began in 1974 and it has now been virtually eliminated by supplementation with this element. Despite the successful selenium intervention and the many associations between poor selenium nutrition and the increased risk for Keshan disease, certain evidence indicates that factors other than selenium status may be involved in the etiology of the disease (Sunde, 1997).

On the other hand, high selenium consumption can cause selenosis in animals and humans. The minimum dietary requirement is 0.1 μg Se/g diet, and dietary levels above 2 μg Se/g diet are chronically toxic, resulting in a factor of 20 between the dietary selenium requirement and the onset of selenium toxicity. Localized seleniferous areas have been identified in China, Ireland the western USA and some other countries. There are three types of selenium poisoning in livestock: acute, chronic of the blind staggers type, and chronic of the alkali disease type. Se accumulator plants play an important role in the incidence of selenosis in grazing stock. Acute blind staggers occurs when animals ingest sufficient quantities of Se (experimentally or accidentally) with death often occurring in few hours. Chronic blind staggers occurs in animals that consume a limited amount of selenium accumulators during a period of weeks or months. Death results from respiratory failure. Animals that consume grains containing 5-40 μg/g selenium over weeks or months develop alkali disease. This chronic Se poisoning is characterized by dullness, loss of hair from the mane and tail of horses, roughness of coat, cirrhosis of the liver, and anemia. The toxicity of Se can be greatly modified by the dietary levels of arsenic, silver, mercury, copper, and cadmium, with each element apparently exerting its protecting action by its own mechanism (Levander, 1986).

Selenium toxicity in humans is uncommon. Acute toxicity in humans mostly results from occupational exposure of workers in copper smelting or selenium rectifier plants (Combs and Combs, 1986). Chronic selenium toxicity in humans was reported in Enshi County, Hubei province, China (Yang et al., 1983). The main symptoms and signs of chronic selenium toxicity are loss of hair, changes in nail structure, lesions of skin and abnormalities of the nervous system. These toxicity symptoms are present with Se intakes

ranging from 3200 to 6700 μg Se/day. The biochemical mechanism of Se toxicity is completely unknown, but is often postulated to be mediated by interaction with critical protein thiols.

Since selenium has double faces -- essentiality and toxicity, it is important to establish the Se daily requirement and a safe upper level. Animal studies showed 0.1-0.2 μg Se/g of diet was adequate for mammalian species (Yang et al., 1989; Spallholz, 1994). Studies in Se deficiency and selenosis areas suggested that for adult males and females, the required intakes of selenium to prevent clinically manifest signs of deficiency were 21 and 16 μg/day, respectively. The desirable or appropriate Se intakes for adult males and females were 40 and 30 μg/day, respectively. It was suggested that the upper safe level of selenium was about 400 μg/day (Yang et al., 1988; Levander, 1997).

## BIOSYNTHESIS OF SELENOPROTEINS

Almost all the selenium in animal tissues is associated with protein, and its remarkable biological effects in eukaryotes may be related to unique functions of various selenoproteins (Burk, 1994 ). Several selenoproteins have been identified, but only one form of selenium, selenocysteine, is present in the selenoproteins in animals. Selenocysteine is an unusual amino acid and it has been identified in both prokaryotic and eukaryotic selenoproteins (Böck et al, 1991; Berry and Larsen, 1993). Most selenocysteine-containing proteins characterized to date are redox enzymes, with selenocysteine functioning at the catalytic center. Replacing selenium with sulfur causes a sharp decline in activity (Axley et al., 1991; Berry et al., 1991a; Rocher et al., 1992). Cotranslational incorporation of selenocysteine into proteins is directed by a specifically

"programmed" UGA codon. Selenocysteine is encoded by UGA which is normally a stop codon in mRNA and has been shown to be specific for this selenoamino (Burk and Hill, 1993). Proteins containing this form of selenium are referred to as selenoproteins. Cellular glutathione peroxidase was the first selenoprotein found in mammalian tissues and was demonstrated to protect against oxidative stress (Rotruck et al., 1973). This peroxidase can effectively reduce peroxides and thus protect cells from damage due to reactive oxygen species. This provides an explanation at the biochemical level for the requirement of selenium as an essential trace element in mammals and birds. The identification of other mammalian selenocysteine-containing enzymes and proteins during the last few years has provided new insights into the functions of this trace element.

**Biosynthesis and incorporation of selenocysteine in prokaryotes**

The synthesis of selenocysteine and its incorporation into protein is a complex process. In the prokaryotic system, the determinants for recognition of the selenocysteine codon have been investigated by studying E. coli formate dehydrogenase H gene (fdhF) (Böck et al., 1991) . Translation of UGA as selenocysteine (Sec) requires a mRNA stem-loop structure with a specific sequence on the loop located immediately downstream of this codon from the coding region of the mRNA (Zinoni et al., 1990). Recently, extensive mutational analysis of the fdhF selenocysteine insertion sequence (SECIS) in E. coli indicated that the entire stem-loop RNA structure was not required for the UGA-directed selenocysteine incorporation in vivo. Only its upper stem-loop structure of 17 nucleotides

is necessary when it is located at a proper distance (11 nucleotides) from the UGA codon (Liu et al., 1998).

In addition, four unique gene products, selA, selB, selC and selD, were identified to be required for selenoprotein synthesis and selenocysteine incorporation (Leinfelder et al., 1988). The SelA gene product is selenocysteine synthase. SelB is an elongation factor of 68 kDa protein necessary for selenocysteine incorporation. It has a 43 kDa region similar to EF-Tu, the elongation factor that serves for all other amino acid-tRNAs. The SelC gene product is a $tRNA^{[Ser]Sec}$. Its anticodon sequence directly matches the UGA codon in mRNA. SelD, a 37 kDa protein, is an enzyme that catalyzes selenide and ATP to produce selenophosphate, which is required for sec-$tRNA^{[Ser]Sec}$ synthesis (Leinfelder et al., 1990).

There are four steps involved in prokaryotic selenoprotein synthesis. First of all, selC product $tRNA^{[Ser]Sec}$ is aminoacylated with serine by seryl-tRNA ligase and ATP to form seryl-$tRNA^{[Ser]Sec}$. Secondly, the side-chain oxygen of serine in seryl-$tRNA^{[Ser]Sec}$ is activated by selenocysteine synthase (selA) to form X-O-seryl-$tRNA^{[Ser]Sec}$. Thirdly, the activated oxygen of X-O-seryl-$tRNA^{[Ser]Sec}$ is replaced by selenium, producing selenocysteinyl-$tRNA^{[Ser]Sec}$. The selenium donor is selenophosphate which is synthesized by selenophosphate synthase (selD). Finally, selenocysteinyl-$tRNA^{[Ser]Sec}$-selB complex attached to the mRNA stem loop facilitates incorporation of selenocysteine into the protein. (Burk, 1991; Burk and Hill, 1993).

In addition to the above four gene products, the stem-loop structure with a specific sequence on the loop located immediately downstream of the UGA selenocysteine codon in prokaryotic selenoprotein mRNAs is also required (Heider et al., 1992). This acts as a

selB recognition motif, leading to the formation of quaternary complex of selB, GTP, selenocysteinyl-tRNA$^{[Ser]Sec}$ and the mRNA stem-loop (Heider and Böck, 1992). These elements are necessary and sufficient to drive selenocysteine incorporation at the UGA codon of prokaryotic selenoprotein mRNAs rather than at termination (Zinoni et al., 1990).

**Prokaryotic selenoproteins**

There are several prokaryotic selenium-dependent enzymes in which selenium is present in the chemical form of selenocysteine. They are glycine reductase (Cone et al., 1976; Turner and Stadtman, 1973), several formate dehydrogenases (Jones and Stadtman, 1981), and hydrogenases (Yamazaki, 1982). All these enzymes are expressed in strict anaerobes. Selenoprotein A, one of the protein components of clostridial glycine reductase complex, is a heat-stable, acidic glycoprotein of 12 kDa that contains one selenocysteine and two cysteine residues. This reductase catalyzes the reductive deamination of glycine to acetate and ammonia with the concomitant esterification of one equivalent of orthophosphate, which reacts with ADP to form one ATP. There are several formate dehydrogenases in microorganisms. The common property of all of the formate dehydrogenases is their marked sensitivity to molecular oxygen. It is involved in gas formation and deliver the electrons from formate to nitrate reductase. Hydrogenases from anaerobic and facultative bacteria generally are oxygen sensitive, but the inactivation due to oxygen exposure usually is reversible (Axley and Stadtman, 1989).

**Biosynthesis and incorporation of selenocysteine in eukaryotes**

Selenoprotein synthesis in the eukaryotic system, unlike in prokaryotes, is incompletely characterized. Essential stem-loop structures have been described in eukaryotic selenoprotein mRNAs in the 3' untranslated region (3' utr), and designated as SECIS motif (Berry et al., 1991b; Low and Berry, 1996). The distance between the UGA selenocysteine codon and the SECIS element can be as much as 2.7 kb and still support selenocysteine insertion into protein (Berry et al., 1993). The minimum distance, however, is not known but 111 bases support selenocysteine insertion while 51 bases do not (Martin et al., 1996). The positions of the SECIS element relative to the UGA selenocysteine codon in eukaryote mRNAs are different from that observed in prokaryotes where the SECIS element occurs immediately downstream of the UGA codon (Heider and Böck, 1993; Böck, 1994).

Mutagenesis studies in SECIS elements have shown that certain conserved features are essential for function (Berry et al., 1993; Shen et al., 1995). These include an AUGA sequence in the 5' arm of the stem, an UGA sequence in the 3' arm and an AAA sequence in the terminal loops. Recently, studies on UGA readthrough suggested that the precise sequences of the SECIS elements are relatively unimportant as long as length and thermostability of the base-paired structures are retained (Kollmus et al., 1996). Structural analysis of 20 animal selenoprotein mRNAs (two 5'-DI, thirteen GPX, two SeP, and one selenoprotein W) showed SECIS elements fit a single model which consists of two helices with an internal loop and an apical loop that is contiguous with one of the helices. (Walczak et al., 1996). So far, except for selenoprotein P, all the selenoproteins contain single selenocysteine in each peptide chain. Selenoprotein P cDNA has 10 TGAs codons

in its open reading frame (UGAs in mRNA) and two stem loops were predicted in its 3' untranslated region. Both of the stem loops contain elements of the SECIS motif (Berry et al., 1991b; Hill et al., 1991). Recently, Martin et al. (1998) reported that mutations at the invariant nucleotides in the consensus RNA motif are tolerated to various degrees in different elements, depending on the identity of the single nonconserved nucleotide. Furthermore, the sequences adjacent to the minimal element, although not required for function, can affect function through their propensity to base pair.

The mechanisms involved in SECIS recognition by the eukaryotic translational machinery remain unknown. SECIS RNA secondary structure models revealed conservation of four consecutive non-Watson-Crick base pairs, with a central GA/AG tandem. Mutation of these non-Watson-Crick base pairs showed that identifying a noncanonical RNA motif is crucial to SECIS function in mediating selenoprotein translation (Walczak et al., 1998).

An extensive search has been conducted for a putative eukaryotic SELB-like elongation factor and other potentially required proteins involved in the combined recognition of UGA and the SECIS element to allow selenocysteine insertion rather than termination at the UGA codon. One candidate for a SELB analog is a 48 kDa polypeptide recognized by autoantibodies present in autoimmune chronic active hepatitis (Gelpi et al., 1992). The second candidate is a 50 kDa protein from bovine liver extract, which protects selenocysteyl-tRNA against alkaline hydrolysis (Yamada et al., 1994) and also binds to UGA-programmed ribosomes (Yamada et al., 1995). However none of the above showed any SECIS specific binding. The third candidate is a SECIS element binding proteins (SBP) reported in cellular GPX SECIS element. The estimated

molecular masses of 55 kDa and 65 kDa (Shen et al., 1995). In Krol's laboratory, a 60-65 kDa SBP was reported from mobility shift assays of GPX and type I iodothyronine 5' deiodinase mRNAs (Hubert et al., 1996). This protein can specifically recognize both GPX and 5' DI SECIS elements.

Selenocysteyl-tRNA is a central component of selenoprotein biosynthesis in eukaryotes. The sec-tRNAs have been sequenced and found to consist of 90 nucleotides, making them the longest eukaryotic tRNAs (Lee et al., 1990; Diamond et al., 1993). The human gene was mapped to chromosome 19 (McBride et al., 1987), localized to bands q13.2-q13.3 and ordered with respect to other genes in this region (Mitchell et al., 1992). The mouse $tRNA^{[Ser]Sec}$ gene has also been mapped and localized; it occupies a similar chromosomal position as that found in humans (Bösl et al., 1995; Ohama et al., 1994). Eukaryotic sec-tRNA may play a dual role, serving as both carrier of molecules for synthesis of selenocysteine from serine and incorporation of selenocysteine into protein (Hatfield et al., 1994).

Eukaryotic $tRNA^{(ser)sec}$ is first charged with serine by conventional seryl-tRNA synthase and has been recovered in three forms with either serine, phosphorylated serine or selenocysteine attached (Lee et al., 1989). It has been presumed that selenocysteyl-tRNA in mammals was biosynthesized from seryl-tRNA by conversion of the serine moiety through the intermediate, phosphoser-tRNA (Lee et al., 1989). Other evidence suggests that a selenocysteine synthase, similar to E. coli SELA, directly converts seryl-$tRNA^{(ser)sec}$ to sec-$tRNA^{(ser)sec}$ using $HSe^-$ as a selenium donor. ATP and another protein similar to the E. coli SELD (Mizutani et al., 1992) are required in this process. However, $^{31}P$ NMP (Nuclear Magnetic Resonance) spectroscopy indicated that the labile selenium

donor compound is selenophosphate (Veres et al., 1992). The enzyme selenophosphate synthetase (Kim et al., 1995) may be involved in sec-tRNA conversion and thus selenophosphate would serve as an intermediate compound. The precise mechanism of selenocysteine biosynthesis in mammalian cells has not been resolved. However, the selenocysteylation process requires the long aminoacyl acceptor stem in $tRNA^{(ser)sec}$, which is unique to this tRNA (Lee et al., 1996).

## MAMMALIAN SELENOPROTEINS

Selenocysteine is recognized as the 21$^{st}$ amino acid in ribosome-mediated protein synthesis and its specific incorporation, as discussed above, is directed by the UGA codon. More than 80% of the selenium in the rat is present as selenocysteine (excluding nonspecific incorporation as selenomethionine) (Hawkes et al., 1985). This form was first identified in 1976 in protein A of the glycine reductase complex of *Clostridium sticklandii* ( Cone et al., 1976). Proteins containing selenocysteine are selenium dependent and only the proteins containing selenocysteine are considered as selenoproteins.

Selenoproteins with known enzymatic activity are redox enzymes and contain selenocysteine in their active sites. As noted earlier, replacing selenium with sulfur causes a large decrease in activity (Axley et al, 1991; Berry et al., 1991a; Rocher et al., 1992). Thus, effective function of these enzymes depends on their selenium content. Tissue extracts from rats which were administered $^{75}Se$ point to the presence of 10-15 high-abundance selenoproteins (Behne et al., 1988), but only a few of them have been characterized.

**Glutathione Peroxidase Family**

Cellular glutathione peroxidase (cGPX) was discovered in 1957 (Mills, 1957) and was noted to be dependent on selenium in 1973 (Rotruck et al., 1973). It has been used extensively to assess selenium nutritional status in human and animals. cGPX is present in almost all tissues, but its specific activity varies greatly between species and tissues. Liver and erythrocytes have the highest cGPX activity. Approximately 25% of rat total body selenium is present in liver cGPX (Behne and Wolters 1983).

cGPX is a tetramer containing four identical 22 kDa subunits. Each subunit contains one selenocysteine residue. This enzyme catalyzes the reduction of hydroperoxides to alcohols or water. Glutathione is required as the reducing substrate (Rotruck et al., 1973; Tappel et al., 1984). Also, a novel function of cGPX defends against peroxynitrite-mediated oxidations, as a peroxynitrite reductase was reported (Sies et al., 1997). However it can not reduce the hydroperoxides of phospholipids. cGPX exhibits some of the characteristics of a storage depot for selenium, and thus may serve as a biological selenium buffer which regulates selenium metabolism (Sunde, 1994).

Extracellular glutathione peroxidase (eGPX) or plasma glutathione peroxidase was recognized as a different enzyme from cGPX in 1986 (Takahashi and Cohen, 1986). It's activity in plasma is a convenient index of selenium nutritional status. eGPX consists of four identical 23 kDa subunits, each of which contains one selenocysteine. There is a 44% amino acid sequence homology to cGPX in humans (Takahashi et al., 1990) and thus cGPX and eGPX are closely related. The function of this enzyme is unclear. It may also metabolize hydrogen peroxide and fatty acid hydroperoxides as cGPX does. Since

the reducing substrate, glutathione, is very low in extracellular fluids, this enzyme might have functions other than as a glutathione peroxidase.

Phospholipid Hydroperoxide Glutathione Peroxidase (phGPX) was purified from porcine heart and liver (Schuckelt et al., 1991) and human testis (Esworthy et al., 1994). It is a monomer of 20 kDa containing one selenocysteine. It is expressed at highest level in testis. phGPX is a unique enzyme in the glutathione peroxidase family, because it is able to interact directly with peroxidized phospholipids (Ursini et al., 1985) and cholesterol (Tomas et al., 1990) and reduce their hydroperoxide moieties. Therefore, it has been considered the main enzymatic defense against oxidative destruction of biomembranes.

Gastrointestinal Tract-specific Glutathione Peroxidase (GI-GPX) is the newest member of GPX family discovered (Chu et al., 1993). It is mainly expressed in the epithelium of the gastrointestinal tract and appears to be the major glutathione-dependent peroxidase in rodent GI tract. It contributes to at least fifty percent of the GPX activity in rodent small intestinal epithelium. This enzyme is a cytosolic protein containing four identical 22 kDa subunits. Each subunit has one selenocysteine. The function of GI-GPX is assumed to be the same as at of cGPX which catalyzes the reduction of hydrogen peroxide, linoleic acid hydroperoxide, tert-butyl hydroperoxide and cumene hydroperoxide to alcohols. This enzyme appears to protect animals from toxicity of ingested lipid hydroperoxides. GI-GPX gene is mapped to mouse chromosome 12 between D12*Mit*4 and D12*Mit*5, near the Ccs locus which contains a colon cancer susceptibility gene (Chu et al, 1997).

**Iodothyronine deiodinase (DI) family**

Thus far, three types of iodothyronine deiodinase have been classified as selenoenzymes. They are important enzymes regulating the formation and degradation of the active thyroid hormone, 3,5,3'-triiodothyronine (T3). Selenium is involved in thyroid hormone metabolism. Hypothyroidism resulting from iodine deficiency worsens with selenium deficiency (Goyens et al., 1987). Similar to the GPXs, selenocysteine is located at the active sites of the deiodinases.

Type 1 iodothyronine deiodinase (D1) is an integral membrane protein of microsomes with molecular weight of 55 kDa containing two 27 kDa identical subunits (Berry, et al., 1991a; Toyoda et al., 1995). This enzyme occurs primarily in thyroid, liver, kidney, and pituitary (Arthur and Beckett, 1994). The function of D1 is to catalyze monodeiodination of the prohormone thyroxine (3,5,3',5'-tetraiodothyronine, T4) outring (ORD) to the active hormone 3,5,3'-triiodothyronine ( T3) or T4 inner ring (IRD) to the inactive metabolite 3,3',5'-triiodothyronine (reverse T3). D1 also catalyzes deiodination of both the IRD of T3 and the ORD of rT3' to form T2, and the deiodination of different iodothyronine sulfates, eg. IRD of T3S and ORD of T2S (Visser et al., 1982).

Type 2 iodothyronine deiodinase (D2) was recently identified as a selenoenzyme in the *Rana catesbeiana* (Davey et al., 1995), human and rat (Croteau et al., 1996) by cDNA cloning. D2 contains a conserved in-frame TGA codon. D2 is a low Km iodothyronine deiodinase converting T4 to the active hormone T3, or rT3 to diiodothyronine (T2), but it does not catalyze inner-ring deiodination of the iodothyronines (Larsen and Berry, 1995). D2 is mainly expressed in extrathyroidal tissues (brain, pituitary gland, and brown adipose tissue) to modulate thyroid hormone action,

and it may be of physiological importance in thyroid hormone economy in the human fetus and adult (Croteau et al., 1996).

Type 3 iodothyronine Deiodinase (D3) was first cloned and confirmed from *Xenopus laevis* tadpoles (St. Germain et al., 1994), and subsequently in rats (Croteau et al., 1995). These cDNAs have very high sequence homology to mammalian D1 cDNAs. D3 catalyzed inner-ring deiodination of T4, T3 and T2 but not of the corresponding sulfated iodothyronines (Santini et al., 1992). The products of these reactions are inactive metabolites. In the rat, D3 activity levels are highest in adult brain, skin, and placenta and in fetal liver, muscle, brain, and the central nervous system. This enzyme has been proposed to protect fetal tissues from high levels of T4 and T3 during development by converting them to the inactive iodothyronines rT3 and T2, respectively (Burrow et al., 1994).

**Thioredoxin reductase (TR)**

Thioredoxin reductase is the newest selenoprotein characterized in mammals (Tamura and Stadtman, 1996). It was originally purified from human lung adenocarcinoma cells. This protein is a homodimer of 57 kDa subunits containing one selenocysteine in each subunit. The purified protein contains FAD and exhibits NADPH-dependent thioredoxin reductase activity. The catalytic properties are similar to those of mammalian liver thioredoxin reductase (TR), but it did not show antibody crossreaction with the liver TR. Therefore, the selenocysteine-containing TR from the adenocarcinoma cells may be a variant form distinct from rat liver TR.

**Selenoprotein P (SeP)**

Selenoprotein P was first identified as a selenium-containing protein in 1977 (Herrman 1977) and it was furthered confirmed to contain selenium in the form of selenocysteine (Motsenbocker and Tappel, 1982). It is a glycosylated single polypeptide chain of 41 kDa, and it contains more than 50% of the plasma selenium in rats (Read et al., 1990) and in humans (Harrison et al., 1996). There are ten TGAs in the open reading frame, indicating that the protein contains ten selenocysteine residues in its primary structure. The deduced 366 amino acid sequence is histidine and cysteine-rich and contains 9 of its selenocysteines in the terminal 122 amino acids. Purification and characterization of SeP found that it contains 7.5 ± 1.0 selenium atoms/molecule as selenocysteine (Hill et al., 1991). This is the only selenoprotein characterized thus far that contains more than one selenocysteine per polypeptide chain. Human selenoprotein P gene is mapped to chromosome 5 q31. (Hill et al., 1996).

SeP is highest in liver and plasma, but the function of the protein is unknown. A correlation between increased SeP levels and increased protection against diquat-induced liver necrosis and lipid peroxidation without any concomitant increased GPX activity suggests an oxidant defense function (Burk et al., 1991).

**Selenoprotein W (SeW)**

SeW is a low molecular weight protein (10 kDa) originally reported as a missing component in selenium-deficient animals suffering from white muscle disease (Pedersen et al., 1972). It was first purified from rat skeletal muscle (Vendeland et al., 1993). Cloned cDNA sequences from rats (Vendeland et al., 1995), mice, sheep, monkeys and

humans (Gu et al., 1997; Whanger et al., 1997) contain one in-frame TGA codon for selenocysteine at residue 12. Sequence analysis of rat SeW revealed 50 bp of 5'UTR, 267 nucleotides encoding an 89-amino acid polypeptide, and 370 bp of the 3'UTR. Deduced amino acid sequences are 83% identical among five species (Gu et al., 1997). Rat and mouse amino acid sequences are 100% identical; and monkey and human are 100% identical as well. SeW is present in most of the tissues in Se adequate animals. It is highest in skeletal muscle, followed by brain and testis (Sun et al., 1998; Yeh et al., 1995; 1997). Like SeP, the function of this protein is unknown. Since GSH is tightly bound to SeW (Beilstein et al., 1996) and all the selenoenzymes are involved in redox reaction, this selenoprotein may have antioxidant function.

# CHAPTER 2

## EFFECT OF DIETARY SELENIUM ON SELENOPROTEIN W AND GLUTATHIONE PEROXIDASE IN 28 TISSUES OF THE RAT

Y. Sun, P-C. Ha**, J. A. Butler, B-R Ou***, J-Y Yeh, and P. Whanger
Department of Agricultural Chemistry
Oregon State University,
Corvallis, OR 97331

Running title: Selenoprotein W in rat tissues

Contact person: P. D. Whanger, Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331; phone 541-737-1803; fax 541-737-0497; email: whangerp@bcc.orst.edu

This paper was published with the approval of the Oregon State Agricultural Experiment Station as Technical paper number 11,171. This research was supported by Public Health Research Grant number DK 38306 from the National Institute of Diabetes and Digestive and Kidney Diseases.

**Present address: Chinese Academy of Preventive Medicine, Institute of Nutrition and Food Hygiene, 29 Nan Wei Road, Beijing, 100050, China.

***Present address: Department of Animal Science, Tunghai University, Taichung, Taiwan, China.

**ABSTRACT**

The influence of deficient (0.004 $\mu$g/g), adequate (0.1 $\mu$g/g) and excessive (4.0 $\mu$g/g) levels of dietary selenium (Se) on the selenoprotein W (SeW) content and glutathione peroxidase (GPX) activity was investigated in 28 tissues of the rat. GPX activity was found in all 28 tissues examined, and dietary selenium resulted in increased activities in all tissues, except for the spinal cord. Except for the brain, 0.1 $\mu$g Se per g diet resulted in significantly greater GPX activity in all tissues as compared to rats fed the deficient diet. When 4.0 $\mu$g Se per g diet was fed, however, this resulted in significantly greater activity in the brain as compared to the rats fed the deficient diet. SeW was non detectable in liver, thyroid, pancreas, pituitary and eyes regardless of the level of Se fed. SeW was not detected in heart, lungs, prostate, esophagus, small intestine, tongue, skin, diaphragm and skeletal muscle from Se deficient rats, but was present in these tissues when the two higher levels of Se were fed. In other tissues such as the kidney and seminal vesicles SeW was detected only in rats fed 4.0 $\mu$g Se per g diet. These results indicate that the distribution of SeW among rat tissues is more widespread than once thought, and suggest that the regulation of SeW by Se is markedly different between various tissues .

Key words: Selenoprotein W, Western blots, Glutathione peroxidase, Rat tissues.

## INTRODUCTION

It has now been established that the essential effects of selenium (Se) in mammals are the result of several biologically active Se compounds. They include the family of glutathione peroxidases (GPX) which are the classical GPX (Rotruck et al., 1973), a plasma GPX (Takahashi, et al., 1987), a GPX present predominantly in the gastrointestinal tract (Chu et al., 1993), and the monomeric phospholipid hydroperoxide GPX (Schuckelt et al., 1991). A second important enzymatic function of Se was identified when types I (Behne et al., 1990), II (Davey et al., 1995), and III (Croteau et al., 1995) iodothyronine deiodinases were identified as selenoenzymes. The most recent selenoenzyme identified was thioredoxin reductase which was isolated from human lung adenocarcinoma (Tamura and Stadtman, 1996). A few other selenoproteins have been identified but their biological functions have not yet been identified. They include selenoprotein P, the main Se compound in plasma (Read et al., 1990) and selenoprotein W (SeW) originally isolated from muscle (Vendeland et al., 1993). SeW is a selenoprotein with a molecular weight slightly less than 10 kDa.

The results of several tracer experiments indicate that many other Se containing compounds exist in addition to the selenoproteins already identified (Behne et al., 1988; Danielson and Medina, 1986; Evenson and Sunde, 1988; Hawkes et al., 1985). After in vivo labeling of rats with $^{75}Se$ and separation of the tissue proteins by SDS-polyacrylamide gel electrophoresis and autoradiography of the labeled compounds, 13 Se-containing proteins were found (Behne et al., 1988). In a subsequent study, the distribution of $^{75}Se$ was investigated in 27 tissues from rats (Behne et al., 1996). In a number of these tissues Se was found in 10 kDa proteins. In a prior study from our

laboratory, SeW was found only in muscle, brain, spleen and testis of the tissues examined from rats (Yeh et al., 1995). Thus, the purpose of the present experiment was to reexamine the tissue distribution of SeW to determine if it is more wide spread than we had initially anticipated. Levels of 0.004, 0.1 and 4 $\mu$g dietary Se per g diet were used. The purpose of these experiments was two fold. One was to investigate the distribution of SeW in a wide range of tissues and secondly to determine the influence of deficient, adequate and excessive levels of dietary Se on SeW in various tissues. For comparative purposes, the activity of GPX was also determined in these tissues.

## MATERIALS AND METHODS

### Animals

Twelve male weaning rats were divided into three groups of four each and fed either the Se deficient diet or this diet with either 0.1 or 4.0 $\mu$g Se as sodium selenate per g for 8 weeks. In order to evaluate SeW content and GPX activity in reproductive organs from female rats, four weaning female rats were fed the diet with 0.1 $\mu$g Se per g also for 8 weeks. The basal Se deficient diet was shown by analysis to contain 4 ng Se per g diet. The composition of this diet is described elsewhere(Yeh et al., 1997b), but briefly it contained (in g/kg) 300 torula yeast (Rhinelander, Wis), 510 sucrose, 90 purified cellulose (Solka Floc, Brown Co., Berlin, NH) 50 corn oil, 35 AIN-93M mineral mix without Se , 10 AIN-76 vitamin mix (American institute of Nutrition, 1977), 3 DL-methionine and 2 choline citrate. At the end of the experiment, all animals were

anesthetized with sodium pentobarbital (80 mg/kg, I.P.) and blood was taken via cardiac puncture. The tissues were removed, frozen immediately in liquid nitrogen and stored at -80 °C. SeW content and classical GPX activity were measured. This research with animals was reviewed and approved by the animal care committee at Oregon State University.

**Western blot analysis**

Tissues were homogenized and the protein content measured in the supernatants by the dye-binding protein assay using bovine serum albumin (Bio-Rad, Richmond, CA) as a standard. Samples (200 $\mu$g protein) were electrophoretically separated on SDS-polyacrylamide 7.5 to 15% gradients gels as described (Laemmli, 1970). Proteins were transferred onto nitrocellulose membranes (0.2 $\mu$m, S & S, Keene, NH) in transfer buffer as described by Towbin et al (Towbin et al., 1979). After transfer, membranes were blocked and subsequently incubated with rabbit anti-selenoprotein W polyclonal antibody (Yeh et al., 1995). Following three washes, membranes were incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad) and the specific binding of anti-SeW antibody onto membrane detected by ECL detection system (Amersham, Arlington, IL). The membrane was then exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY). Developed films were scanned with a Personal Densitometer SI analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA).

**Glutathione peroxidase activity**

The cellular GPX activity was measured by a coupled enzyme method using hydrogen peroxide as the substrate (Paglia and Valentine, 1967) with a DU Series 64 spectrophotometer (Beckman Instruments, Fullerton, CA).

**Statistical analysis**

The multiple t test was used to determine differences between the dietary groups. A significance level of 5% was adopted for all comparisons. .

**RESULTS**

There were no differences in the growth of the rats fed the various diets. As expected, the final body weights of the male rats were significantly greater than the females (280 ± 15 vs 180 ± 12 g fed the diet with 0.1 $\mu$g Se per g).

A western blot of tissue preparations from rats fed the diet with 0.1 $\mu$g Se per g is shown as an example in figure 2-1. Of the tissues depicted in this figure, bands corresponding to SeW were found in muscle, brain, spleen, testes, skin and lungs. The bands were the darkest for muscle, brain and testis.

The SeW content and GPX activities in internal organs from the rats fed the three levels of Se are shown in table 2-1. GPX activity increased with each increase of dietary Se in kidney, thymus and pancreas. However, no further increase of GPX activity occurred when 4.0 $\mu$g Se per g were fed as compared to 0.1 $\mu$g Se per g in heart, liver, lung, spleen and thyroid. No SeW was detected in liver, thyroid or pancreas regardless of

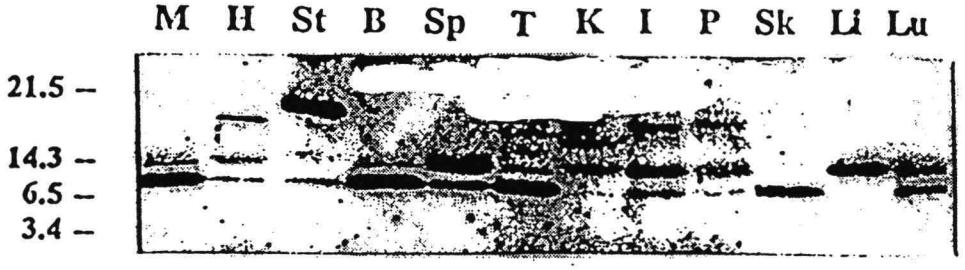



Figure 2-1. Tissue distribution of SeW in various tissues from rats fed 0.1 $\mu$g Se per g diet. The SeW band is the one located between 6.5 and 14.3 kDa. The symbols for the various tissues are M: muscle; H: heart; St: stomach; B: brain; Sp: spleen; T: testis; K: kidney; I: intestine; P: pancreas; Sk: skin; Li: liver; and Lu: lung. Western blots were conducted as described in the methods and materials section.

Table 2-1. Selenoprotein W and glutathione peroxidase in internal organs of rats fed three dietary levels of selenium.

| | Dietary Selenium | | |
|---|---|---|---|
| Tissues | Deficient | 0.1 μg/g | 4 μg/g |
| Adrenals | | | |
| GPX | 455 ± 114[a] | 1163 ± 165[b] | 1502 ± 44[b] |
| SeW | 585 ± 58[a] | 646 ± 265[a] | 929 ± 87[b] |
| Heart | | | |
| GPX | 38 ± 5[a] | 610 ± 49[b] | 533 ± 62[b] |
| SeW | ND | 25 ± 15[a] | 66 ± 25[a] |
| Kidney | | | |
| GPX | 24 ± 5[a] | 869 ± 109[b] | 1146 ± 209[c] |
| SeW | ND | ND | 673 ± 25 |
| Liver | | | |
| GPX | 8 ± 2[a] | 992 ± 113[b] | 1224 ± 400[b] |
| SeW | ND | ND | ND |
| Lung | | | |
| GPX | 21 ± 2[a] | 383 ± 41[b] | 295 ± 169[b] |
| SeW | ND | 37 ± 25[a] | 136 ± 62[b] |
| Spleen | | | |
| GPX | 132 ± 31[a] | 589 ± 198[b] | 669 ± 112[b] |
| SeW | 320 ± 68[a] | 1410 ± 13[b] | 1156 ± 191[b] |
| Thymus | | | |
| GPX | 49 ± 7[a] | 275 ± 33[b] | 371 ± 83[c] |
| SeW | 148 ± 20[a] | 250 ± 19[b] | 283 ± 40[b] |
| Thyroid* | | | |
| GPX | 22 | 98 | 85 |
| SeW | ND | ND | ND |
| Pancreas | | | |
| GPX | 17 ± 2[a] | 55 ± 2[b] | 133 ± 38[c] |
| SeW | ND | ND | ND |

GPX activity is expressed as nm NADPH ox/min/mg protein; SeW is expressed in scan units. Values are means ± SE for 4 rats; ND=not detectable. Values in a horizontal row with different letters– a, b, c– are significantly different ($P < 0.05$).

* Single determination on pooled samples from four rats

the amount of Se included in the diet and SeW was detected only in the kidney from rats fed the highest amount of Se. SeW was non detectable in lungs from Se deficient rats but was present when Se was added to the diet. The lungs from rats fed the diet with 4.0 $\mu$g Se per g was significantly higher than in this organ from the rats fed the next lowest level of Se. The heart is similar to the lungs in that no SeW was detected in this organ from Se deficient rats, but was present when Se was added to the deficient diet. However, in contrast to the lungs, the SeW level was not significantly different in this organ from rats fed diets with the two levels of Se. The response in thymus and spleen is similar in that SeW was higher in rats fed the diet with 0.1 $\mu$g per g than in these organs from the deficient rats, but no further increase was found with the highest level of Se fed as compared to 0.1 $\mu$g Se per g. The adrenals showed a different pattern in that no difference was found between deficient rats and those fed diet with 0.1 $\mu$g Se per g, but an increase occurred when 4.0 $\mu$g Se per g was fed.

GPX activity was found in all of the reproductive tissues (table 2-2). In all of the male reproductive tissues 0.1 $\mu$g Se per g resulted in a significant increase of GPX activity as compared to these tissues from the deficient rats with no further increase of activity when the highest level of Se was fed. SeW was undetectable in prostate and seminal vesicles from deficient animals and in seminal vesicles from rats fed the diet with 0.1 $\mu$g Se per g, but was detectable in this tissue with the highest amount of Se fed. The epididymis and testis showed similar patterns in that SeW was significantly higher from rats fed the diet with 0.1 $\mu$g Se per g than from the deficient rats, but no further

Table 2-2. Selenoprotein W and glutathione peroxidase in reproductive organs of rats fed three dietary levels of selenium.

| | Dietary Selenium | | |
|---|---|---|---|
| Tissues | Deficient | 0.1 $\mu$g/g | 4.0 $\mu$g/g |
| Epididimus | | | |
| GPX | $32 \pm 9^{a}$ | $168 \pm 26^{b}$ | $182 \pm 10^{b}$ |
| SeW | $444 \pm 64^{a}$ | $798 \pm 211^{a,b}$ | $1099 \pm 194^{b}$ |
| Prostate | | | |
| GPX | $16 \pm 3^{a}$ | $149 \pm 33^{b}$ | $182 \pm 60^{b}$ |
| SeW | ND | $1489 \pm 9^{a}$ | $1509 \pm 13^{a}$ |
| Seminal Vesicles | | | |
| GPX | $65 \pm 8^{a}$ | $123 \pm 18^{b}$ | $159 \pm 37^{b}$ |
| SeW | ND | ND | $419 \pm 16$ |
| Testis | | | |
| GPX | $40 \pm 1^{a}$ | $115 \pm 6^{b}$ | $131 \pm 19^{b}$ |
| SeW | $1688 \pm 99^{a}$ | $3020 \pm 307^{b}$ | $3147 \pm 184^{b}$ |
| Female | | | |
| Uterus | | | |
| GPX | | $423 \pm 39$ | |
| SeW | | $266 \pm 111$ | |
| Ovary | | | |
| GPX | | $210 \pm 45$ | |
| SeW | | ND | |

Values are means ± SE of 4 animals. Values in a horizontal row with different letters – a, b, c – are significant different ($P < 0.05$).
GPX activity is expressed as nm NADPH oxidized/min/mg protein
SeW is expressed as scan units.

increase occurred with the higher level of Se fed. GPX was present in uterus and ovary of the female rats but SeW was detectable only in the uterus.

The eyes and diaphragm are the only organs where there was a significant increase of GPX activity with each increase of dietary selenium (Table 2-3). Se status had no effect upon the activity of this selenoenzyme in the spinal cord. In tissues like the cerebellum and cortex (and presumably the pituitary) there was no difference in GPX activity between deficient rats and those given 0.1 $\mu$g Se per g, but a significant increase when 4.0 $\mu$g Se per g was included in the diet. This is in contrast to all the other tissues (brown adipose, esophagus, stomach, small intestine, tongue, skin and skeletal muscle) where there was a significant increase of GPX activity with 0.1 $\mu$g Se per g diet as compared to the deficient animals, but no further increase with the highest level of Se fed. Regardless of the level of Se fed, SeW was not detected in pituitary and eyes, and was undetectable in brown adipose, esophagus, stomach, small intestine, tongue, skin, skeletal muscle and diaphragm from Se deficient animals. Se status had no effect upon SeW in cerebellum or cortex. In other tissues such as brown adipose, esophagus, stomach, small intestine, tongue, skin and skeletal muscle SeW was undetectable in deficient animals and the levels were not different in rats fed diets with 0.1 $\mu$g Se versus 4.0 $\mu$g Se per g. Diaphragm showed a different pattern in that SeW was not detectable in deficient rats, but was present in this tissue at higher levels in rat fed the diet with 4.0 $\mu$g Se per g than those fed the diet with 0.1 $\mu$g Se per g. The spinal cord showed another pattern in that no difference was found between the deficient rats and those fed the diet with 0.1 $\mu$g Se per g, but this organ from rats fed 4.0 $\mu$g Se per g contained significantly higher levels.

Table 2-3. Selenoprotein W and glutathione peroxidase in brain, digestive tract and other tissues of rats fed three dietary levels of selenium.

| | Dietary Selenium | | |
|---|---|---|---|
| Tissues | Deficient | 0.1 μg/g | 4.0 μg/g |
| Brown Adipose | | | |
| GPX | 26 ± 9[a] | 130 ± 8[b] | 168 ± 43[b] |
| SeW | ND | 1215 ± 848 | 1525 ± 1212 |
| Cerebellum | | | |
| GPX | 61 ± 9[a] | 69 ± 16[a] | 122[b] |
| SeW | 456 ± ± 143[a] | 569 ± 222[a] | 685 ± 114[a] |
| Cortex | | | |
| GPX | 57 ± 13[a] | 63 ± 14[a] | 83 ± 10[b] |
| SeW | 428 ± 228[a] | 685 ± 127[a] | 704 ± 115[a] |
| Pituitary* | | | |
| GPX | 120 | 104 | 361 |
| SeW | ND | ND | ND |
| Esophagus | | | |
| GPX | 21 ± 6[a] | 333 ± 54[b] | 277 ± 98[b] |
| SeW | ND | 268 ± 48[a] | 286 ± 41[a] |
| Stomach | | | |
| GPX | 17 ± 6[a] | 558 ± 138[b] | 624 ± 197[b] |
| Se-W | ND | 120 ± 43[a] | 145 ± 70[a] |
| Small Intestine | | | |
| GPX | 28 ± 8[a] | 183 ± 42[b] | 269 ± 68[b] |
| SeW | ND | 316 ± 35[a] | 337 ± 48[a] |
| Spinal Cord | | | |
| GPX | 86 ± 16[a] | 67 ± 32[a] | 83 ±58[a] |
| SeW | 40 ± 6[a] | 58 ± 2[a] | 201 ± 83[b] |

Table 2-3 (Continued)

| | | | |
|---|---|---|---|
| Tongue | | | |
| GPX | 26 ± 5[a] | 218 ± 3[b] | 268 ± 6[b] |
| SeW | ND | 466 ± 38[a] | 732 ± 76[b] |
| Skin | | | |
| GPX | 14 ± 2[a] | 196 ± 52[b] | 202 ± 51[b] |
| SeW | ND | 1591 ± 99 | 1516 ± 174 |
| Eyes | | | |
| GPX | 12 ± 1[a] | 32 ± 7[b] | 68 ± 10[c] |
| SeW | ND | ND | ND |
| Diaphragm | | | |
| GPX | 31 ± 3[a] | 599 ± 163[b] | 925 ± 60[c] |
| SeW | ND | 525 ± 83[a] | 1084 ± 8[b] |

Values are mean ± SE of 4 animals. Values in a horizontal row with different letters – a, b, c – are significantly different ($P < 0.05$).
GPX activity is defined as nm NADPH oxidized/min/mg protein
SeW is expressed as scan units.
ND= not detectable.
*Single determination on a pool from four animals.

## DISCUSSION

These results indicate that the tissue distribution of SeW in rats is more widespread than we originally thought. Of the tissues examined previously, SeW was found only in muscle, brain, spleen and testis (Yeh et al., 1995). SeW was not detected in liver, thyroid, pancreas (table 2-1), eyes or pituitary (table 2-3) regardless of the Se status of the animal. This selenoprotein was found in all of the other tissues examined when Se was included in the diet (tables 2-1~3). Se status had a different effect upon Se-W content in the various tissues. In most tissues where SeW was undetectable in deficient animals, it was present when 0.1 $\mu$g Se per g was present in the diet, but in some tissues like the kidney (table 2-1) and seminal vesicles (table 2-2) it was detected only in rats fed the diet with 4.0 $\mu$g Se per g. Further research is needed to determine what level of dietary Se is required to reach saturation of Se-W and GPX in these tissues because the present results should not be taken to imply that 4 $\mu$g Se per g diet is required to obtain maximum responses in these tissues.

Of 27 tissues examined by $^{75}$Se labeling, a 10 kDa band was found in brain, diaphragm, epididymis, eye, kidney, lungs, pituitary, prostate, seminal vesicles, skeletal muscle, skin, small intestine, spleen, stomach, testis, thymus, thyroid and tongue (Behne et al., 1996). SeW was found in all of these tissues except for pancreas, thyroid and pituitary (tables 2-1 and 2-3). Tissues where no 10 kDa bands were found (Behne et al., 1996) and no SeW were detected include the eye, liver and ovary (tables 2-1~3). However, the tissues where no 10 kDa bands were found but SeW was detected include the adrenals, brown adipose tissue, esophagus, heart, spinal cord and the uterus.

Therefore, these results suggest that there are low molecular weight Se-containing proteins in mammalian tissues which are not SeW.

The disagreements between the two methods could be due to several factors. The rats used in the labeling study had been depleted of Se for six generations (Behne et al., 1996), and as shown in tables 2-1~3 Se-W was undetectable in many tissues from Se deficient animals. Second, the turnovers of the Se containing proteins are not likely to be similar. Even though the rats were killed 6 and 21 days after injection of radioactive Se, this may have not been sufficient for some of the Se containing proteins. If a Se containing protein has a very slow turnover rate, then it would be missed using this method. This is not to indicate that this method has not been useful in Se research because it has been used to identify new selenoproteins such as type I iodothyronine deiodinase (Bene et al., 1990) and the prostatic epithelial selenoprotein (Kalchlosch et al., 1995).

GPX activity was found in all 28 tissues examined in this study (tables 2-1~3). This is the first report on the investigation of GPX activity in such a wide range of tissues. Liver and blood have been the most common tissues used for GPX assays. Other workers have determined the GPX activity in plasma, liver, and muscle (Waschulewski and Sunde, 1988); heart, liver, testis, lung and kidney (Lei et al., 1995); brain (Burk et al., 1991; Huang et al., 1994); brain and liver (Buckman et al., 1993); heart (Xia et al., 1985); liver and kidney (Burk et al., 1995; Viljoen et al., 1989); lung (Jenkinson et al., 1989); thyroid (Arthur et al., 1990; Cammack et al., 1995) and pancreas (Yeh et al., 1997a). The results in the present investigation indicate that GPX responds differently in various tissues. In most tissues a significant increase was found with 0.1 $\mu$g Se per g diet

as compared to deficient rats with no further increase with higher Se intake. However in tissues such as the kidney and pancreas (Table 2-1) and eyes and diaphragm (table 2-3) there was a significant increase of GPX activity with each increase of dietary Se. It is interesting that Se status had no influence on GPX activity in the spinal cord but Se affected the SeW levels in this organ (table 2-3). This suggests that the regulation of GPX and SeW by Se in this organ is markedly different.

With the limited data obtained so far, it is evident that there are some species differences in the tissue distribution of SeW. SeW is present in the heart of sheep (Yeh et al., 1997a) and primates (Gu et. al, Oregon State University, unpublished work) at the same concentration as the muscle whereas in the rat it is very low even when Se is given (Yeh et al., 1997b) (table 2-1). SeW responds differently to Se in various tissues. For example, SeW in the testis responded very rapidly to low levels of Se (0.01 $\mu$g Se/g) but that in the muscle did not respond until much higher levels of Se were used (Yeh et al., 1997b). Consistent with the present data there is no correlation of GPX activity with SeW content between the various tissues (Yeh et al., 1997a; Yeh et al., 1997b).

Like selenoprotein P (Burk et al., 1995), the metabolic function of SeW is not known. Consistent with our prior data (Vendeland et al., 1995), the levels of this selenoprotein respond to Se intake. The cDNA for SeW has been sequenced and there is a UGA in the open reading frame corresponding to the insertion of selenocysteine (Vendeland et al., 1995). Since many selenoenzymes are involved in antioxidant functions, it has been suggested that SeW may play such a role. This was strengthened when glutathione was demonstrated to be bound to it (Beilstein et al., 1996). The wide

distribution of SeW among tissues suggest an important metabolic role for this selenoprotein.

In summary, GPX activity was found in all 28 tissues examined in the rat. SeW was not detected in five (liver, thyroid, pancreas, pituitary and eyes) tissues regardless of the Se status, but it was detected in all other tissues when excess Se was given. This is not to imply that excess Se is required to increase SeW to the maximal levels in some tissues, but further research is needed to determine the dietary levels of this element needed to result in saturation for this selenoprotein. The distribution of SeW in tissues from rats is much wider than once thought.

**ACKNOWLEDGMENT**

We are grateful to Dr. Harold Engel , School of Veterinary Medicine, Oregon State University, for assistance in the identification of some of the tissues.

# CHAPTER 3

## SELENOPROTEIN W, SELENIUM AND GLUTATHIONE PEROXIDASE IN RAT AND SHEEP BRAINS AND IN BRAIN CELL CULTURES

Y. Sun*, J. Butler*, N. Forsberg**, and P. Whanger*
Departments of Agricultural Chemistry* and Animal Sciences**
Oregon State University,
Corvallis, OR USA 97331

Running title: Selenoprotein W in Brain

Contact person for proofs and reprint requests: P. D. Whanger; Phone: 541-737-1803; Fax: 541-737-0497; email: whangerp@bcc.orst.edu

This paper is published with the approval of the Oregon State Agricultural Experiment Station as Technical Paper number_____. This research was supported by Public Health Research Grant number DK 38341 from the National Institute of Diabetes and Digestive and Kidney Diseases and USDA competitive grant number 94-37204-0494.

**ABSTRACT**

The effects of various selenium (Se) levels on selenoprotein W (SeW) content, Se concentration and glutathione peroxidase (GPX) activity in various parts of the brain from sheep and rats, and in glial and neuronal cells from rats, were determined. Se concentration and GPX activity were significantly lower in whole brains from Se deficient lambs than from those receiving Se, but the SeW levels were not different. This is in contrast to the heart and muscle where SeW levels were significantly lower in deficient lambs. As the Se status of lambs increased, GPX and SeW increased in the brain cortex, cerebellum and thalamus. Different patterns from the lambs were noted for the increase of GPX and SeW levels in the cortex and cerebellum from rats fed various levels of Se. SeW increased at a faster rate in glial cells than either in L8 muscle or neuronal cells but a different pattern was found for GPX activity. Although GPX activity increased with Se content, the differences between cells were not as great as with SeW. The rate of decline was different for SeW levels and GPX activity upon removal of Se from the media with the three types of cells. SeW and GPX activity decreased at about the same rate in L8 muscle cells, GPX activity decreased faster than SeW in neuronal cells, but SeW decreased faster than GPX activity in glial cells when Se was removed from the medium. Thus, Se appears to be metabolized differently by various parts of the brain and by different brain cell types.

## INTRODUCTION

Although most work on selenium (Se) metabolism has been directed towards other organs, metabolism of this element by the brain appears to have some unique features. Se as either selenite or selenomethionine (Semet) is taken up in various amounts by all parts of the brain in rats (Clausen, 1991; Huang et al., 1994), indicating that several regions of this organ are involved in Se metabolism. When radioactive Se was given as selenoprotein P, the brain, but not other tissues of Se deficient rats took up significantly more radioactive Se than did controls (Burk et al., 1991).

In contrast to other tissues, the brain has a remarkable ability to retain Se. Forty days after injection, retention of Se from radioselenite was greatest in the brain (Behne et al., 1988). In nearly all tissues examined in the hierarchy of Se retention, the supply to other selenoproteins had priority over that for glutathione peroxidase (GPX), suggesting a more essential function for the other selenoproteins. A detailed study of selenoprotein expression in rat brain and liver was reported (Buckman et al., 1993). In contrast to the liver, there was a sequestration of Se and greater conservation of GPX in brain when dietary Se was limited. In a study with sheep fed Se deficient and supplemented diets, the selenoprotein W (SeW) levels were significantly lower in all tissues examined (skeletal muscle, heart, tongue, lung, spleen, kidney and liver) except for the brain (Yeh et al., 1997c). There was a 50% reduction in the Se concentration and a 30% reduction in the GPX activity in the brain of deficient animals, suggesting preferential retention of SeW. Because of this apparent tenacity of the brain for SeW, the present investigations were undertaken to explore the unique aspects of Se metabolism by the brain. Rats and

lambs, and glial and neuronal cells from rat brain in culture were used for these investigations.

## MATERIALS AND METHODS

### Lambs

Se deficient lambs were obtained by procedures previously described (Schubert et al., 1961). Briefly Se deficient ewes were fed Se deficient alfalfa (0.02 mg Se/kg) pellets (Yeh et al., 1997b) for 3 to 6 months prior to parturition. Some of the lambs (up to 80%, usually 40 to 60%) developed white muscle disease (WMD), that is animals with pathological lesions due to Se deficiency, between 3 and 9 weeks of age. The remainder did not develop WMD and served as internal controls. The onset of WMD was clinically diagnosed by elevated plasma creatine phosphokinase levels (Whanger et al., 1976) and the lambs were necropsied shortly thereafter, usually within a week of diagnosis. Normal (control) lambs were those born to ewes fed salt containing Se. The lambs were anesthetized with Sleepaway™ (0.5 ml/kg, Fort Dodge Laboratories, Fort Dodge, Iowa) and killed by exsanguination via the carotid artery. Brain, skeletal muscle (semitendinosus) and heart samples were removed and immediately frozen on dry ice. The brain was separated into thalamus, cortex and cerebellum before they were frozen.

### Rats

Sprague Dawley rats, purchased from Simonsen Laboratories (Gilroy, CA) were used. All animals had free access to food and water and a record of food consumption

was recorded. Rats were fed a torula yeast basal diet. It contained 30% torula yeast (Rhinelander Paper Co., Rhinelander, WI), 51% sucrose, 9% purified cellulose (Solka Floc, Brown Co., Berlin, NH), 5% corn oil, 3.5% AIN-93 mineral mix without Se and 1% AIN-76 vitamin mix (American Institute of Nutrition, 1977), 0.3% L-methionine and 0.2% choline citrate. This basal diet was shown by analysis to contain about 4 μg Se per kg diet. Three groups of male weaning rats (5/group) were used and fed either the deficient diet or this diet supplemented with either 0.1 or 4.0 mg Se per kg diet for 6 weeks. The animals were anesthetized with sodium pentobarbital (80 mg per kg i.p.) and blood removed by cardiac puncture. Rats were decapitated while under anesthesia. Tissues were removed and frozen immediately on dry ice. Before freezing, the brain was divided into cortex and cerebellum. The research with animals was reviewed and approved by the Animal Care and Use Committee at Oregon State University.

**Cell Cultures**

Undifferentiated L8 myoblasts were cultured in Dulbecco's modified Eagle's media (DMEM) plus 10 % calf serum. After the cells reached confluence, they were incubated in differentiation medium (DMEM plus 2 % calf serum) to induce differentiation (Yeh et al., 1997c). Serum free medium for differentiated cells includes linoleic acid (conjugated with BSA), insulin, dexamethasone, fetuin, transferrin and selenium. The neuronal and glial cells were grown in media described by Bottenstein (Bottenstein, 1985). Briefly, for the neuronal cells, insulin, transferrin, progesterone, putrescine and a low level of Se are required for growth in Dulbecco's modified Eagle's medium. The requirements for the glial cells are slightly different and include

transferrin, hydrocortisone, fibroblast growth factor, biotin, Se, insulin and epidermal growth factor in Dulbecco's modified Eagle's medium. All cells were grown in media containing either $10^{-8}$ M, $10^{-7}$ M or $10^{-6}$ M Se as selenite. After the cells had reached confluence, they were switched to a medium without added Se, and the rate of decline of GPX activity and SeW content determined at various times afterwards.

**Procedures**

Western blots were used to quantitate SeW levels as described by Yeh et al (Yeh et al., 1997c). The relative amounts of SeW were estimated by densitometric scanning of the X-ray film using a Personal Densitometer SI and analyzed by the Image QuanNT program (both from Molecular Dynamics, Sunnyvale, CA). GPX activity was assayed by the coupled enzyme procedure of Paglia and Valentine (1967). After digestion with nitric and perchloric acids, the Se content was determined fluorimetrically by a semi-automated method (Brown and Watkinson, 1977) using an Alpkem II system (Alpkem. Corp., Milwaukee, OR).

**Supplies**

The cell lines were obtained from American Type Culture Collection (Rockville, MD). Calf serum (CS) was purchased from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium, penicillin/streptomycin solution and trypsin were purchased from GIBCO (Grand Island, NY). Rainbow molecular weight marker and the ECL Western blotting detection system were purchased from Amersham (Arlington Heights, IL). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and bovine serum

albumin (BSA) were purchased from Bio-Rad (Richmond, CA). Cell culture petri dishes were purchased from Corning (Corning, NY). Nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH).

**Statistical analysis**

The data were examined for equal variance and normal distribution prior to statistical analysis. Mean values were compared by analysis of variance (2 way-ANOVA) with Fisher's least-significant difference (LSD) method for comparing groups. Linear regression was used (Steel and Torrie, 1980). A significance level of 5% was adopted for all comparisons.

**RESULTS**

Se concentration was lower in the brain from WMD lambs compared to the brain from internal controls, which was lower than the Se content in brain of the control lambs (table 3-1). There were no significant differences in the GPX activity in brain from WMD and internal control lambs, but both were significantly lower than the activity in brain of the control lambs. Except for the thalamus in WMD and internal control lambs, each of the corresponding increases of selenium in the brain regions were statistically significant when WMD was compared to internal controls and subsequently compared to controls. In contrast, there were no significant differences in the SeW content in the various parts of the brain between WMD and internal control lambs, but this content was significantly higher in control lambs than in the other two groups. The GPX activity in the cortex and thalamus was similar to the whole brain in that there were no differences

Table 3-1. Selenium Concentration, Selenoprotein W Content and Glutathione Peroxidase Activity in Brains from Sheep Fed Two levels of Dietary Selenium

| Component | Status | | |
|---|---|---|---|
| | WMD | Internal Ctrl | Control |
| Whole brain | | | |
| Selenium* | 5.4±0.3[a] | 7.4±0.4[bc] | 11.6±0.3[bd] |
| GPX | 103±5[a] | 117±6[a] | 134±5[b] |
| Selenium* | | | |
| Cortex | 5.4±0.5[a] | 7.3±0.7[bc] | 13.1±0.6[bd] |
| Cerebellum | 5.8±0.6[a] | 8.5±0.8[bc] | 11.3±0.7[bd] |
| Thalamus | 5.1±0.5[a] | 6.4±0.7[ac] | 10.4±0.6[bd] |
| Selenoprotein W[⌀] | | | |
| Cortex | 1768±172[a] | 2262±217[a] | 3542±183[b] |
| Cerebellum | 2011±166[a] | 2494±210[a] | 3355±178[b] |
| Thalamus | 1468±125[a] | 1666±158[a] | 2466±134[b] |
| GPX activity[τ] | | | |
| Cortex | 86±7[a] | 103±9[ac] | 127±7[bd] |
| Cerebellum | 126±10 | 137±13 | 144±11 |
| Thalamus | 97±7[a] | 110±9 | 130±8[b] |

*: Concentration of selenium is expressed as nmole per g dry tissue.
⌀: Values for SeW are expressed as scan units per 200 μg pretein.
τ: Activity of GPX is expressed as nmoles NADPH oxidized/min.mg protein.
Values are mean of 5-9 animals ± standard error.

between WMD and internal controls but significant increases in the control lambs. However, there were no significant differences in the GPX activity in cerebellum from any of the three groups.

Different patterns from the brain were found in the muscle and heart of these animals (table 3-2). There were no differences in either the Se concentration or GPX activity in muscle and heart between the WMD and internal control lambs, but these values were significantly lower than those in control lambs. SeW was not detectable in muscle and hearts from WMD or internal control lambs, but was present in these tissues of the control lambs. In all three groups, the Se concentration in heart was significantly higher than in the skeletal muscle, but the GPX activity, except the control lambs, was not significantly different between these two organs. The GPX activity in the heart of control lambs was significantly higher than this activity in the muscle.

Slightly different patterns were found in rats in comparison to lambs (table 3-3). There were no differences in the SeW content in the cortex from brains of rats fed the diet with 0.1 mg Se/kg compared to those fed the diet with 4 mg Se/kg, but both were higher than this content in the cortex from rats fed the dificient diet. In contrast, a corresponding slight increase occured in the cerebellum with increases of Se in the diet but none of these were significantly different. Different patterns were found for GPX activity in the two brain regions in rats. No differences in GPX activity were found either between the cortex or cerebellum of the two lowest dietary groups or between these tissues from rats fed the two lower dietary groups of Se. However, the GPX activity in the rats fed the diet with 4.0 mg Se per kg was higher than in these brain parts from rats fed the two lower levels of

Table 3-2. Selenium, GPX Activity and Selenoprotein W Content in Muscle and Heart from WMD and Control Lambs

| | Muscle | | | Heart | | |
|---|---|---|---|---|---|---|
| | Se* | GPX$^{\varnothing}$ | SeW$^{\tau}$ | Se* | GPX$^{\varnothing}$ | SeW$^{\tau}$ |
| WMD | 0.6±0.2$^{ac}$ | 30±3$^{a}$ | ND | 1.2±0.2$^{ad}$ | 28±4$^{a}$ | ND |
| Int.Ctrl | 0.6±0.2$^{ac}$ | 33±2$^{a}$ | ND | 1.3±0.2$^{ad}$ | 33±2$^{a}$ | ND |
| Control | 8.0±2.1$^{bc}$ | 86±35$^{bc}$ | 3351±671 | 13.6±2.1$^{bd}$ | 274±35$^{bd}$ | 4338±930 |

*: Concentration of Se is expressed as nmole/g dry weight.
$\varnothing$: GPX activity is expressed as nmoles NADPH oxidized/min. mg protein
$\tau$: SeW is expressed in scan units per 200 μg protein.
Values are means ± standard error, n = 5 - 9.
$^{ab}$: significant difference within columns ($P<0.05$). $^{cd}$: significant difference between rows ($P<0.05$). ND: not detectable.

Table 3-3. Selenoprotein W content and Glutathione Peroxidase Activity in Cortex and Cerebellum From Rats Fed Three Levels of Dietary Selenium

| Component | Dietary level of Se (μg/kg) | | |
|---|---|---|---|
| | 0 | 0.1 | 4.0 |
| Selenoprotein W* | | | |
| Cortex | 838±84[a] | 1103±84[b] | 1113±73[b] |
| Cerebellum | 1105±113 | 1224±113 | 1369±98 |
| Glutathione Peroxidase** | | | |
| Cortex | 57±8[a] | 63±8[a] | 83±7[b] |
| Cerebellum | 61±7[a] | 69±7[a] | 122±6[b] |

* Values are given in scan units per 200 μg protein.
** Activity is expressed as nmoles NADPH oxidized /min. mg protein.
Values are mean of 5 animals ± standard error. Values within a row with different superscripts are significantly different ($P < 0.05$).

Se, and this activity was significantly higher in the cerebellum than in the cortex in rats fed the diet supplemented with the highest amount of Se.

The SeW content increased to a greater extent in the glial cells than in the L8 muscle cells with increasing levels of Se in the media (figure 3-1). No differences were found in SeW content either between the two brain cell lines with $10^{-8}$ M Se or with muscle cells with $10^{-7}$ M Se compared to those incubated with $10^{-8}$ M Se. However, this content was significantly higher in glial cells than in muscle cells incubated with the two highest levels of Se. The SeW content in the group incubated with $10^{-6}$M Se was about 2 fold greater in the glial cells than in the muscle cells, suggesting a greater response in glial cells to Se.

A comparison of the SeW content was made between the glial and neuronal cells (figure 3-2). A corresponding increase of SeW occurred with each increase of Se in the media in the glial cells, but this did not occur in the neuronal cells. The SeW content was highest in the cells grown with$10^{-7}$ M Se, suggesting that $10^{-6}$ M Se may be above requirements for neuronal cells.

Different patterns in GPX activity occurred with the three types of cells grown in media with three levels of Se (figure 3-3). A corresponding increase of GPX activity occurred in the muscle cells with each increase of Se in the media, but this activity did not differ in glial cells grown on the two lowest Se levels with a significant greater than the highest level of Se was used. However, the GPX activity was significantly lower in neuronal cells grown on the lowest level of Se as compared to the next highest level used, which was not different from the activity in the cells grown with the highest level of Se.

The decline of SeW content and GPX activity was similar in the L8 muscle cells when Se was removed from the media (figure 3-4). There were no significant differences in the rate of loss between the level of this selenoprotein and GPX activity. The regression equations for each of these were $y = 1182 - 92x$ for SeW and $y = 1509 - 119x$ for GPX.

The decline of GPX activity was significantly greater than that of SeW in the neuronal cells (figure 3-5). The regression equation for SeW was $y = 3530 - 47x$ and that for GPX was calculated to be $y = 2143 - 103x$.

A different pattern of SeW and GPX decline was found in the glial cells than in neuronal cells(figure 3-6). SeW content depleted significantly faster than did the GPX activity. The regression equation for SeW was calculated to be $y = 1723 - 267x$ and that for GPX activity to be $y = 1315 - 43x$. Thus, it is apparent that SeW content and GPX activity decreased at different rates between the three cell lines when Se was removed from the media.

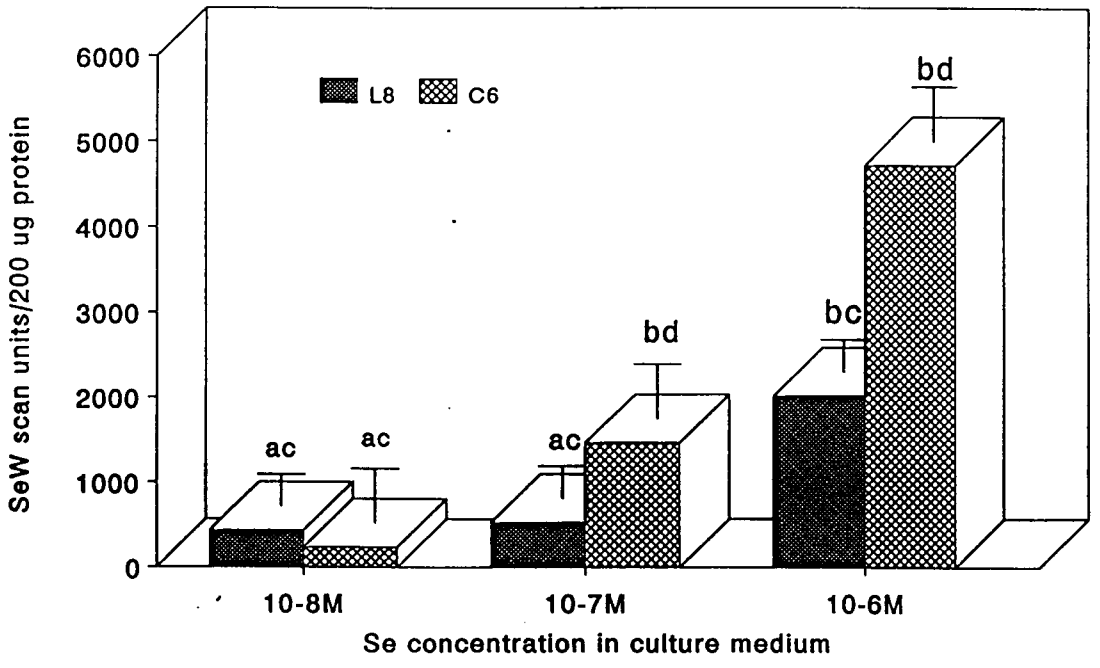



Figure 3-1. SeW content in rat L8 muscle and C6 glial cells incubated with various levels of Se. The bars represent means of 3 incubations ± SEM and those with different superscripts are significantly different ($P<0.05$).

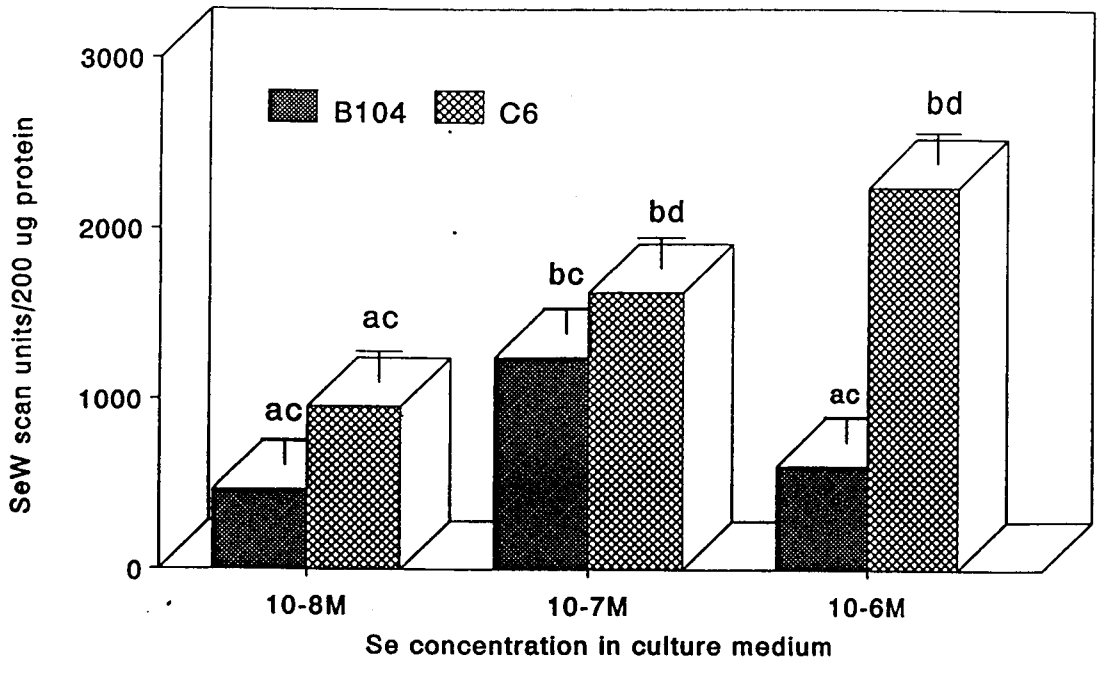



Figure 3-2. SeW content in rat C6 glial and B104 neuroblastoma cells incubated with various levels of Se. SeW levels are expressed as indicated in figure 3-1 legend. The bars represent means of 3 incubations ± SEM and those with different superscripts are significantly different ($P<0.05$).

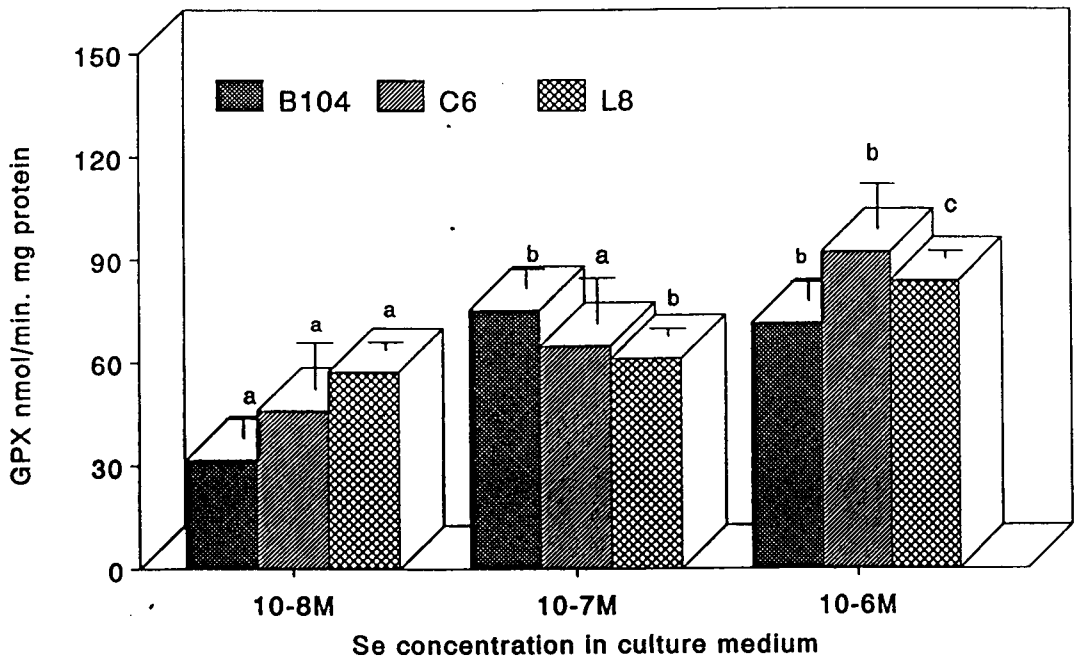



Figure 3-3. GPX activity in rat L8 muscle, C6 glial and B104 neuroblastoma cells incubated with various levels of Se. GPX activity is expressed as nmole NADPH oxidized per mg protein. The bars represent means of 3 incubations ± SEM and those with different superscripts are significantly different ($P<0.05$).

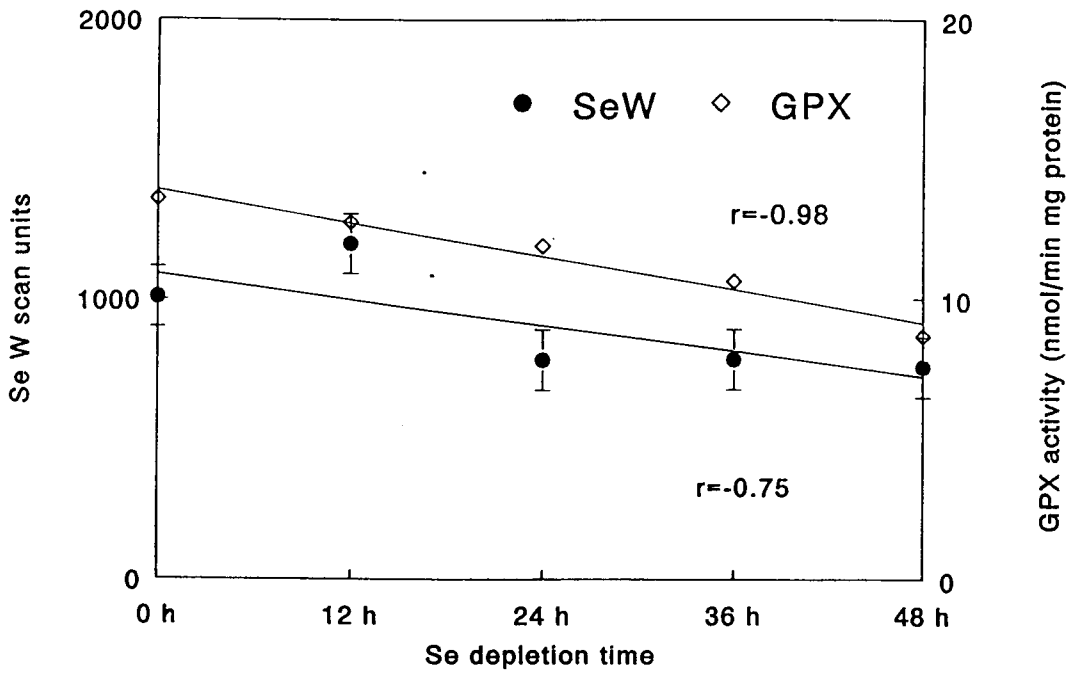



Figure 3-4. The decline of SeW content and GPX activity in rat L8 muscle cells after removal of Se from the media. SeW levels are expressed as indicated in legend of figure 3-1 and GPX activity as indicated in legend of figure 3-3. Each point means of 3 incubations ± SEM. The rate of decrease of SeW and GPX activity is not significantly different ($P>0.05$).

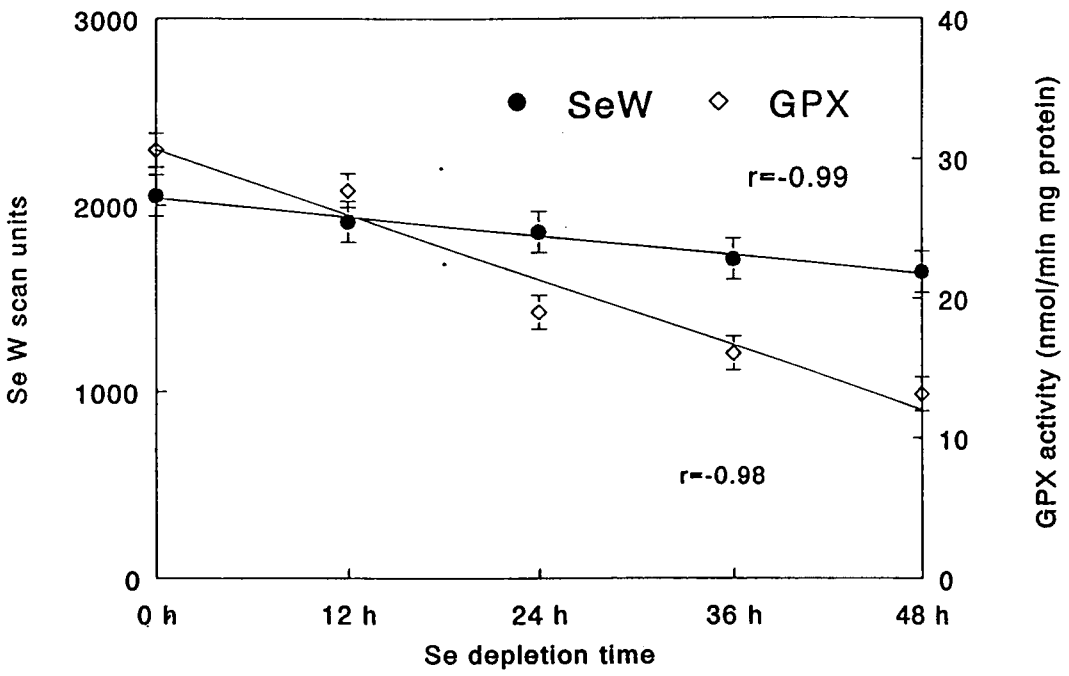



Figure 3-5. The decline of SeW content and GPX activity in rat B104 neuroblastoma cells after removal of Se from the media. SeW level and GPX activity are expressed as indicated in legends for figures 3-1 and 3-3. Each point means of 3 incubations ± SEM. The rate of decrease of SeW and GPX activity is significantly faster ($P < 0.05$) than that for SeW.

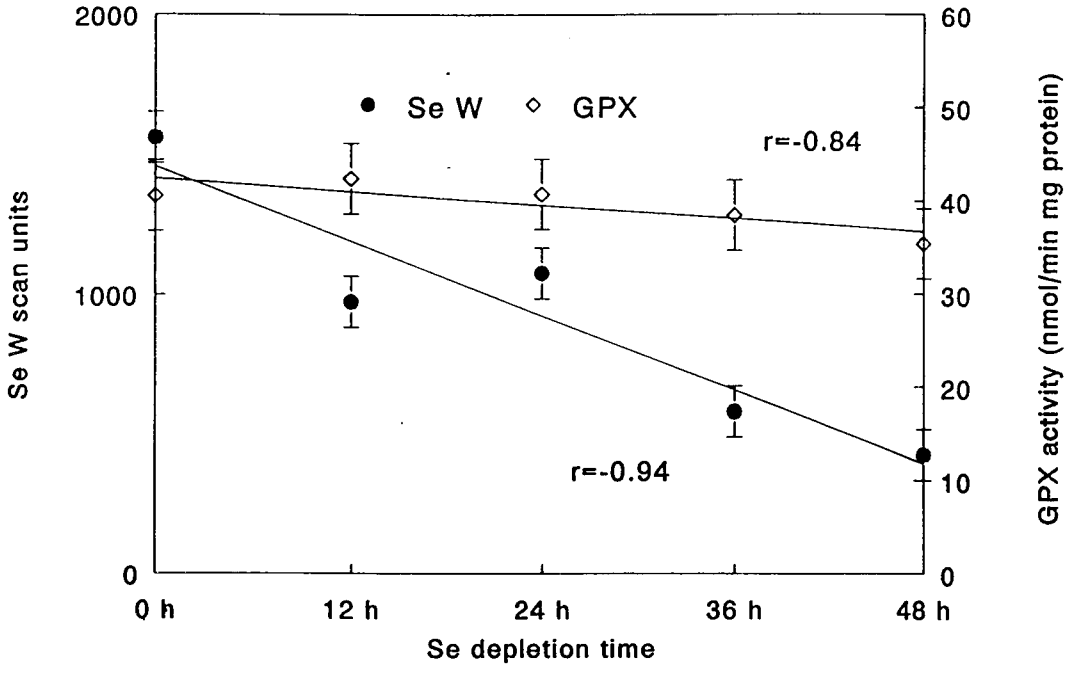



Figure 3-6. The decline of SeW content and GPX activity in rat C6 glial cells after removal of Se from the media. SeW level and GPX activity are expressed as indicated in legend of figures 3-1 and 3-3. Each point means of 3 incubations ± SEM. The rate of decline of SeW content is significantly different ($P < 0.05$) than that for the GPX activity.

## DISCUSSION

Our results indicate that there is a difference in the Se concentration in the brain of WMD and internal controls, and between these and control lambs (table 3-1). This is in contrast to the pattern observed in the muscle and heart where there was no difference in the Se concentration between WMD and internal controls (table 3-2), which is in agreement with prior work from our laboratory (Whanger et al., 1977). The brain (table 3-1) was similar to the muscle and heart (table 3-2) in that there was no significant difference in the GPX activity in WMD and internal control lambs. The results with GPX in the muscle and heart is also consistent with our prior work (Whanger et al., 1977). The data obtained previously (Yeh et al., 1997b) indicates a strong tenacity of the brain for SeW because GPX and Se were not retained in the brain at a constant level during Se depletion whereas this selenoprotein was retained.

The GPX activity, Se concentration and SeW content in different parts of the brain indicate that Se is not metabolized similarly in all regions of this organ (tables 3-1 and 3-3). Similar to the whole brain the GPX activities in cortex and thalamus were not different between WMD and internal control lambs, but there was a significant increase in this tissue from control lambs. In contrast, there were no differences in GPX activity in cerebellum between any of these groups. There were no differences in SeW content in the three brain regions from WMD versus internal control lambs, but there were significant increases in these regions from control lambs. Like wise, the rat data indicate that different regions of the brain can vary. For example, the Se status affected SeW in the cortex but not in the cerebellum (table 3-3). These data suggest that critical information

will be missed when a composite sample of the whole brain is used to study Se metabolism.

The cell culture work is consistent with the results with different parts of the brain. The two brain cell lines metabolized Se differently (figures 3-1 & 3-2). Since SeW is higher in muscle than in brain of animals (Yeh et al., 1995; Yeh et al., 1997a; Yeh et al., 1997b), the data in figure 3-1 showing a greater response of SeW to Se supplementation was unexpected. Extensive work on SeW with muscle cells in culture has been conducted in our laboratory (Yeh et al., 1997c). Se has been shown to affect both the levels of SeW and mRNAs for this selenoprotein. The two brain cell lines, glial and neuronal, used in our study indicate a difference in Se metabolism between them as demonstrated by SeW levels (figures 3-1and 3-2) and GPX activity (figure 3-3) and further indicates that a study of Se metabolism by different regions of the brain is warranted.

The depletion of SeW and GPX activity was different in the three cell lines studied in the present investigations (figures 3-4, 5, 6). The SeW levels and GPX activity declined at about the same rate in the muscle cells (figure 3-4) but the Se patterns were reversed in the neuronal (figure 3-5) and glial cells (figure 3-6), lending additional support to the concept that different regions of the brain do not metabolize Se identically.

The differences in response of the various cells to Se as demonstrated by SeW content and GPX activity are consistent with the data obtained with various tissues in the whole animal (Yeh et al., 1997c; Bermano et al., 1995 and Lei et al., 1995). The reasons for different responses of selenoproteins (and selenoenzymes) in various tissues are not known. One hypothesis could be that promoters for some selenoproteins respond

differently to Se in various tissues. Reduced Se status could elicit degradation of mRNA for some selenoproteins but not for other selenoproteins (Christensen and Burgener, 1992 and Toyoda et al., 1990). An alternative hypothesis is that selenocysteine insertion sequences in the mRNA which are necessary for Se insertion (Berry et al., 1993) may also affect stability.

Cell cultures have been extremely useful in studying Se metabolism. This was used to demonstrate that selenite was more effective than Semet for increasing GPX activity (Beilstein and Whanger, 1987; Whanger et al., 1979). The conversion of Semet to selenocysteine was shown to be influenced by the methionine content of the media, which mimics the results found in the intact animal (Butler et al., 1989). Various Se compounds have been studied in transsulfuration defective cells (Beilstein and Whanger , 1992) and again higher levels of Semet were required to induce the same amount of GPX activity as either selenite or selenocysteine. The addition of Se to the incubation medium resulted in increases of both SeW content and the mRNA levels in L8 muscle cells (Yeh et al., 1997c). The results with muscle cells are consistent with those obtained by others with kidney cells where mRNAs for GPX and 5'-deiodinase were shown to be regulated by Se concentration in the cultured media (Closs et al., 1995).

Like selenoprotein P (Burk and Hill, 1993; Burk et al., 1991), the function of SeW is unknown. Selenoprotein P and SeW are the two most studied selenoproteins without known functions. Since all of the known selenoenzymes catalyze oxidation-reduction reactions (Burk and Hill, 1993), it has been suggested that selenoprotein P and SeW may function as antioxidants (Burk and Hill, 1993; Yeh et al., 1997a; Yeh et al., 1997b). This possibility for SeW has been strengthened by data showing that reduced glutathione is

bound to two of the four species of this selenoprotein from rat muscle (Beilstein et al., 1996). Previously, it was postulated that SeW plays a critical role in muscle metabolism (Yeh et al., 1997a; Yeh et al., 1997b), but the present data and that showing that SeW did not deplete from the brain of deficient sheep (Yeh et al., 1997a) are suggestive of a more essential role for this selenoprotein in brain. Although pathological lesions have been reported in the heart and muscle of Se deficient animals (Schuber et al., 1961), This has never been reported in the brain of deficient animals. The data showing that the brain (table 3-1; ref. Yeh et al., 1997b) has such a tenacity for SeW may be the reason such lesions have not been reported in brain of Se deficient animals. Therefore, it appears that the investigation of Se metabolism in the brain is an extremely fruitful field to pursue.

# CHAPTER 4

## GLUTATHIONE PEROXIDASE ACTIVITY AND SELENOPROTEIN W LEVELS IN DIFFERENT BRAIN REGIONS OF SELENIUM DEPLETED RATS

Y. Sun, J. A. Butler, and P. D. Whanger
Toxicology Program and Department of Agricultural Chemistry
Oregon State University, Corvallis, Oregon 97331 USA

Running title: Selenoprotein W in rat brain

Correspondence about this manuscript should be made with Dr. P. D. Whanger at the above address; by phone, (541)737-1803; by fax, (541)737-0497; by e-mail, whangerp@bcc.orst.edu

Published with the approval of Oregon State University Experiment Station as technical paper number _____. This research was supported by Public Health Service Research Grant number DK 38341 from the National Institute of Diabetes and Digestive and Kidney Diseases.

**ABSTRACT**

Previous studies in sheep and rats showed that selenoprotein W (SeW) levels decreased in all tissues except brain during selenium (Se) depletion. To further investigate this depletion in different parts of the brain, second generation Se depleted rats were used. Dams consumed the Se deficient basal diet during gestation and lactation. Control rats were fed 0.1 ppm Se in the diet after weaning. Tissues were collected at weaning, 2, 6, 10, and 14 weeks afterwards. Glutathione peroxidase (GPX) activities were measured for comparative purposes. This activity in muscle, skin, spleen and testis increased about four-fold with Se repletion and reached a plateau after 6 or 10 weeks. GPX activity decreased to almost one tenth of the original activity with continuous Se depletion. GPX activities increased two-fold in brain regions (cortex, cerebellum, thalamus) with Se supplementation, but did not significantly decrease with Se depletion. Of the three parts of the brain, cerebellum had the highest GPX activity, and cortex had the lowest. SeW levels in skin, spleen, muscle and testis were undetectable by Western blot in weanling rats, but became detectable after 6 weeks of Se repletion. In contrast, the expression of SeW in cortex, cerebellum and thalamus was not significantly affected by Se depletion or repletion. Cortex had the highest SeW expression among the three parts of the brain.

## INTRODUCTION

Selenium was recognized in 1957 as an essential element in mammalian species. Since then several selenoenzymes have been identified. They are the glutathione peroxidase (GPX) family containing at least four different selenoenzymes (Rotruck et al., 1973; Takahashi and Cohen, 1986; Ursini et al., 1985; Chu et al., 1993), the iodothyronine deiodinase family containing type I, II and III deiodinases (Behne et al., 1990; Berry et al., 1991a; Davey et al., 1995; Croteau et al., 1995) and thioredoxin reductase (Tamura and Stadtman, 1996). All of these selenoenzymes contain one selenium atom per polypeptide chain although some of the GPXs are multimeric proteins. Selenoprotein P (Yang et al., 1987; Hill et al., 1991) and selenoprotein W (SeW) are known selenoproteins but their functions have not been identified. Selenoprotein P, the major Se compound in plasma, contains 10 selenocysteine residues. SeW is a low molecular weight protein (10 kDa) originally reported as a missing component in selenium-deficient animals suffering from white muscle disease (Pedersen et al., 1969). It was first purified from cytosol of rat skeletal muscle (Vendeland et al., 1993). The cDNA sequence of rat SeW is composed of 672 nt. The 5' untranslated region consists of 51 bases followed by the 267-base coding sequence. The presence of one selenocysteine codon, TGA, corresponds to amino acid residue 13 in the predicted sequence and confirms that the protein is indeed a selenoprotein (Vendeland et al., 1995; Gu et al., 1997). The 3' untranslated region is required for selenocysteine cotranslation (Berry et al., 1991b; Gu et al., 1997).

SeW is present in most tissues from rats fed a Se adequate diet, but is highest in skeletal muscle, brain, testis and spleen (Sun et al., 1998; Yeh et al., 1995). In selenium

deficiency, SeW was depleted from most of rat and sheep tissues except brain (Yeh et al., 1997; Sun et al., chapter 3 in this thesis). This raises the question that SeW might be important in brain functions. Other groups reported that Se distribution and selenoprotein expression are regulated differently among different tissues when Se is limited (Bermano, et al., 1996; Bermano, et al., 1995). The organ specific modulation of SeW expression may relate to the most important sites of function in Se deficiency and influence the response of the selenium-deficient animal during selenium repletion. Therefore, this experiment was conducted to further investigate the effects of depletion and repletion of selenium on SeW expression in different parts of the brain of second generation Se depleted rats. GPX activity in different parts of the brain was also determined for comparative purposes. In addition, GPX and SeW were determined in other tissues of rats for comparison to the brain.

## MATERIALS AND METHODS

### Animals

Second generation selenium depleted rats were used to examine the expression of SeW in different tissues. Pregnant Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were fed a selenium deficient diet during gestation and lactation. Weaning rats (45 male and 45 female) were fed either the Se deficient diet or this diet supplemented with 0.1 mg sodium selenite per kg. The basal diet was composed of 30 % torula yeast (Rhinelander Paper Co, Rhinelander, WI), 51.5 % sucrose, 9 % purified cellulose (Solka Floc, Brown Co., Berlin, NH) 5 % corn oil, 3.5 % AIN-93M mineral mix without

selenium and 1 % AIN-76 vitamin mix (American Institute of Nutrition, 1977), 0.3 % L-methionine and 0.2 % choline citrate. The animals were anesthetized with sodium pentobarbital (80 mg/kg i.p.) and rat tissues (brain, muscle, skin, spleen, and testis) were removed and frozen immediately afterward at - 80°C. Rat brains were dissected as cortex, cerebellum, thalamus and medulla oblongata. Rat tissues (5 rats each group) were collected at weaning, 2, 6, 10 and 14 weeks afterwards. SeW levels and GPX activities were measured on all tissues examined. Since no differences were found due to gender, only the value for the male rats are presented.

**Western blot analysis**

Tissues were homogenized in 5 volumes of homogenization buffer (20 mM Tris [pH 7.5], 0.25 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM 2-mercaptoethanol and 25 μg/ml leupeptin). The homogenates were centrifuged at 14,000 x g for 20 minutes at 4°C to obtain cytosolic extracts. Protein content was analyzed by the Lowry method (Lowry et al., 1951) with bovine serum albumin as standards. Tissues extracts (200 μg cytosol protein) were electrophoresed on 7.5 % to 15 % gradient SDS-polyacrylamide gels as described by Laemmili (1970) and proteins were transferred onto nitrocellulose membranes (0.2 μm, BA-S83; Schleicher & Schuell, Keene, NH) overnight at 4°C according to the method of Towbin et al., (1979). The membranes were incubated with 5 % non-fat milk in TTBS (50 mM Tris [pH 7.5], 0.5 M NaCl, 0.05 % Tween-20) 1 hr for blocking, then hybridized with purified SeW polyclonal antibody against the peptide sequence corresponding to amino acid residues 13 - 31 of rat selenoprotein W (1:500 in TTBS containing 1 % non- fat milk) for 1.5 hrs (Yeh et al, 1995). The

membranes were washed 3 times in TTBS, 5 min each; then incubated in secondary antibody (1:2000 goat anti-rabbit IgG conjugated with horse-radish peroxidase in TTBS containing 1 % non-fat milk) for 1 hr. After washing the membranes with TTBS to eliminate excess secondary antibody, blots were incubated with ELC chemiluminescent reagent (Amersham Life Science, Inc., Arlington Heights, IL) and exposed to Hyperfilm ECL (Amersham, IL). Developed films were scanned with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA).

**Glutathione peroxidase activity assay**

Cellular GPX was measured by the coupled enzyme method using hydrogen peroxide as a substrate (Paglia and Valentine, 1967) with a Beckman DU Series 64 spectrophotometer (Beckman Instruments, Inc. CA). GPX activity was adjusted to sample protein content and was expressed as nmole NADPH oxidized/min. mg protein.

**Statistical analysis**

Data were examined for equal variance and normal distribution prior to statistical analysis. Mean values were compared by analysis of variance (ANOVA) with Fisher's least-significant difference (LSD) method (Steel and Torrie, 1980). A significant level of 5 % was adopted for all comparisons.

# RESULTS

## GPX activity in rat tissues

As shown in Figure 4-1, GPX activities in muscle (A), spleen (B), skin (C) and testis (D) were gradually reduced with Se depletion. By 14 weeks after weaning, GPX activity was significantly decreased from 9.6 to 1.4 nmole/min. mg protein in muscle; from 20.3 to 7.9 nmole/min. mg protein in testis; from 79.9 to 2.6 nmole/min. mg protein in skin, and from 111.4 to 20.2 nmole/min mg protein in spleen.

When Se was supplied at 0.1 mg/kg diet after weaning, GPX activities were increased about 5-fold in all the above tissues and reached a plateau. GPX activity in muscle (A) and spleen (B) reached a plateau after 6 weeks Se supplementation, whereas in skin (C) and testis (D) a plateau was reached after 10 weeks Se supplementation (Figure 4-1).

After 14 weeks Se depletion, GPX activities in different parts of the brain (cortex, cerebellum and thalamus) were not significantly different compared to the original activities (at weaning), but it did increase with Se supplementation and reached a plateau in 10 weeks (figure 4-2). In addition, cerebellum had the highest GPX activity among these brain parts when Se was supplied, whereas GPX activity was not significantly different in these brain regions when Se was depleted. Cerebellum (B) had the highest GPX activity increase (100 %) after Se supplementation, cortex (A) had the least (40 %), and thalamus (C) had about 60 % increase in GPX activity after 10 weeks of Se supplementation.

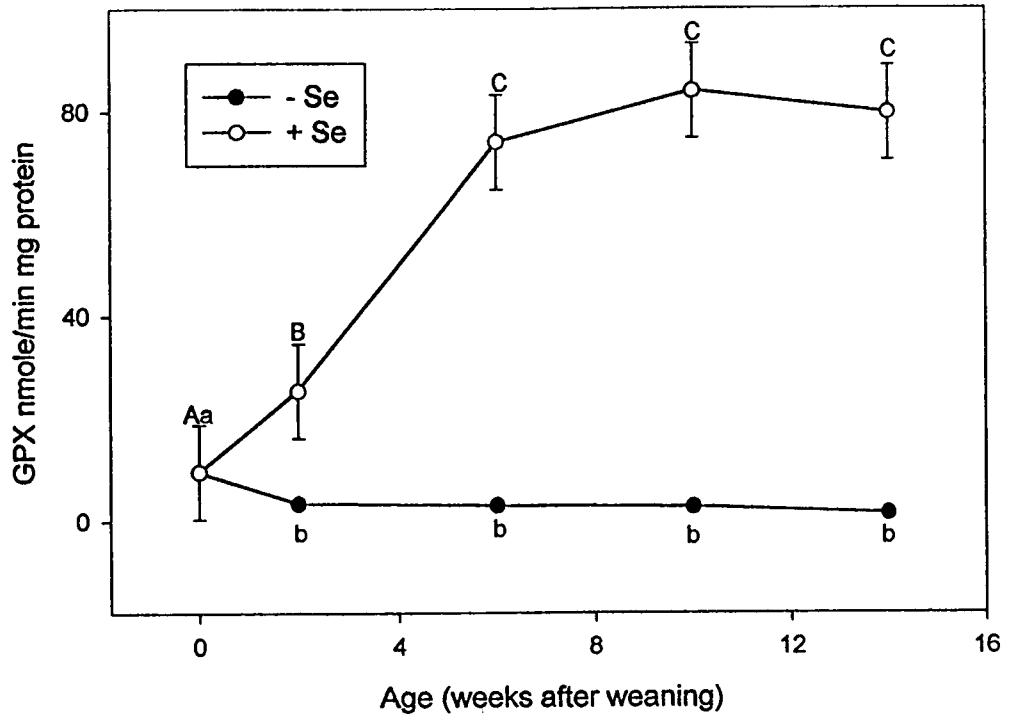
**SeW expression in rat tissues**

There were no significant decrease of SeW levels in cortex (A), cerebellum (B) and thalamus (C) with depleting of Se ($p>0.05$) (figure 4-3). There were no significant differences in SeW levels in cortex and cerebellum between Se depleted or repleted rats, even after 14 weeks ($p>0.05$). However, the SeW level in the thalamus was significantly higher ($P < 0.05$) in repleted than depleted rats both at 6 and 14.

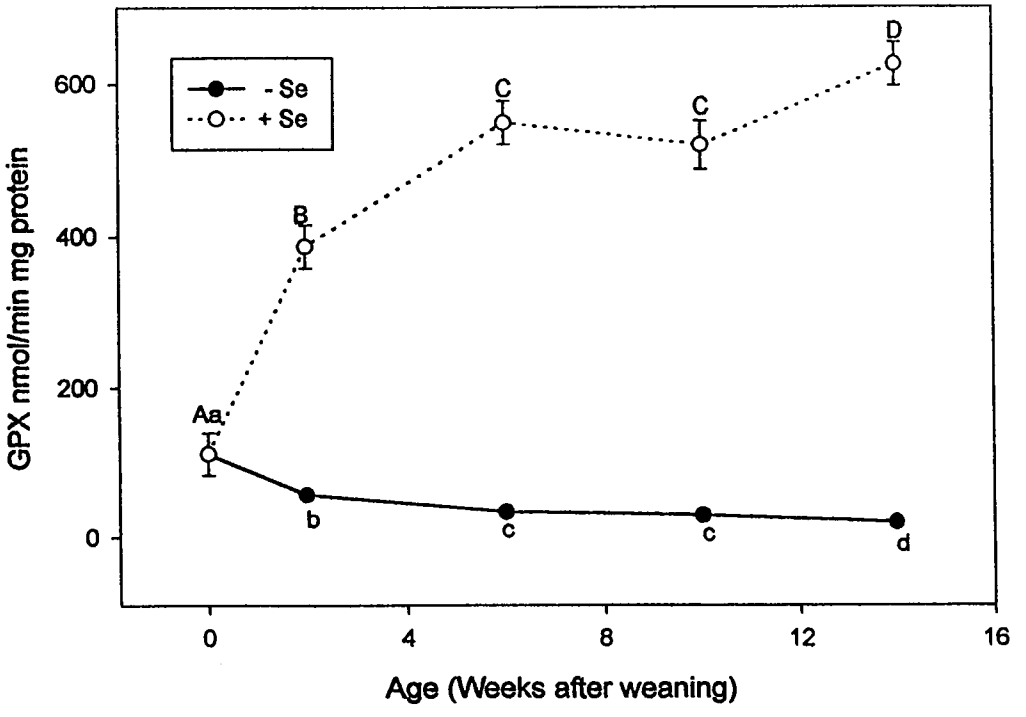
SeW was determined by western blots. SeW was undetectable in muscle, skin, spleen and testis in rats depleted with selenium (0 week), but it started to be detectable after 6 weeks of Se supplementation (figure 4-4). SeW levels in muscle, spleen, and skin were significantly higher ($P < 0.05$) at 14 weeks than at 6 weeks, but there was no difference in the testis between 6 and 14 weeks.

Figure 4-1. GPX activity in muscle (A), spleen (B), skin (C), and testis (D) from Se deficient and supplemented rats. GPX activity was increased about 5-fold in all these tissues and reached a plateau. In muscle and spleen, it reached a plateau after 6 weeks Se supplementation. In skin and testis, it reached a plateau after 10 weeks Se supplementation. GPX activity is expressed as nmole NADPH oxidized per min per mg protein. The bars represent means of 5 animals ± SEM. Values with different letters are significantly different ($P < 0.05$).

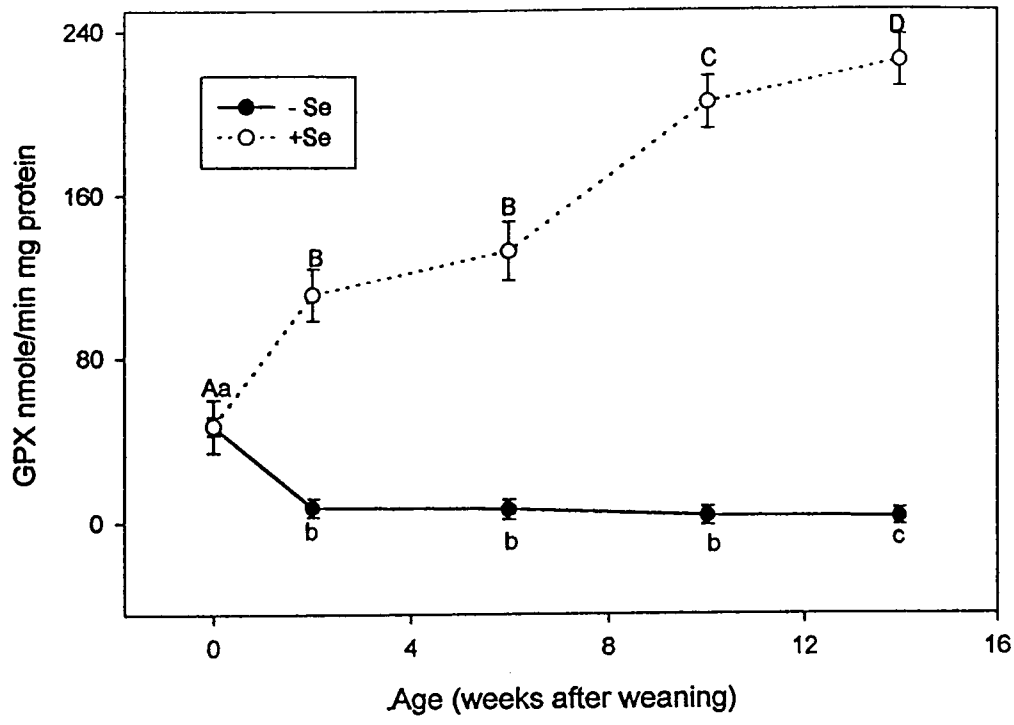
A.


- Se
+ Se
GPX nmole/min mg protein
80
40
0
Aa
B
C
C
C
b
b
b
b
0
4
8
12
16
Age (weeks after weaning)

B.


- Se
+ Se
GPX nmol/min mg protein
600
400
200
0
Aa
B
C
C
D
b
c
c
d
0
4
8
12
16
Age (Weeks after weaning)

C.


240
160
80
0
GPX nmole/min mg protein
- Se
+Se
Aa
B
B
C
D
b
b
b
c
0
4
8
12
16
Age (weeks after weaning)

D.

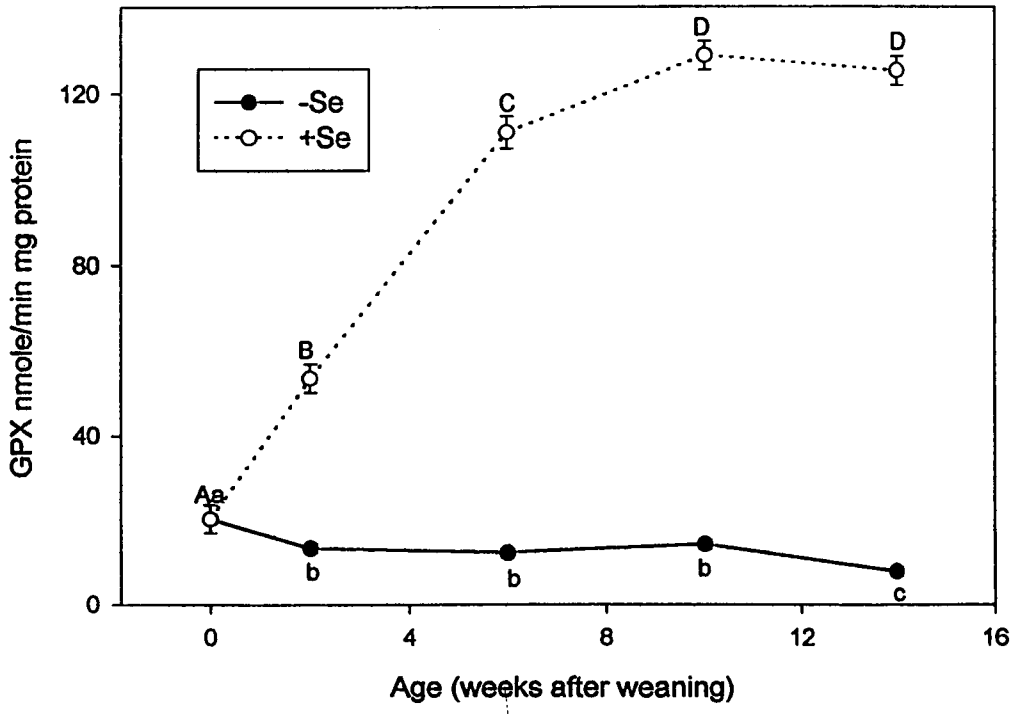
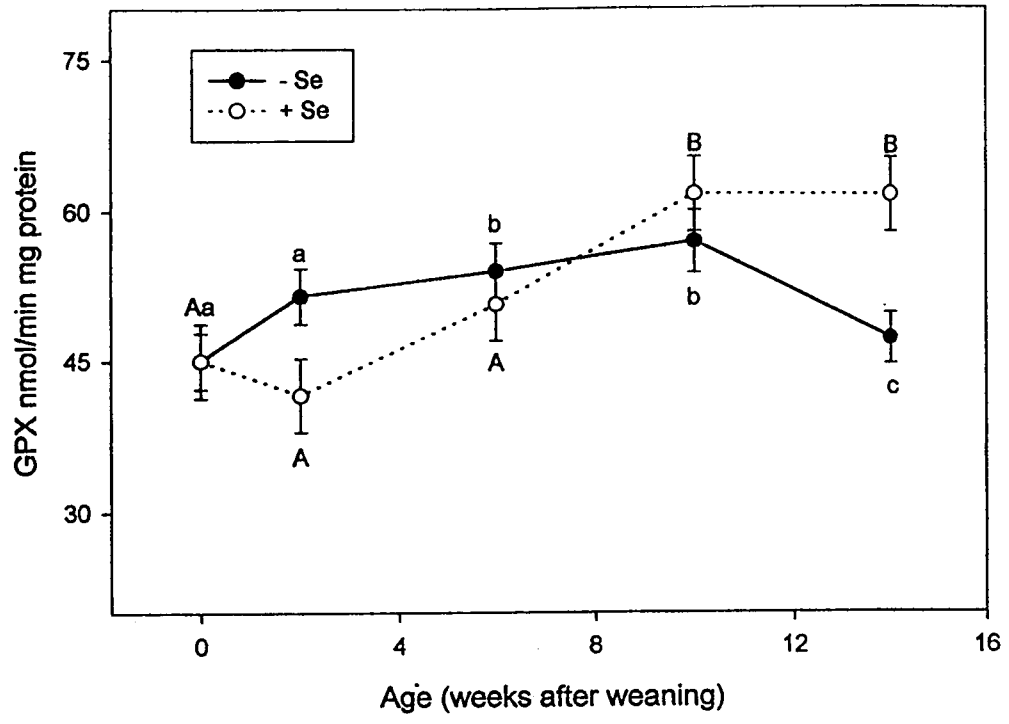
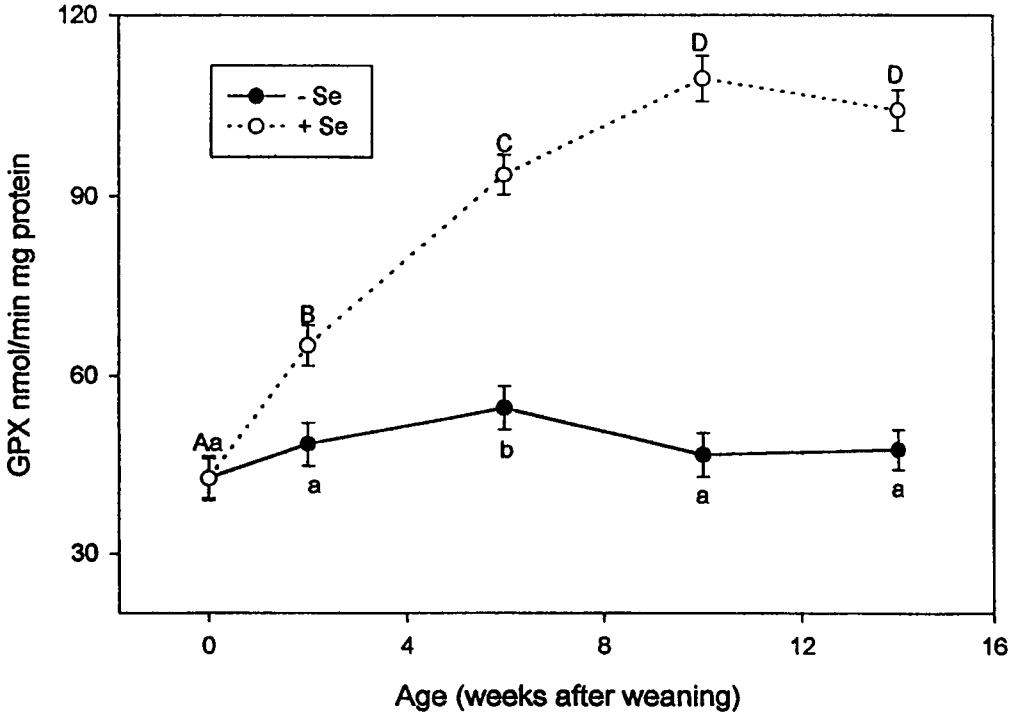

120
80
40
0
GPX nmole/min mg protein
-Se
+Se
Aa
B
C
D
D
b
b
b
c
0
4
8
12
16
Age (weeks after weaning)

Figure 4-2. GPX activity in cortex (A), cerebellum (B), and thalamus (C) from Se deficient and supplemented rats. After 14 weeks Se depletion, GPX in these parts of the brain was not significantly different as compared to original activity ($p > 0.05$). A. GPX in cortex increased 40 %; B. GPX in cerebellum increased 100 %; C. GPX activity in thalamus increased 60 %. GPX activity reached a plateau after 10 weeks of Se supplementation. GPX activity is expressed as nmole NADPH oxidized per min per mg protein. The bars represent means of 5 animals ± SEM. Values with different letters are significantly different ($P < 0.05$).

A.


- Se
+ Se
GPX nmol/min mg protein
75
60
45
30
0
4
8
12
16
Age (weeks after weaning)
Aa
a
b
B
B
A
b
c
A

B.


- Se
+ Se
GPX nmol/min mg protein
120
90
60
30
0
4
8
12
16
Age (weeks after weaning)
Aa
a
b
a
a
B
C
D
D

C.

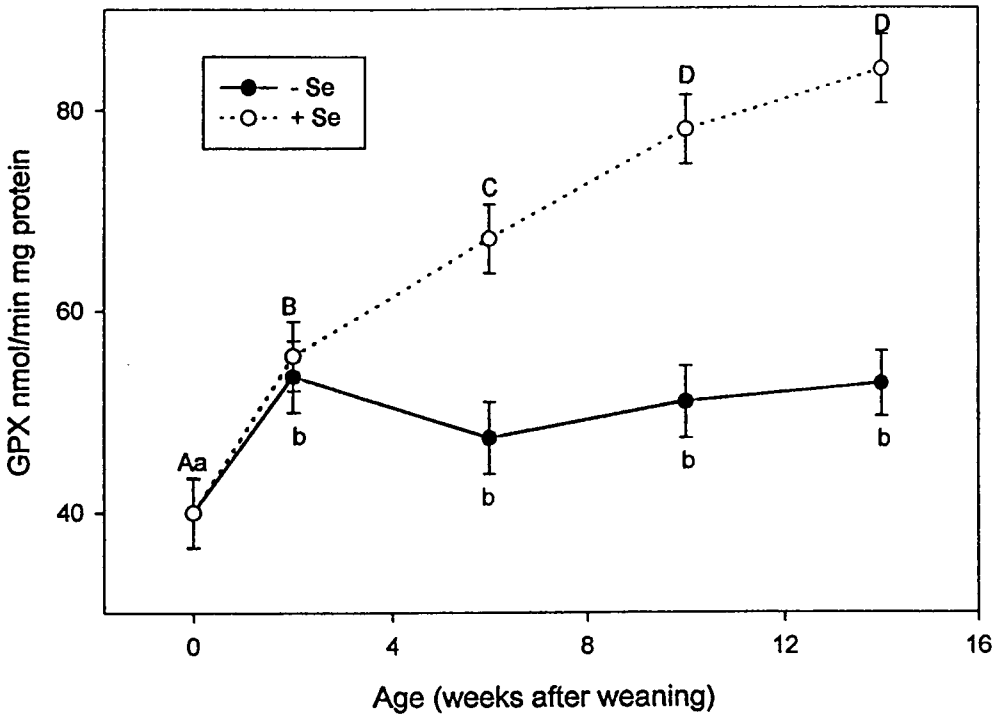
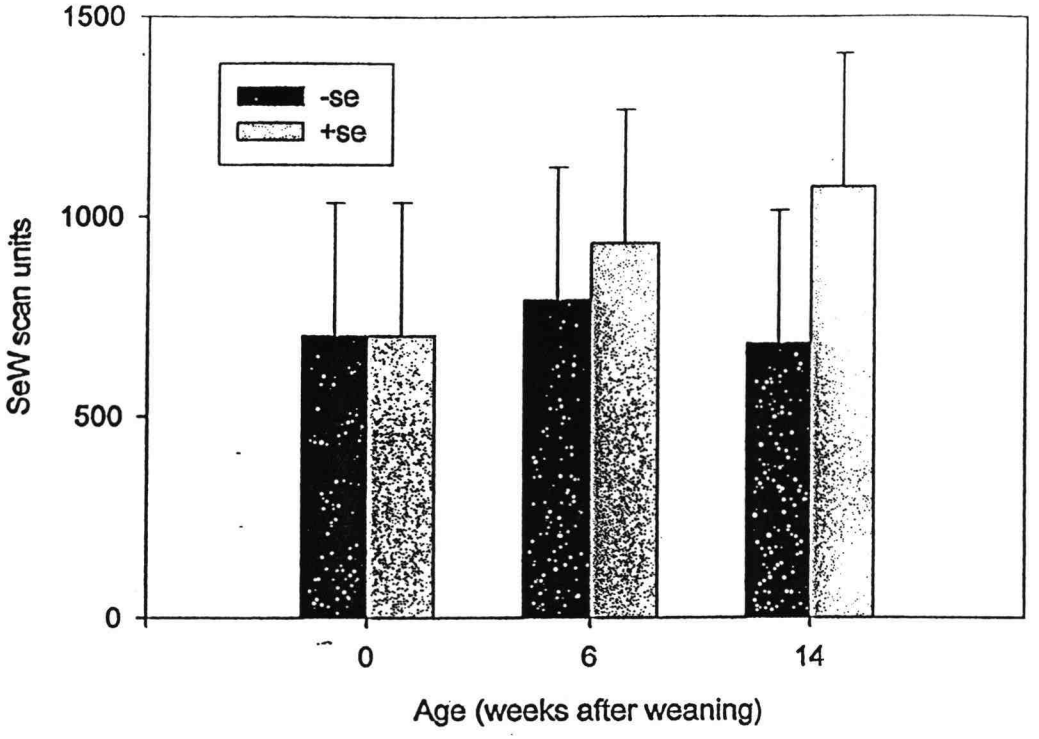
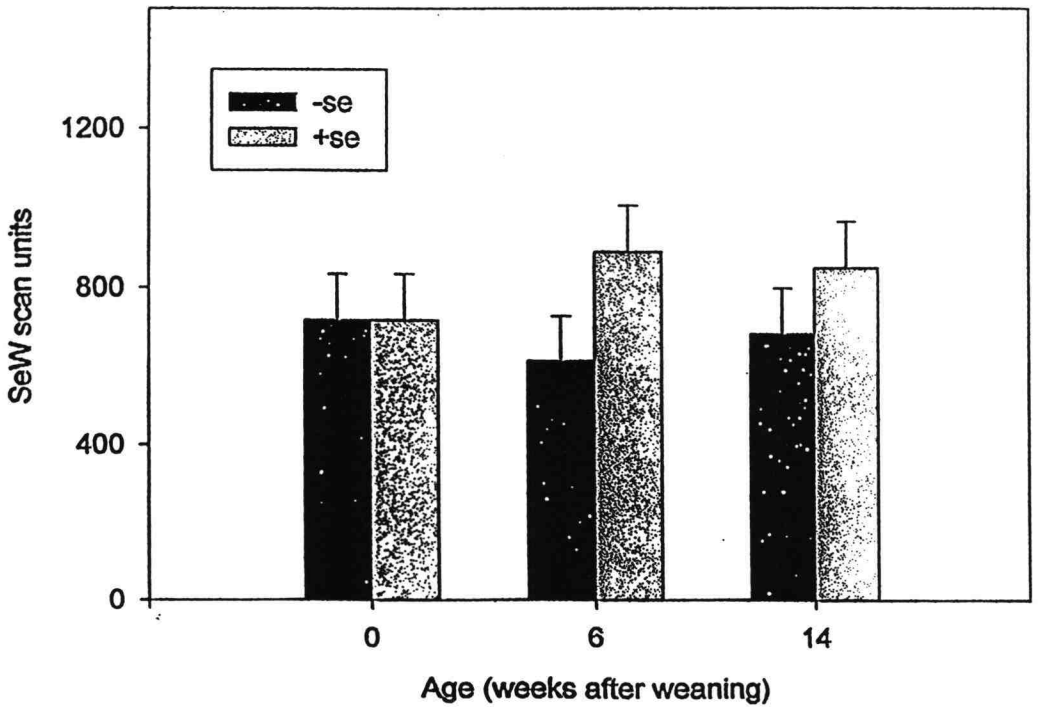

- Se
+ Se
GPX nmol/min mg protein
80
60
40
0
4
8
12
16
Age (weeks after weaning)
Aa
B
C
D
D
b
b
b
b

Figure 4-3. SeW expression in cortex (A), cerebellum (B), and thalamus (C) from Se deficient and supplemented rats. SeW was not significantly different within these three regions when Se was depleted for 14 weeks ($p > 0.05$). A & B: SeW had no significant increase in cortex and cerebellum after 14 weeks Se supplementation ($p > 0.05$). C. SeW was significantly increased in thalamus after Se supplementation. SeW levels are expressed as scan units. The bars represent means of 3 animals ± SEM. Values with different letters are significantly different ($P < 0.05$).

A.


1500
1000
500
0
SeW scan units
-se
+se
0
6
14
Age (weeks after weaning)

B.


1200
800
400
0
SeW scan units
-se
+se
0
6
14
Age (weeks after weaning)

C.

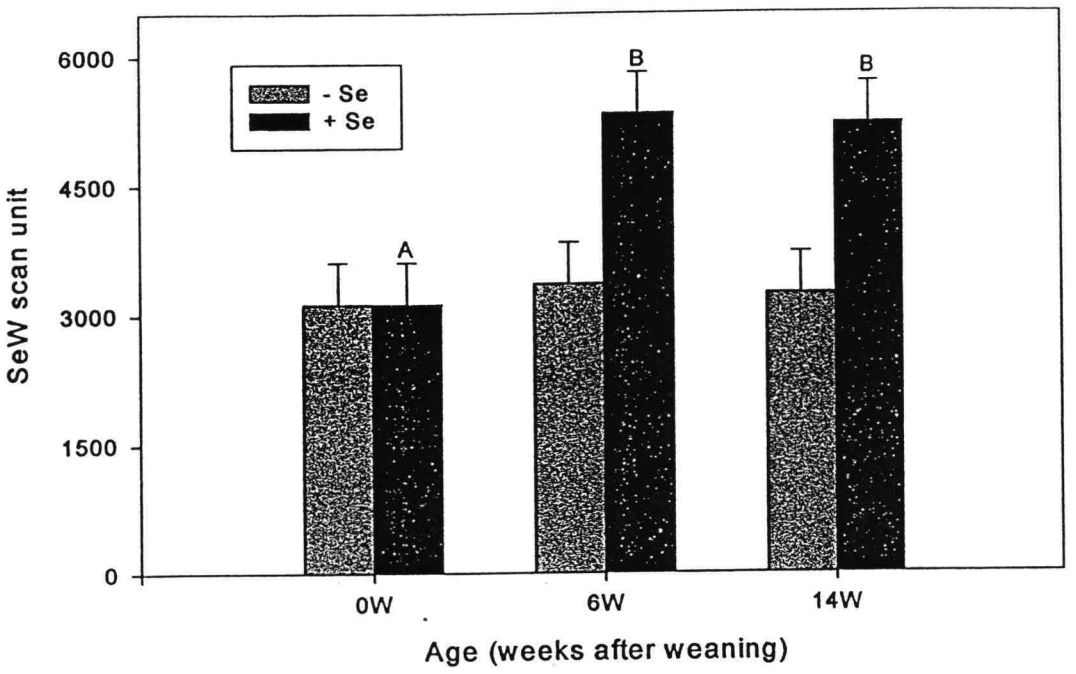
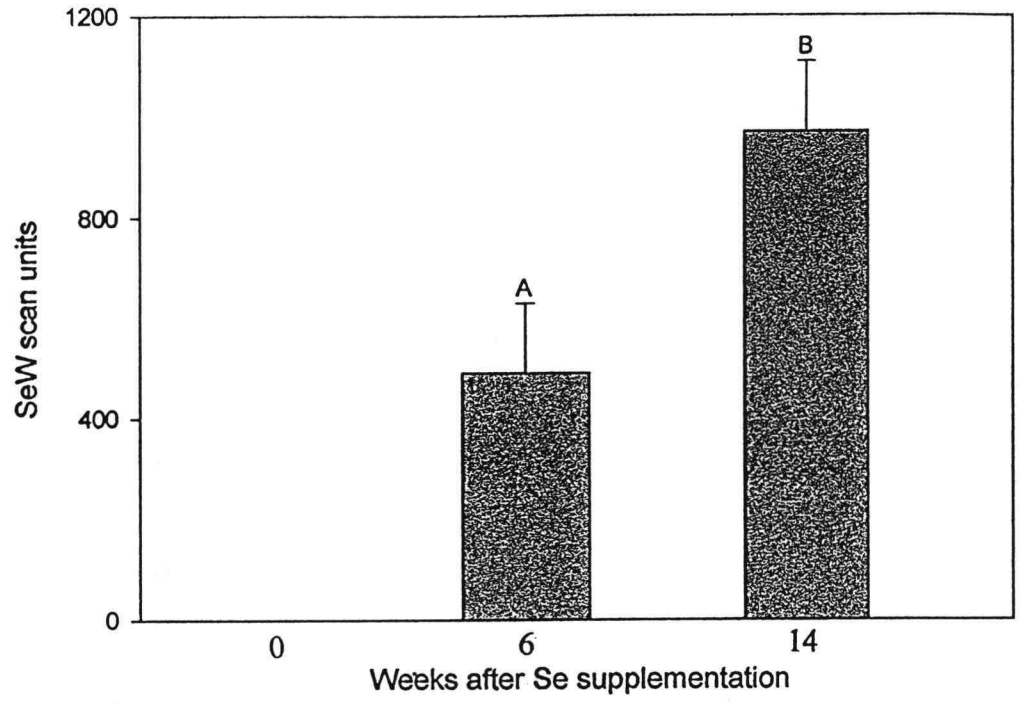
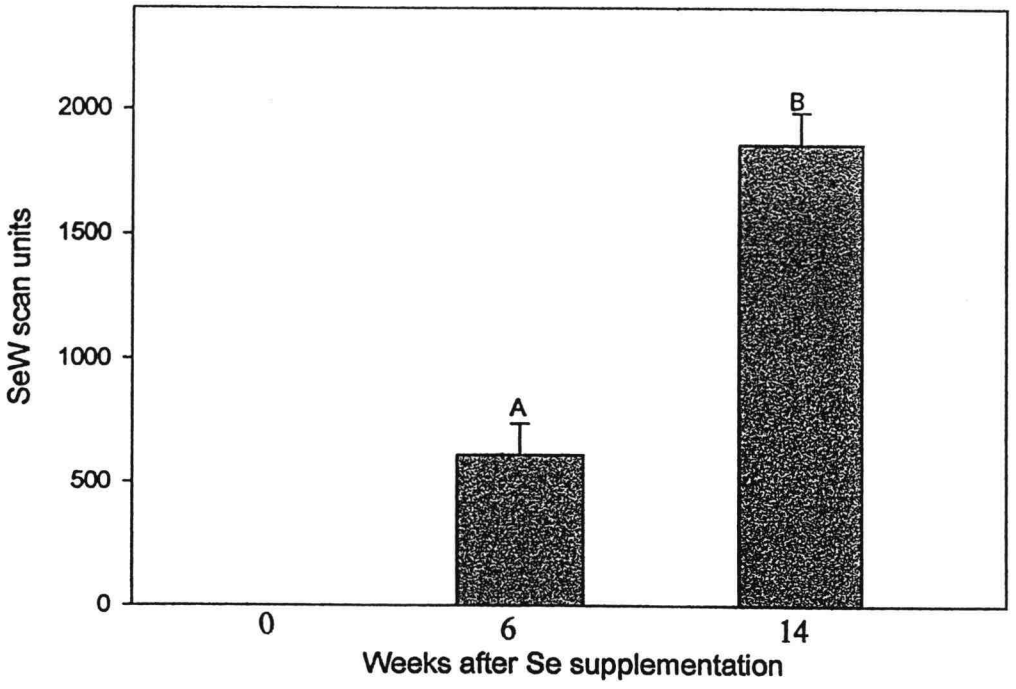

6000
4500
3000
1500
0
SeW scan unit
- Se
+ Se
A
B
B
0W
6W
14W
Age (weeks after weaning)

Figure 4-4. SeW levels in muscle (A), spleen (B), skin (C) and testis (D) after Se supplementation. Values with different letters are significantly different ($P < 0.05$).

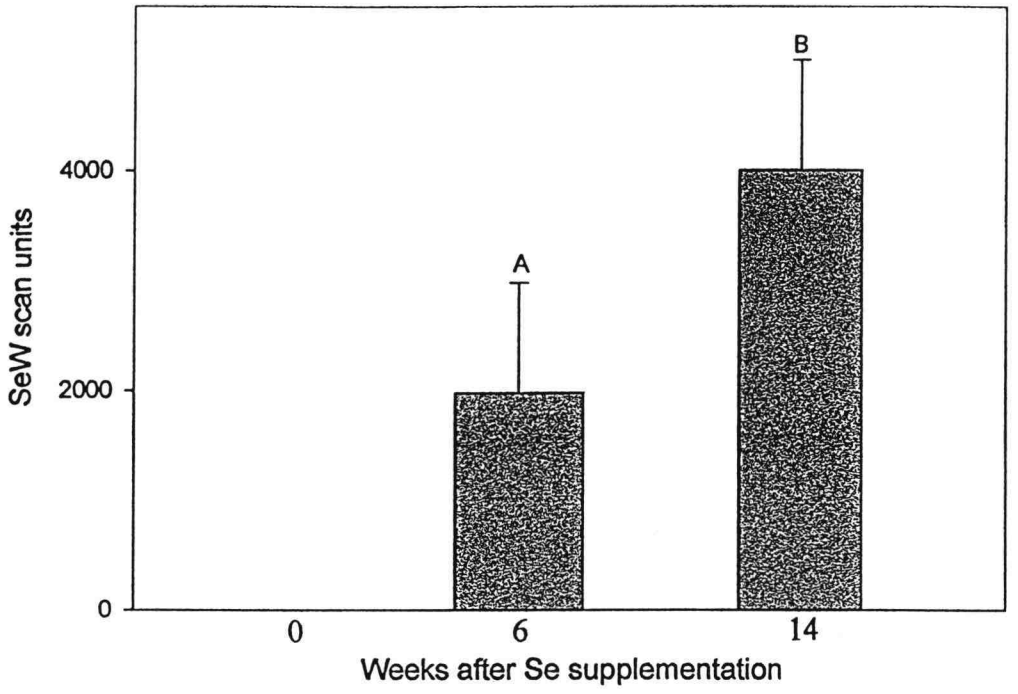
A.


1200
800
400
0
SeW scan units
A
B
0
6
14
Weeks after Se supplementation

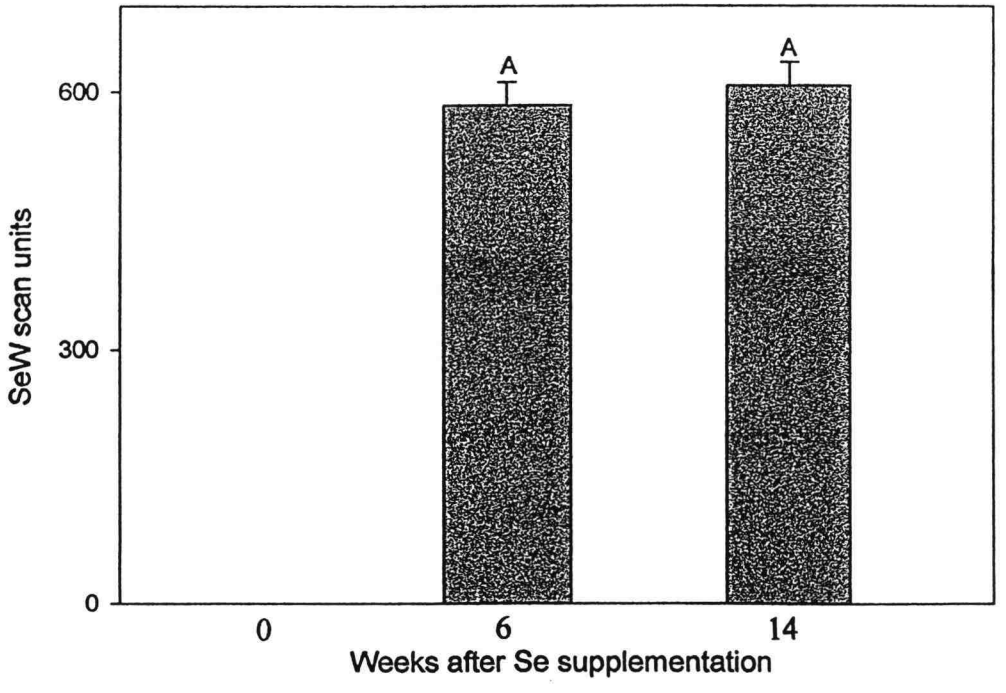
B.


2000
1500
1000
500
0
SeW scan units
A
B
0
6
14
Weeks after Se supplementation

C.


B
A
4000
2000
0
SeW scan units
0
6
14
Weeks after Se supplementation

D.


A
A
600
300
0
SeW scan units
0
6
14
Weeks after Se supplementation

## DISCUSSION

The biological functions of the trace element selenium are mediated by selenoproteins. SeW is one of the selenoproteins in mammalian species for which the function is as yet unknown. Previous studies showed that SeW expression was modulated differently in brain and other tissues of the rat or sheep tissues when Se was depleted (Yeh et al., 1997; Sun et al., chapter 3 of this thesis). This study on Se depletion in rats was conducted to determine any differences in SeW expression among different parts of the brain.

Behne et al. (1988) reported that the brain and endocrine organs are able to retain Se at the expense of other tissues such as liver, kidney, and muscle when supplies of the micronutrient are limited. Additionally, within a particular organ, certain selenoproteins are preferentially synthesized when supplies of Se are inadequate for optimal expression of all selenoproteins (Sunde, 1994). Therefore, there is a tissue specific regulation of selenoprotein expression which may indicate the most important functional sites of function for this protein.

Selenoproteins are selenium-dependent and require specific mechanisms for the control of their expression especially when supplies of Se are limited. In rat muscle, spleen, skin and testis, GPX activity was dramatically decreased when Se was depleted from the diet, and it quickly increased after Se repletion. SeW in these tissues were undetectable at weaning and afterwards, while it gradually increased after Se supplementation. SeW and GPX expression in these four tissues followed the same pattern during Se depletion or when replenished in the diet.

There were no decreases in GPX activity or SeW expression In cortex, cerebellum and thalamus when Se was depleted from the diet. When Se was supplied, GPX activity increased 40 %, 100 %, and 60 %, respectively, in the cortex, cerebellum, and thalamus. Cerebellum had the highest GPX activity among the three parts of the brain. This result is consistent with the findings in a sheep experiment (Sun et al., chapter 3 of this thesis). After Se repletion, there was no significant increase in SeW expression in cortex and cerebellum, but a significant increase in the thalamus (figure 4-3). Therefore, the expression of SeW in cortex and cerebellum was not significantly affected by Se depletion or repletion, but significant changes in the thalamus. The regulation of selenoprotein synthesis by various parts of the brain appears to be different for various selenoproteins and this organ may regulate its selenoprotein levels to compensate for deficiency or excess of selenium. These results provide evidence for the existence of distinct mechanisms for the control of expression of different selenoproteins both within and between muscle and brain in the rat.

There are 8 well characterized mammalian selenoenzymes. All of these have a redox function. SeW is a selenoprotein with unknown functions, but glutathione was recently demonstrated to be bound to SeW through a disulfide (or selenodisulfide) bond (Beilstein et al., 1996). Thus, it would be reasonable to conclude that SeW may possess antioxidant properties, but its function needs to be further investigated.

In a previous publication, a gender difference was found in SeW level in rat tissues (Yeh et al., 1998). However no differences were found in the present study (data not shown). The reason for this disagreement is not known, but could be due to a number of factors including second generation Se depleted rats, type of diet fed or other factors.

The rats in the earlier study were fed commercial chow diet and had not been depleted of selenium.

# CHAPTER 5

## SELENOPROTEIN W OVEREXPRESSED AND UNDEREXPRESSED IN CULTURED RAT GLIAL CELLS

Y. SUN, Q-P. GU, P. D. WHANGER
Toxicology Program and Department of Agricultural Chemistry
Oregon State University, Corvallis, Oregon 97331 USA

Running title: Selenoprotein W expression in cultured cells

Correspondence about this manuscript should be made with Dr. P. D. Whanger at the above address; by phone, (541)737-1803; by fax, (541)737-0497; by e-mail, whangerp@bcc.orst.edu

Published with the approval of Oregon State University Experiment Station as technical paper number _____. This research was supported by Public Health Service Research Grant number DK 38341 from the National Institute of Diabetes and Digestive and Kidney Diseases.

**ABSTRACT**

Selenium deficiency results in undetectable levels of selenoprotein W (SeW) in muscle but has very little effect upon its content in the brain and thus glial cells were studied. Mass spectrometry studies by other investigators showed that glutathione (GSH) is bound to SeW which led to the speculation it has an antioxidant function similar to other selenoproteins. Full length cDNA of SeW was cloned to inducible LacSwitch expression vector and stablely transfected to C6 rat glial cells. After induction, SeW and its mRNA were expressed 22- and 11-fold higher than control, respectively. The cDNA coding region of SeW was cloned to the vector in the antisense direction and stablely transfected in C6 cells for underexpression of the protein. SeW expression was reduced to 20% of the control cells after induction. Glutathione peroxidase activity and GSH levels were not significantly different between induced and control cells. There was a greater survival rate of overexpressed cells when incubated with 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) than control cells, suggesting SeW possibly has an antioxidant function. Supported by NIH grant #DK38341.

## INTRODUCTION

Selenium (Se) is an essential element for animals and humans. Se deficiency in lambs and calves results in white muscle disease, a disorder characterized by degeneration of both skeletal and cardiac muscle (Schubert et al., 1961). Severe dietary selenium deprivation in humans is associated with an endemic juvenile cardiomyopathy called Keshan disease (Chen et al., 1980). Therefore, Se is important in muscle metabolism. The biological function of this trace element is the incorporation of selenocysteine into selenoproteins. Several mammalian selenoproteins have been identified and their biological functions are involved in redox reaction. The glutathione peroxidase (GPX) family includes four selenium-dependent enzymes. They are cellular GPX (Rotruck et al., 1973), extracellular GPX (Takahashi and Cohen, 1986), phospholipid hydroperoxide GPX (Ursini et al., 1985) and gastrointestinal GPX (Chu et al., 1993). All four selenoenzymes protect against toxicity of activated oxygen species by converting peroxides to alcohols in both cellular and extracellular compartments of tissues. Iodothyronine 5'-deiodinase family includes type I (Berry et al., 1991), type II (Davey et al., 1995) and type III (Croteau et al., 1995) deiodinases. The newly discovered selenoenzyme in mammals is thioredoxin reductase (Tamura and Stadtman, 1996). The purified protein contains flavin adenine dinucleotide (FAD) and exhibits nicotinamide adenine dinucleotide phosphate (NADPH)-dependent thioredoxin reductase activity. Thus, all of the above selenoenzymes are involved in redox reactions. The antioxidant property of Se has been demonstrated as an important function at physiological levels of Se.

All of these mammalian selenoenzyme genes have been cloned and sequenced, and except for selenoprotein P contain one selenium atom per polypeptide chain. Selenocysteine is at the active site of these selenoenzymes and dietary selenium regulates enzymatic activities.

Selenoprotein W is a low molecular weight protein (10 kDa) originally reported as a missing component in selenium-deficient animals suffering from white muscle disease (Pedersen et al., 1972). It was first purified from rat skeletal muscle (Vendeland et al., 1993). Cloned cDNA sequences from rats (Vendeland et al., 1995), mice, sheep, monkeys and humans (Gu et al., 1997; Whanger et al., 1997) contain one in-frame TGA codon for selenocysteine at residue 12. Sequence analysis of rat SeW revealed 50 bp of 5' untranslated region (UTR), 267 nucleotides encoding an 89-amino acid polypeptide, and 370 bp in the 3'UTR. Deduced amino acid sequences are 83% identical among five species (Gu et al., 1997). Rat and mouse amino acid sequences are 100% identical as well as 100% identical between monkey and human. SeW is present in most of the tissues in Se adequate animals. It is highest in skeletal muscle, followed by brain and testis (Sun et al., 1998; Yeh et al., 1995; 1997). Although the function of selenoprotein W has not been determined, recently it was reported that glutathione (GSH) is tightly bound to it (Beilstein et al., 1996). Since all the selenoenzymes are involved in redox reaction, it is reasonable to suspect that this selenoprotein may have antioxidant function.

In this study, to clarify the ability of SeW to protect cells against the oxidative stress induced by a radical initiator in culture, transfectants of rat glial cells were isolated that overexpressed or underexpressed SeW. It was shown that SeW overexpression could prevent cell death due to oxidative damage.

# MATERIALS AND METHODS

## Materials

Rat C6 glial cell line was purchased from American Type Culture Collection (ATCC, MD). Cell culture media Ham's F-12 and Dulbecco's modified Eagle's media (DMEM), antibiotics G418 and Hygromyocin B, DNA mass ladder and DNA size ladder were obtained from Gibco BRL, (Grand Island, NY). Calf serum (CS) was purchased from Hyclone (Logan, UT). Cell culture petri dishes were purchased from Corning (Corning, NY). 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA, Inc. (Richmond, VA). LacSwitch inducible Mammalian Expression System was purchased from Stratagene, CA. All the restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA). Plasma Purification Kit was purchased from QIAGEN, Inc. (Chatsworth, CA). Geneclean II Kit was obtained from Bio-101, Inc. (Vista, CA). PCR amplification kit was purchased from Promega (Madison, WI). DIG Oligonucleotide Tailing Kit, diogoxigenin-dUTP, anti-DIG-AP conjugate, blocking reagent, luminescent detection substrate CSPD and cell proliferation Kit I (MTT) were obtained from Boehringer Mannheim Biochemical (Indianapolis, IN). GeneScreen Plus nylon membrane was purchased from DuPont/NEN (Boston, MA). Western Blot reagents were purchased from Bio-Rad Laboratories (Hercules, CA). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH). Hyperfilm and protein rainbow markers were obtained from Amersham (Arlington Heights, IL). All other chemicals were of molecular biology grade and purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Culture**

C6 rat glial cell line was maintained in a humidified atmosphere of 5 % $CO_2$ and 95 % air at 37°C. The culture medium is 90% FD (50% F-12 and 50% DMEM) supplemented with 10% calf serum plus penicillin G 120 μg/ml, streptomycin 200 μg/ml and ampicillin 25 μg/ml. The culture medium for transfected C6 cell was supplemented with hygromycin B at 400 $\mu$g/ml (GIBCO) and G418 at 600 $\mu$g/ml (GIBCO) to maintain the selective pressure. Sense and antisense RNA was induced by IPTG (isopropyl-β-D-thiogalactoside) at 5mM for 72 hours.

**Plasmid construction and stable transfection**

Rat SeW full length cDNA (672 bp) and cDNA coding region (267 bp) were amplified by PCR. The PCR reactions were performed with SeW specific primers with Not I compatible restriction site ends. The PCR products generated for rat SeW were subcloned into Not I site of the vector pOP13 (LacSwitch, Stratagene) in the sense and antisense orientation, respectively. The directional cloning of the entire cDNA (5' leading, coding region and 3' untranslated region) insert was confirmed by PCR and sequencing. The orientation of the cDNA coding region was confirmed by PCR. The expression of the insert is regulated by a eukaryotic Lac-repressor-expressing vector p3'SS.

Transfection of C6 rat glial cells was accomplished using LipofectAmine™ (Gibco BRL) according to the supplier's instructions with either the control (vector without insert) or the vectors that contained SeW full length cDNA or cDNA coding region. Ten $\mu$g of pOP13 with the insert and 5 $\mu$g of p3'SS were co-transfected to 80% confluence of C6 cells in 100 mm culture dish. After 6 hrs transfection, the cells were

cultured in nonselective medium for 24 hr. Hygromycin B and G418 were then added at the optimal concentration (predetermined to be 400 $\mu$g/ml and 600 $\mu$g/ml respectively for C6). For the isolation of single cell transfectants, clonal rings (Nalge) were used, and at least 60 single cell clones of C6 containing the sense or antisense vectors were further expanded under continued antibiotics selection.

**Screening of transfectants**

Both western blots and northern blots were used to screen the clones. After IPTG induction at 5 mM for 72 hr., cells were harvested and sonicated. 200 $\mu$g of cytosol protein was used for western blot analysis (Sun et al., 1998). Protein content was analyzed by the Lowry method (Lowry et al., 1951). Rat SeW polyclonal antibody was generated from synthesized peptide (Yeh et al., 1995).

Total cellular RNA was isolated by single step guanidine isothiocyanate procedure (Chomczynski and Sacchi, 1987). The final RNA pellet was dissolved in diethyl pyrocarvonate treated water and RNA concentration was determined spectrophotometrically at 260 nm. The ratio of 260nm/280nm was used to check the quality of RNA purification. A sample of 20 $\mu$g of formaldehyde denatured RNA was size fractioned by electrophoresis on a 1.5 % agarose-2.2 M formaldehyde gel, and the RNA blotted onto GeneScreen nylon membrane in 10xSSC (1.5M NaCl and 0.15 M sodium citrate, pH 7.0) as transfer medium. The membrane was prehybridized at 65°C for 4 hours in medium containing 16 % SDS, 0.25 M $NaPO_4$, pH 7.2, 1 mM EDTA and 0.5% blocking reagent. Hybridization of the cDNA probe was performed at 65°C overnight in the prehybridization with addition of 30 ng/ml of dig-dUTP labeled probe.

After extensively washing to remove excess probe and non-specific binding, hybridized RNA signals were detected by chemiluminesce detection system based on the procedure described by Krueger (1995).

Northern blot dignoxigenin nuclei acid probe was prepared by PCR from cloned rat SeW cDNA coding region. Primers were designed based on the rat SeW cDNA start and termination signal regions. Dignoxigenin-labeled dUTP was incorporated into probe DNA during PCR amplification under standard conditions. The PCR products were then purified using Chroma Spin™ Columns and the concentration of probe was estimated in comparison with DNA mass ladder electrophoresis.

**MTT assay**

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a tetrazolium salt which can be metabolized to formazan salt by viable cells. Formazan dye can be quantified by spectrophotometer. Therefore, MTT assay was used for determination of cell proliferation and viability. Oxidative stress was applied to overexpressed and underexpressed cells to examine the antioxidation function of SeW. 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), a radical initiator, was used as an oxidant at concentrations of 5 to 80 mM.

The MTT assay was used for measurement of cytotoxicity. It was conducted according to the supplier's instructions. Cells were seeded to 96-well plates at $1x10^4$ cells/well. After 3-4 days induction, the cells reached confluence. Different concentrations (5, 10, 20, 30, 40, 50, 60, 70, 80 mM) of AAPH were applied and incubated for 16 hours in a cell culture incubator. Cells grown in a 96 well tissue culture

plate were incubated with the yellow MTT solution (final concentration 0.5 mg/ml) for 4 hours. After this incubation period, purple formazan salt crystals were formed. Subsequently, 100 μl of 10% SDS in 0.01 M HCl was added to each well and the plate was incubated overnight in humidified atmosphere (37°C, 6.5 % $CO_2$) to solubilize formazan crystals. The solubilized formazan product was spectrophotometrically quantified using an ELISA reader (Molecular Devices, Spectra MAX 250, Sunnyvale, CA) at a wave length of 550 nm with reference wave length at 690 nm. Cell survival rate = $OD_{treated}/OD_{untreated}$x100%.

**Thiobarbituric acid reaction substances (TBARS)**

Cell lipid peroxidation was quantified by TBARS (Yogi, 1984). Malondialdehyde (MDA) is derived from lipid peroxides of polyunsaturated fatty acids with three or more double bonds. The reaction of MDA with thiobarbituric acid (TBA) forms a red-colored pigment, which is fluorescent and can be measured fluorometrically at 553 nm using 515 nm excitation. Cells were cultured in 100 mm culture dishes. After 3-day induction, cells were incubated with 30 mM AAPH for 8 or 16 hr at 37°C, then they were harvested and sonicated in 0.9 % NaCl. A sample of 100 μl homogenate was used for TBARS quantification and the content was expressed on a protein basis.

**Measurements of GPX activity and Glutathione**

The cellular GPX activity was measured by a coupled enzyme method using hydrogen peroxide as the substrate (Paglia and Valentine, 1967) with a DU Series 64 spectrophotometer (Beckman Instruments, Fullerton, CA). Hydrogen peroxide was

used as a substrate. GSH was measured by HPLC as described by Fariss and Read (1987).

## RESULTS

A full length cDNA encoding the rat SeW (5' leading, open reading frame and 3' untranslated region) and the cDNA open reading frame only were constructed to the LacSwitch expression vectors in sense and antisense orientation, respectively, as described under materials and methods. These constructs were used for transfection of rat C6 glial cells by means of LipofectAmine™ (GIBCO BRL); and after selection of transfected cells with G418 and Hygromycin B, three clones of transfectants for SeW overexpression and four clones of transfectants for SeW underexpression were isolated.

### RNA, Protein, GPX and GSH in transfected C6 cells

Figure 5-1 shows the northern blot of these cell clones with or without IPTG induction. Lane 1 is SeW mRNA from rat muscle, which was used as a marker of the location of this mRNA. Lane 2 is SeW mRNA from normal C6 cells. Lane 3 is mRNA from C6 cells transfected with vector without insert. Lanes 4 and 5, 6 and 7, 8 and 9 are three SeW overexpressed transfectant clones. Lanes 4, 6 and 8 were not induced by IPTG; whereas lanes 5, 7 and 9 were induced by IPTG. Based on the intensity of the bands, the SeW mRNA in overexpressed cells (lane 9) was 11-fold higher compared to non-induced cells (lane 8). The clone of lanes 8 and 9 has the highest SeW mRNA and protein expression after IPTG induction. Therefore, this clone was used for all the experiments in this study. Lanes 10-13 were mRNA of four SeW underexpressed clones

induced for 3 days. Of these, the clone in lane 10 has the lowest selenoprotein W expression. This clone was used for all the SeW underexpressed experiments.

After three days induction of SeW overexpressed cells, western blot showed the content of this selenoprotein in these cells were 22-fold greater compared to parental cells or non-induced cells (Figure 5-2A,B). SeW in underexpressed cells was reduced to 20 % of non-induced cells or 10 % of normal C6 cells after 3 days IPTG induction (Figure 5-3A,.B).

GPX activities were measured in these cells to determine if transfection affected its expression. Figure 5-4 indicates that there was no significant difference in GPX activities between induced and non-induced cells or between transfected and normal C6 cells ($p>0.05$). Glutathione assay (figure 5-5) shows that there is no significant difference between transfected and normal C6 cells or between induced and non-induced SeW overexpressed cells ($p>0.05$), but GSH was significantly lower in SeW underexpressed cells compared to non-induced cells ($p<0.05$).

**Susceptibility of the transfectants to lipid hydroperoxide-mediated cell injury**

The established SeW overexpressed and underpressed cells were used to determine whether SeW renders cells less susceptible to killing by lipid peroxidation. Cell viability of the transfectants were assessed by the MTT assay. As shown in figure 5-6A, it was found that AAPH, a radical initiator that can peroxidize membrane lipids (Guo et al., 1995), has a dose-dependent toxic effect on cells. After exposure to 5 - 80 mM AAPH for 16 hours, both SeW overexpressed and control cells displayed a dose-dependent reduction in cell viability, but SeW overexpressed cells had higher survival

rate than control cells. However, no difference in cell survival rate changes occurred in SeW underexpressed cells as compared to control cells (figure 5-6B).

The TBA has been widely used as an indicator of lipid peroxidation in tissues. SeW overexpressed cells treated with 30 mM AAPH for 8 or 16 hour, the TBARS was slightly increased after 16 hours of oxidative treatment, but this never reached significance ($p>0.05$) (figure 5-7).

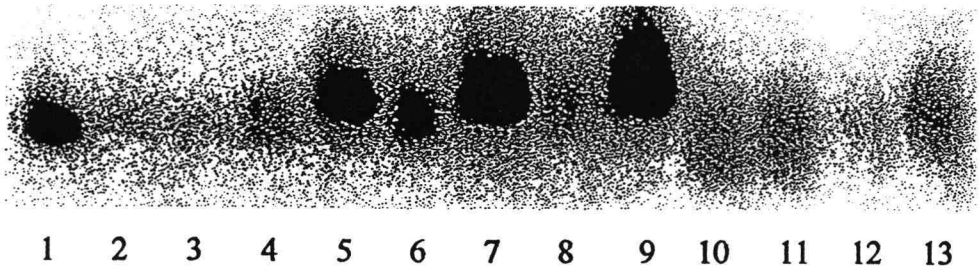



Figure 5-1. Northern blot of SeW mRNA in SeW cDNA transfected cells. Lane 1: rat muscle tissue. Lane 2: normal C6 cells. Lane 3: C6 cells transfected with vectors without insert. Lane 4/5, 6/7, 8/9: three clones of C6 cells transfected with full length cDNA. 4, 6, 8 were clones not IPTG induced. 5, 7, and 9 were IPTG induced for 3 days. Of which clone 8/9 has highest response to induction. Lane 10, 11, 12 and 13 are four antisense transfected cell clones after 3 days induction.

A.


B.

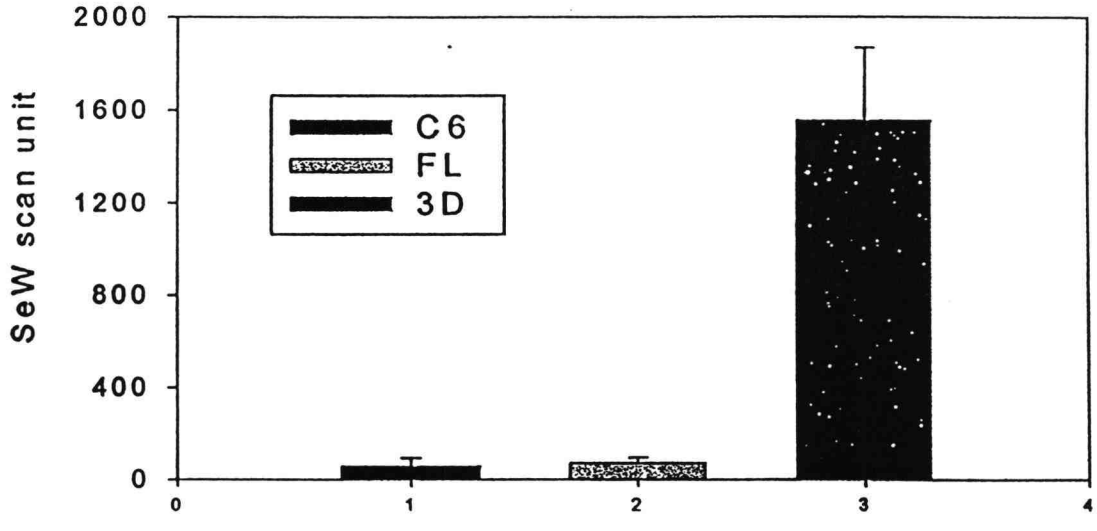
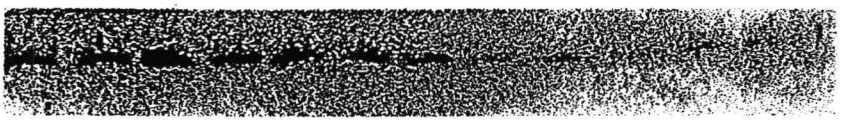



Figure 5-2. A. Western blot of SeW in overexpressed cells. Lane 1-3: normal C6 cells. Lane 4-6: transfectants without induction. Lane 7-9: transfectants after 3 days induction. Lane 10: purified rat SeW as MW marker. B. SeW scan units in different cells. SeW expression was 22-fold increased in overexpressed cells compared to normal C6 cells or transfectants without IPTG induction.

A.




B.

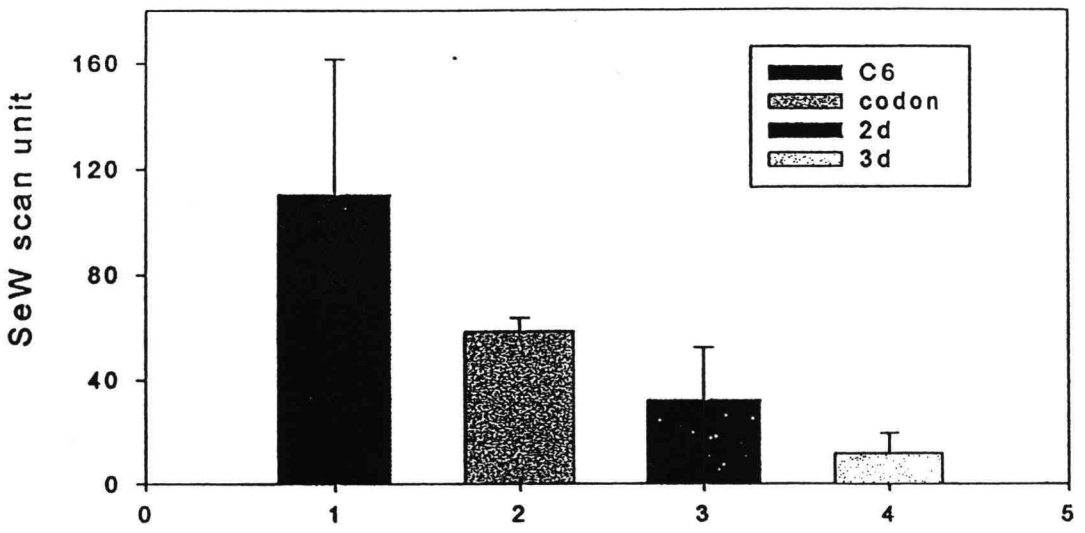



Figure 5-3. A. Western blot of SeW in underexpressed cells. Lane 1-3: normal C6 cells. Lane 4-6: SeW antisense cDNA transfectants without IPTG induction. Lane 7-9: transfectants with 2 days IPTG induction. Lane 10-12: transfectants with 3 days IPTG induction. B. SeW scan units in different cells. SeW expression in underexpressed cells was 10 % of normal C6 cells and 20 % of transfectants without induction.

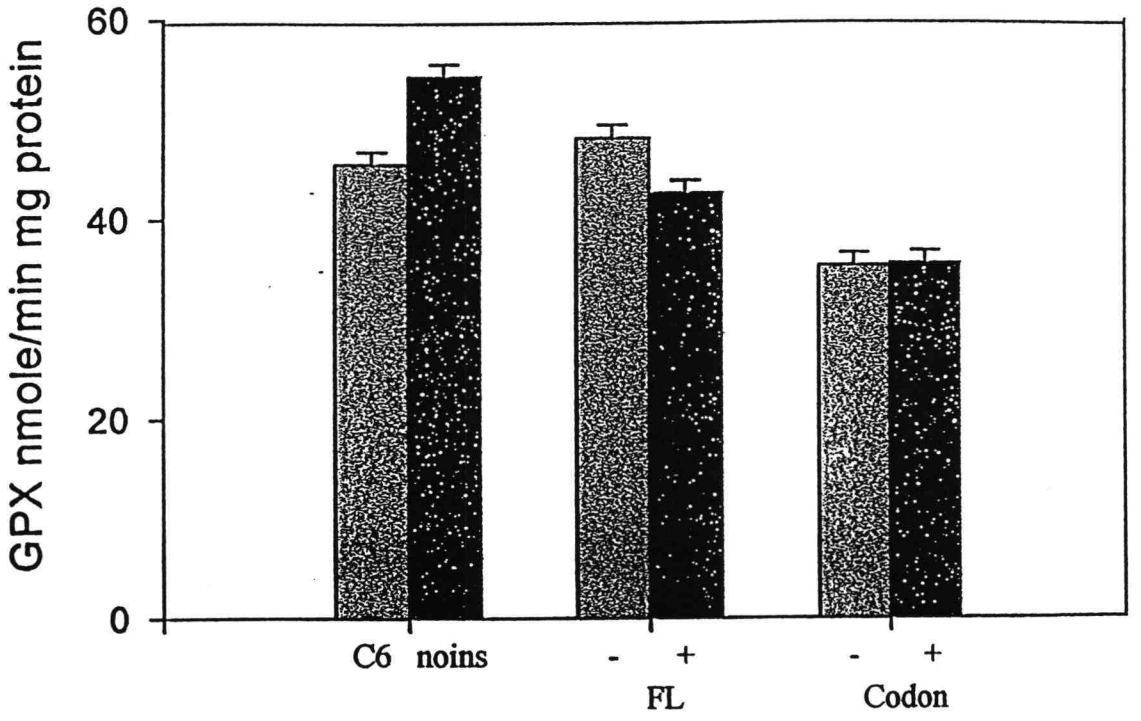



Figure 5-4. GPX activity in different cells. There is no significant difference in GPX activity between C6 and C6 clone transfected with vector without insert, or between full length cDNA transfectant with and without induction, or between antisense transfectant with and without induction ($P > 0.05$). +: IPTG induction. -: without IPTG induction. GPX activity is expressed as nmole NADPH oxidized per min per mg protein. The bars represent means of 3 samples ± SEM.

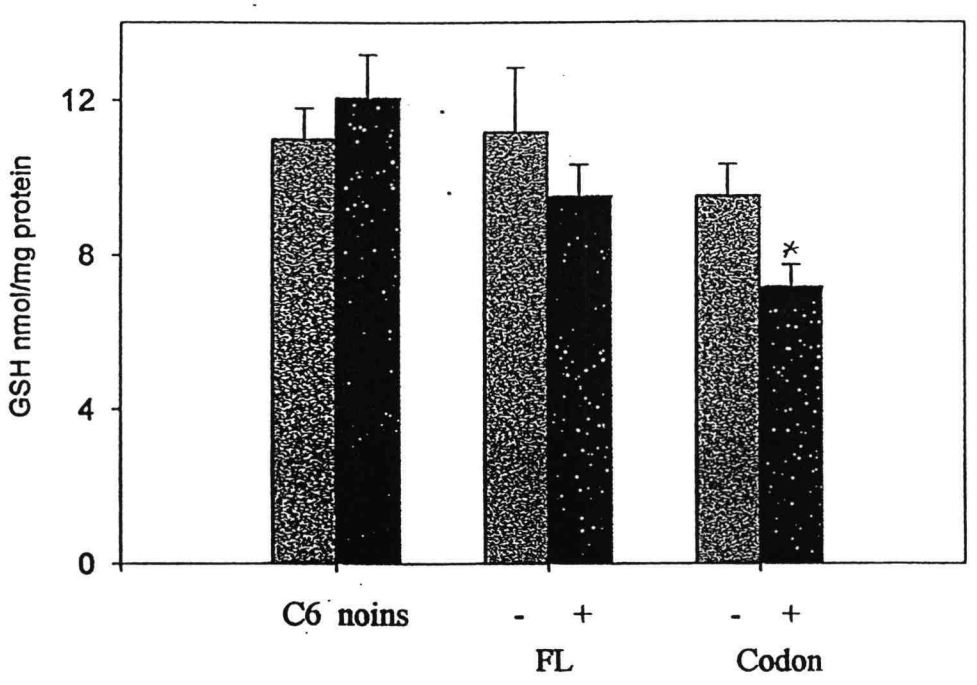
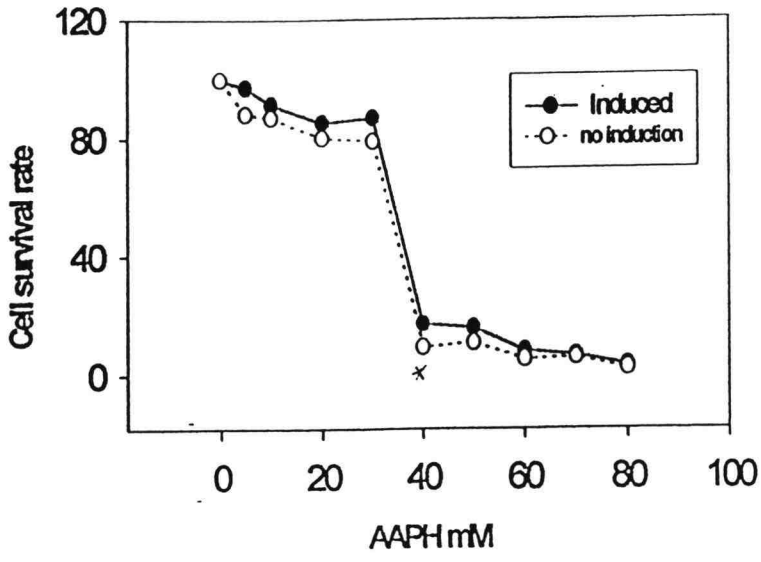



Figure 5-5. Glutathione content in different cells. There is no significant difference in GSH content between C6 and C6 clone transfected with vector without insert, or between full length cDNA transfectant with and without induction. But GSH in antisense transfectant after IPTG induction was significantly decreased compared to non-induced transfectant ($P < 0.05$).

A.




B.

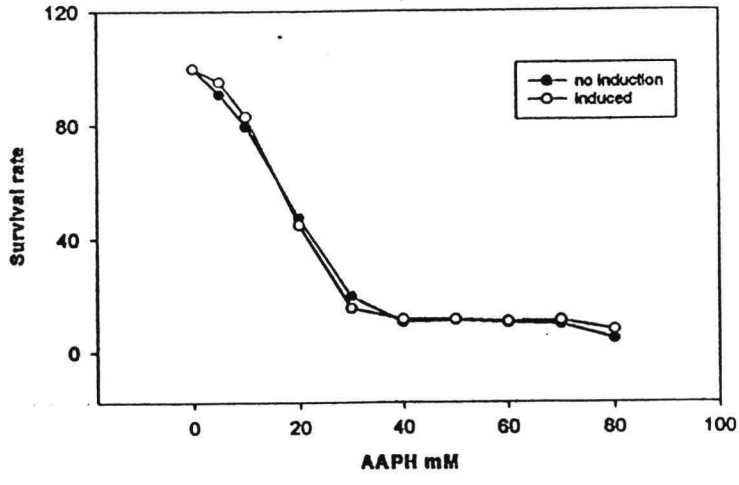



Figure 5-6. A. Cell survival rate in SeW overexpressed cells. Overall cell survival rate in SeW overexpressing cells was higher than control after oxidative stress. Cell survival rate in SeW overexpressing cells was significantly higher than control after 40 mM AAPH treatment for 16 hours. B. Cell survival rate in SeW underexpressed cells. There is no significant difference in cell survival rate between SeW underexpressed cells and control.

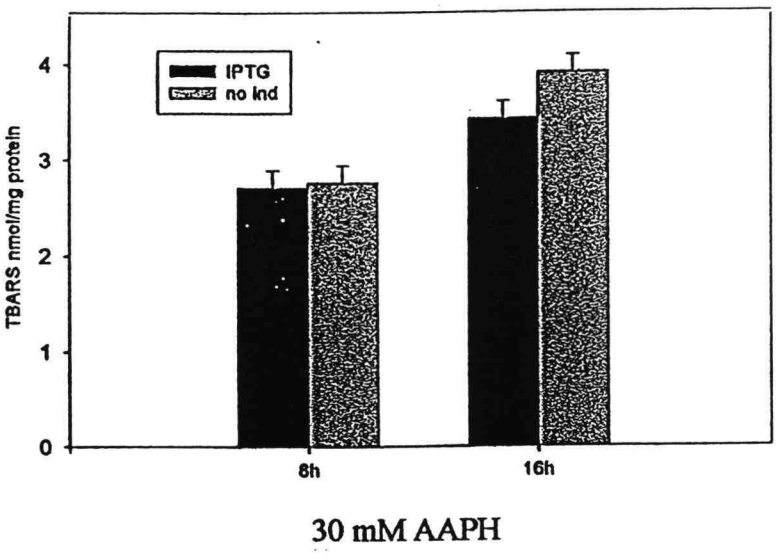



Figure 5-7. Thiobarbituric acid reaction substances in SeW overexpresed cells. There was no significant difference in TBARS between SeW overexpressed and control cells. However, TBARS was slightly decreased in SeW overexpressed cells after 16 hours with 30 mM AAPH treatment.

## DISCUSSION

The ability to reversibly turn genes off and on is a powerful tool in the investigation of various genetic functions. Statagene's LacSwith Inducible Mammalian Expression System consists of a eukaryotic Lac-repressor-expressing vector and a eukaryotic lac-operator containing vector. The genes of interest (SeW full length cDNA or cDNA coding region) were inserted by cloning into lac-operator containing vectors. These vectors were transfected into rat C6 glial cell line in which expression of the inserted gene was repressed until an inducer isopropyl β D thiogalactopyranoside (IPTG) was added to the media. Upon induction, expression of the inserted gene resumes.

Transfection of rat C6 glial cells with SeW expression vectors yielded several transfectant clones which contained 22-fold higher levels of SeW than did parent cells or control transfectants Northern and western blot analyses indicate that the excess SeW was due to increased synthesis of SeW derived from the transfected gene. mRNA increased 11-fold compared to cells transfected with vector only (figure 5-1) and SeW was 22-fold greater than that found in nontransfected cells or in control cells transfected with the vector only (figure 5-2B). Moreover, no significant difference were detected in either cellular glutathione peroxidase activity (figure 5-4) or glutathione content (figure 5-5). These results suggest that neither selenocysteine nor selenocysteyl-tRNA$^{[Ser]Sec}$ synthesis were limiting for SeW overproduction in C6 cells grown in the presence of $10^{-7}$ M selenium. As shown in figure 5-6A, SeW-overexpressed cells were resistant to the oxidative damage induced by a compound, AAPH, which promotes lipid peroxidation It is suggested that the decreased cell death due to lipid peroxides observed in this study resulted from overexpression of recombinant SeW.

Interestingly, no difference was found in the degree of protection from hydrogen peroxide damage after IPTG induction in SeW overexpressed C6 cells, despite a 22-fold enhancement of SeW expression (data not shown). Hydrogen peroxide may not be the optimum compound to use as an oxidant. Other researchers obtained similar findings where PHGPX overexpressed cells were not protected from oxidative stress caused by hydrogen peroxide, but protection was obtained from oxidative stress caused by AAPH (Imai et al., 1996). In contrast, Mirault et al. (1991) showed that GPX overexpressed cells were considerably more resistant than control cells to hydrogen peroxide damage, suggesting different GPXs may have various antioxidant activities.

Four SeW underexpressed transfectants were selected, one of which had the lowest SeW production. The mRNA of SeW in this transfectant is shown in figure 5-1, lane 10, and the western blot in figure 5-3. SeW was reduced to 20% of that in transfected cells without induction or 10% of parent cells. The insert for SeW underexpression vector is 267 bp. On northern blot two bands were seen, one is from cells and the other is from the underexpressed construct. There are several theories on how the antisense RNA work, but the most common one is that the antisense mRNA hybridize to the sense mRNA in which degradation then occurs. The northern blot of this antisense transfection does not support this theory, but SeW was reduced significantly after IPTG induction. Therefore, the mechanism of how the antisense mRNA works in gene underexpression needs to be further investigated.

Similar to overexpressed transfectants, SeW underexpression did not affect GPX activity in transfectants. However GSH was significantly reduced when SeW was underexpressed. Our laboratory reported that GSH is bound to SeW (Beilstein et al.,

1996), and thus it is reasonable to conclude that GSH is reduced when SeW is underexpressed. However, this does not provide any information on the reason GSH did not increase when SeW was overexpressed. It is possible that the species which does not contain GSH was induced.

In summary, two cell lines were produced by transfecting the rat C6 glial cell line with the full length cDNA for SeW in sense orientation or the coding region only in antisense orientation under the transcriptional regulation. After induction with IPTG, SeW increased 22-fold and SeW mRNA by 11-fold compared to control cells. The SeW overexpressed cell is more resistant than control cells to the cytotoxic effects of AAPH, but is not more resistant to hydrogen peroxide.

**ACKNOWLEDGMENT**

We thank Ms. Yvonne Will for her assistance in GSH assay.

# CHAPTER 6

# CONCLUSION

Evidence for a low molecular weight (10 KDa) selenium containing protein called selenoprotein W (SeW) was obtained about two decades ago. However, it was not purified until 1993 (Vendeland et al., 1993). The cDNA sequence of this protein confirmed that this is a selenoprotein. It contains one in-frame UGA codon which encodes a selenocysteine in each peptide (Vendeland et al., 1995). Since then, SeW cDNA has been sequenced in the rat, mouse, sheep, monkey and human. Selenoprotein W gene is highly conserved. The coding region of the nucleotide sequences and their predicted amino acid sequences are 80 and 83 % identical, respectively, among these five species (Gu et al., 1997; Whanger et al., 1997). This conserved feature of the protein suggests that it has important functions in mammalian. Normally, UGA encodes a stop codon which terminates protein translation, but in certain circumstances, this codon can encode a selenocysteine. Selenocysteine insertion sequence (SECIS) in the 3' untranslated region is required to ensure selenocysteine insertion (Berry et al., 1993; Martin et al., 1996). Therefore, experiments with overexpression of SeW using molecular recombinant techniques indicated that both 5' and 3' untranslated regions are required to ensure selenocysteine incorporation.

Antibody raised against rat synthesized peptide can recognize rat, mouse and sheep SeW by western blotting. Antibody raised against human mutant SeW (selenocysteine is substituted by cysteine) can recognize monkey and human tissue SeW by western blot. An

investigation of 28 tissues from rats indicated that SeW is more widely spread than once thought, but was undetectable in liver, thyroid, pancreas, pituitary and eyes regardless of the level of selenium fed. Dietary Se affected SeW in most of the remaining 23 tissues which were investigated (Sun et al., 1998). This indicates that SeW must have important functions in rats. SeW expression is regulated by dietary selenium levels in most of the tissues except the brain. SeW expression increases with increased selenium and it has the highest expression in skeletal muscles, followed by heart, brain, spleen and testis in selenium supplemented rats. Interestingly, SeW in brain is not affected by selenium depletion or repletion which suggested that the brain is unique in selenium metabolism and also suggested that SeW may be important in maintaining brain functions. It will be of interest to investigate the function of this protein in brain. Preliminary data showed that SeW is present in human tissues. Heart had the highest SeW expression, followed by muscle. SeW is also regulated by dietary selenium levels in human. Northen blot of SeW mRNA in human tissues showed that it is widely expressed and it is highest in heart (Gu et al., unpublished data). These results suggested that SeW also has important roles in humans.

Immunostaining of this protein using polyclonal antibody in rat and sheep muscle and brain samples confirmed that SeW is located in cytoplasm. Some of the slides showed that SeW in Purkenje cells tended to be higher than in other brain cells, but this result was not consistent. Therefore, studies on the distribution of SeW in different brain cell types would be a fruitful area to pursue.

Cell culture of rat brain (C6 glial and B104 neuroblastoma) and muscle (L8) cells showed that C6 glial cell has the highest SeW expression, followed by L8 muscle and

B104 neuroblastoma cells. SeW in these cells is regulated by selenium levels in culture medium, but it is regulated at different rates with selenium depletion or repletion. $^{75}Se$ uptake in the three cell lines indicated that they took up more $^{75}Se$ when Se was not added to the culture medium and addition of $10^{-6}$ M Se reduced the uptake of the isotope. Highest $^{75}Se$ uptake occurred in the nuclear fraction, followed by mitochondria, cytosol and microsome in decreasing order. SeW regulation in cultured cells fit the pattern of this protein in animals suggesting that cell culture is a good model for SeW studies.

Currently, the function of SeW is not known. However, previous studies suggested that this protein may have antioxidant functions. First, glutathione is bound to SeW (Beilstein et al., 1996). Secondly, all the other selenoenzymes are involved in redox reactions. Two approaches have been used to investigate the possible antioxidant function of this protein. One is overexpression of SeW in cultured cells and the other is the underexpression of SeW in C6 cells. SeW overexpressed cells showed higher survival rate after oxidative stress compared to control cells, but no difference was found in underexpressed cells compared to controls. SeW overexpression and underexpression in cultured cells is a useful tool to investigate the possible function of this protein. Furthermore, SeW overexpression and underexpression is controllable in these cell lines, which makes the research more practicable. Thus, functional research of SeW using cultured cells would appear to be a worthy goal to pursue in the future.

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# APPENDICES

# APPENDIX 1

## $^{75}$SELENIUM UPTAKE IN DIFFERENT CELL LINES

Y. SUN, P. D. WHANGER
Toxicology Program and Department of Agricultural Chemistry
Oregon State University, Corvallis, Oregon 97331 USA

Running title: $^{75}$Selenium uptake in cell fractions and autoradiography

Correspondence about this manuscript should be made with Dr. P. D. Whanger at the above address; by phone, (541)737-1803; by fax, (541)737-0497; by e-mail, whangerp@bcc.orst.edu

Published with the approval of Oregon State University Experiment Station as technical paper number _____. This research was supported by Public Health Service Research Grant number DK 38341 from the National Institute of Diabetes and Digestive and Kidney Diseases.

**ABSTRACT**

The distribution of radioactive selenium (Se) among cell nuclear, mitochondrial, cytosolic and microsomal was investigated using C6 glial, L8 muscle, B104 neuroblastoma rat cell lines and human U118MG glioblastoma and neuroblastoma IMR-32 cells grown with 0, $10^{-8}$M and $10^{-6}$M Se. Radioactive selenium ($^{75}$Se) as selenite was administered in culture medium at 1 μCi/10 ml medium. $^{75}$Se incorporation in protein was examined by autoradiography and the distribution of isotope in different cell compartments was measured with a Gamma-counter. There were four major selenoproteins identified in rat cells, with molecular weights of 58, 22, 18 and 14 kDa. Three major selenoproteins were found in human cells, with molecular weights of 58, 22 and 15 kDa. The highest $^{75}$Se uptake occurred in the nuclear fraction, followed in decreasing order by mitochondria, cytosol and microsome. Cells took up more $^{75}$Se when no Se was added to culture medium, whereas cells cultured with $10^{-6}$ M Se took up less of the isotope.

## INTRODUCTION

Selenium (Se) is an essential trace element in mammals. Nearly all the Se in animal tissues is associated with protein. Selenium has been reported to be incorporated as selenocysteine in proteins which are called selenoproteins. Several selenoproteins have been identified and characterized at the molecular level. Evidence for a low molecular weight selenoprotein (10 kDa) was obtained in the early 1970's (Pedersen et al., 1972). The absence of this protein has been implicated as the cause of a nutritional muscular dystrophy in selenium-deficient sheep. This protein, now called selenoprotein W (SeW), has been purified (Vendeland et al., 1993) and sequenced (Vendeland et al., 1995). Western blots of this protein in rat and sheep tissues showed that SeW has the highest expression in muscle, followed by heart, brain, spleen and testis when selenium is provided in the diet. Since SeW in brain is not affected by Se depletion and repletion, this tissue has a very unique pathway for Se metabolic mechanisms. Thus, it is of interest to understand selenium uptake and the pattern of selenoproteins in brain and muscle. The purpose of this study was to determine if SeW is one of the major selenoproteins in muscle and brain cells and whether there are other selenoproteins in these two tissues. Since a human muscle cell line was not available, human brain, rat brain and muscle cell lines were examined in this study.

## MATERIAL AND METHODS

### Materials

Rat C6 glial and L8 muscle, human U118MG glioblastoma and IMR-32 neuroblastoma cells were obtained from American Type Cell Collection (ATCC, Rockville, MD). Rat B104 neuroblastoma cells were donated by Dr. David Barnes, Department of Biochemistry and Biophysics, Oregon State University. Cell culture petri dishes were purchased from Corning (Corning, NY). $^{75}Se$ and $^{14}C$ labeled molecular weight markers, Hyperfilm-ECL and Hyperfilm-MP were purchased from Amersham (Arlington Heights, IL). Cell culture reagents were purchased from GIBCO BRL (Grand Island, NY). Western blotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA).

### Cell culture

Five cell lines were cultured in serum free medium. Serum free media for different cell lines are as follows; C6 rat glial and U-118MG human cells: 50 μg/ml transferrin, 10 nM hydrocortisone, 5 ng/ml fibroblast growth factor, 10 ng/ml biotin and 30 nM sodium selenite in Dulbecco's modified Eagle's media (DMEM); B104 rat neuroblastoma and IMR-32 human neuroblastoma cells: 5 μg/ml insulin, 5 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine and 30 nM sodium selenite in 50 % Ham's F-12 and 50 % DMEM; differentiated L8 rat muscle cells: 1 μg/ml linoleic acid conjugated with bovine serum albumin, $10^{-7}$ M insulin, $10^{-7}$ M dexamethasone, 300 mg/ml fetuin, 5 μg/ml transferrin and 25 nM sodium selenite in DMEM.

**Autoradiography**

Cells were cultured in serum free media without added Se for 2 days, 1 μCi $^{75}$Se/10 ml medium was added and incubated for 48 hrs. Cells were rinsed 3 times in ice cold phosphate-buffered saline (PBS) and harvested. Cells were sonicated in 0.4 ml homogenization buffer (20 mM Tris [pH 7.5], 0.25 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM 2-mercaptoethanol and 25 μg/ml leupeptin). The homogenate was centrifuged at 14,000 x g for 20 minutes at 4°C to obtain cytosolic extracts. Protein content was analyzed by the Lowry method (Lowry et al., 1951) with bovine serum albumin as standards. Cell extracts (100 μg cytosol protein) were electrophoresed on 7.5 % to 15 % gradient SDS-polyacrylamide gels as described by Laemmili (1970) and proteins were transferred onto nitrocellulose membranes (0.2 μm, BA-S83; Schleicher & Schuell, Keene, NH) overnight at 4°C according to the method of Towbin et al., (1979). The nitrocellulose membrane was exposed to Hyperfilm-MP for 2 weeks at -80°C. The film was developed by KODAK RPX-OMAT Processor, Model M6B (Eastman Kodak Co., Rochester, NY).

**$^{75}$Se uptake in different cell fractions**

Five different cells were cultured in different serum free media as indicated above with sodium selenite at 0, $10^{-8}$ M or $10^{-6}$ M for 2 days, then 1 μCi $^{75}$Se/10 ml medium added and cultured for 48 hours. Cells were rinsed 3 times in ice cold PBS and harvested. Three 100 mm dishes of cells were combined as one sample and each treatment had 3 samples. Cells were homogenized in 1 ml homogenization buffer (0.3 M sucrose, 20 mM Hepes, 0.5 mM EDTA, pH 7.2) at 400 rpm for 6 strokes. Cell homogenate was centrifuged

at 1000 x g at 4°C for 10 minutes. The resulting pellet is the nuclear fraction and the supernatant was centrifuged again at 15,000 x g at 4°C for 20 min . This second pellet is the mitochondrial fraction and the supernatant was centrifuged at 100,000 x g for 30 min at 4°C. The third pellet is the microsomal fraction and the supernatant is the cytosol. $^{75}Se$ in cell homogenate and different cell fractions was counted using a Beckman Gamma 5500 counter (Beckman Instruments, Inc. Fullerton, CA).

**RESULTS**

Autoradiography of selenium-containing proteins in five different cell lines showed that three rat cell lines (L8 muscle, C6 glial and B104 neuroblastoma) have similar selenoproteins, while two human cell lines (U118MG glioblastoma and IMR-32 neuroblastoma) have similar molecular selenoproteins (figure 1). Human and rat cells have different patterns in selenoprotein molecular weight. There are ten plus selenoprotein bands in each rat cell line, but the most abundant selenoproteins are at molecular weights at 58, 22, 18 and 14 kDa. There are also more than ten selenium-containing protein bands in human cells, but the most abundant selenoproteins are found to have molecular weights of 58, 22 and 15 kDa. Obviously, there is no major band around 10 kDa. Thus, SeW is not a major selenoprotein in these muscle and brain cells.

$^{75}Se$ in the five cell lines examined showed that the nuclear fraction has the highest selenium uptake, followed by mitochondrial, cytosolic and microsomal fractions in decreasing order. Figures 2, 3, 4, 5, and 6 show $^{75}Se$ uptake in rat L8 muscle, C6 glial, B104 neuroblastoma, human U118MG gliablastoma, and IMR neuroblastoma cells, respectively. These figures indicate that all the cell lines tested had similar patterns in Se

uptake. The addition of $10^{-8}$ M Se had very little effect on the uptake of $^{75}Se$, but interestingly, it resulted in slight increases in the nuclear fraction of L8 muscle and neuroblastoma cells. However, when cells were cultured in $10^{-6}$ M sodium selenite, very little uptake of the isotope occured.

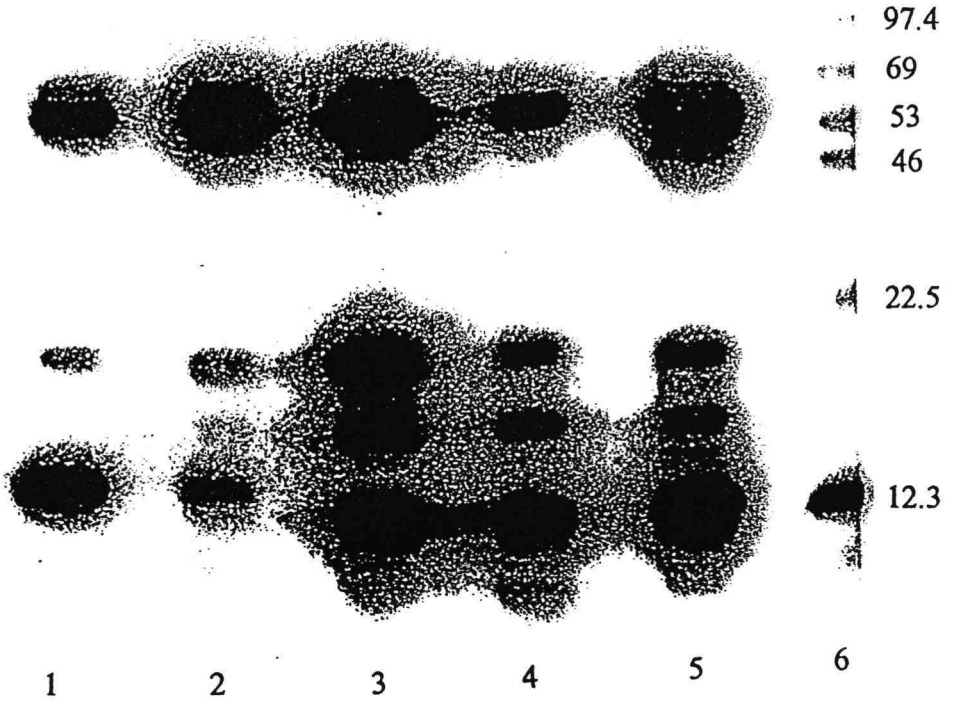


Figure 1. Autoradiography of selenoproteins in five cell lines. Lane 1: human IMR neuroblastoma cells. Lane 2: human U118MG glialblastoma cells. Lane 3: rat B104 neuroblastoma cells. Lane 4: rat C6 glial cells. Lane 5: rat L8 muscle cells. Lane 6: $^{14}C$ labeled protein molecular weight marker.

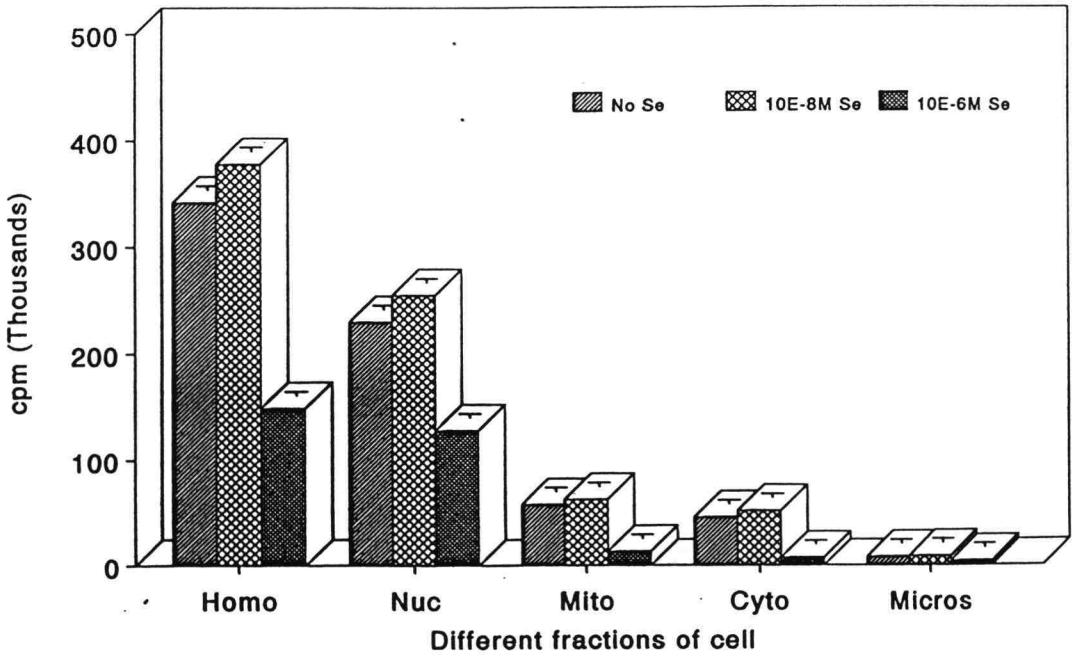


Figure 2. $^{75}Se$ uptake in different fraction of rat L8 muscle cells.

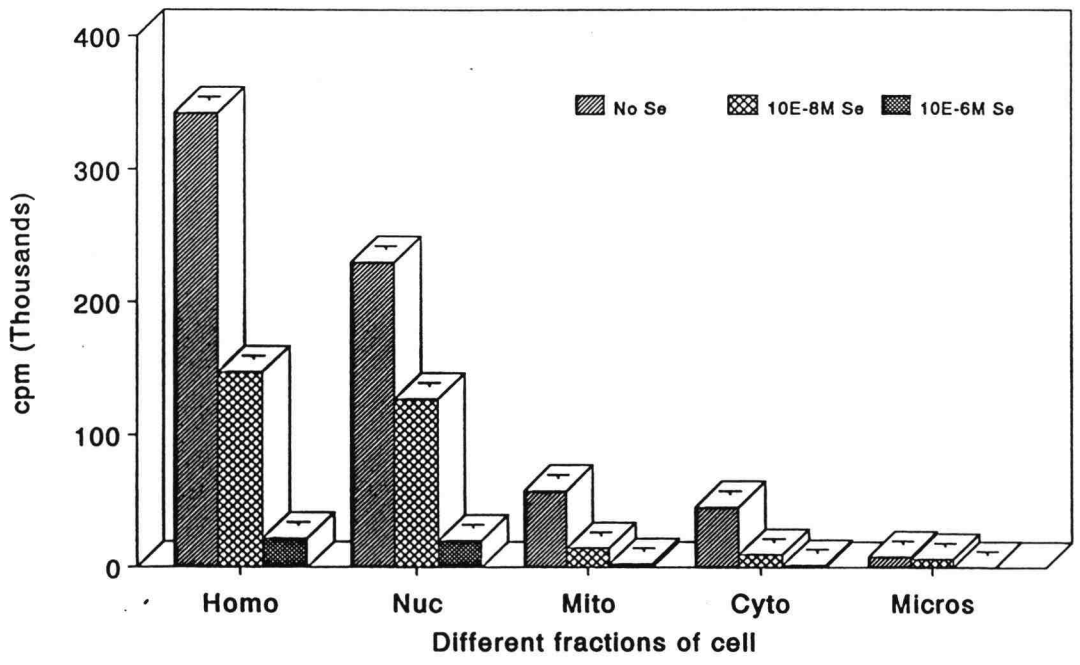


Figure 3. $^{75}$Se uptake in different fraction of rat C6 glial cells.

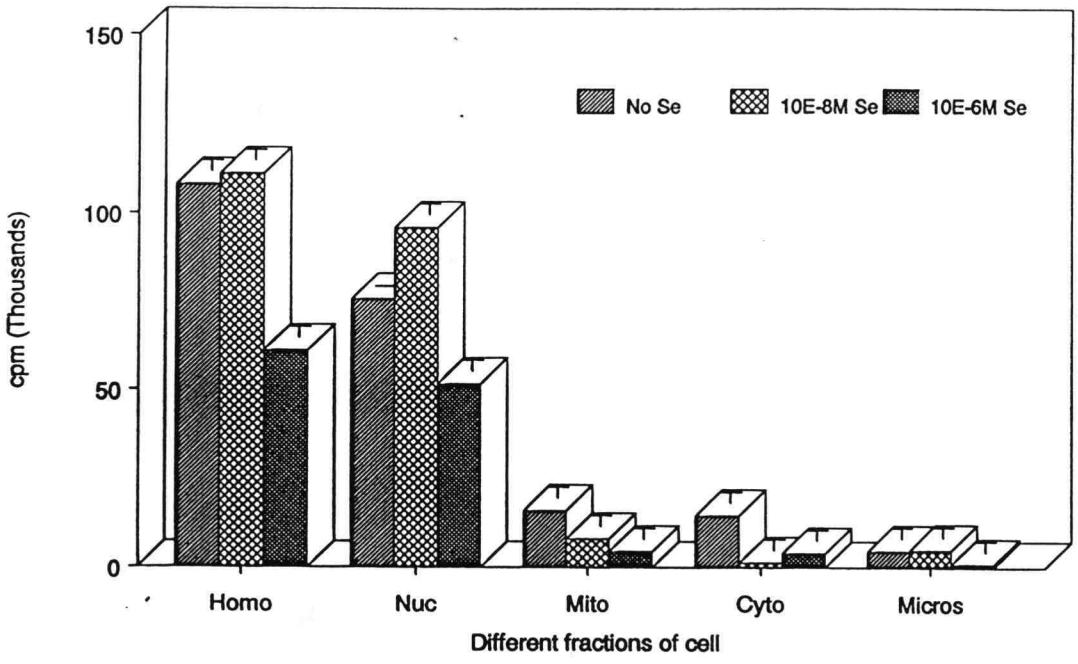


Figure 4. $^{75}Se$ uptake in different fraction of rat B104 neuroblastoma cells.

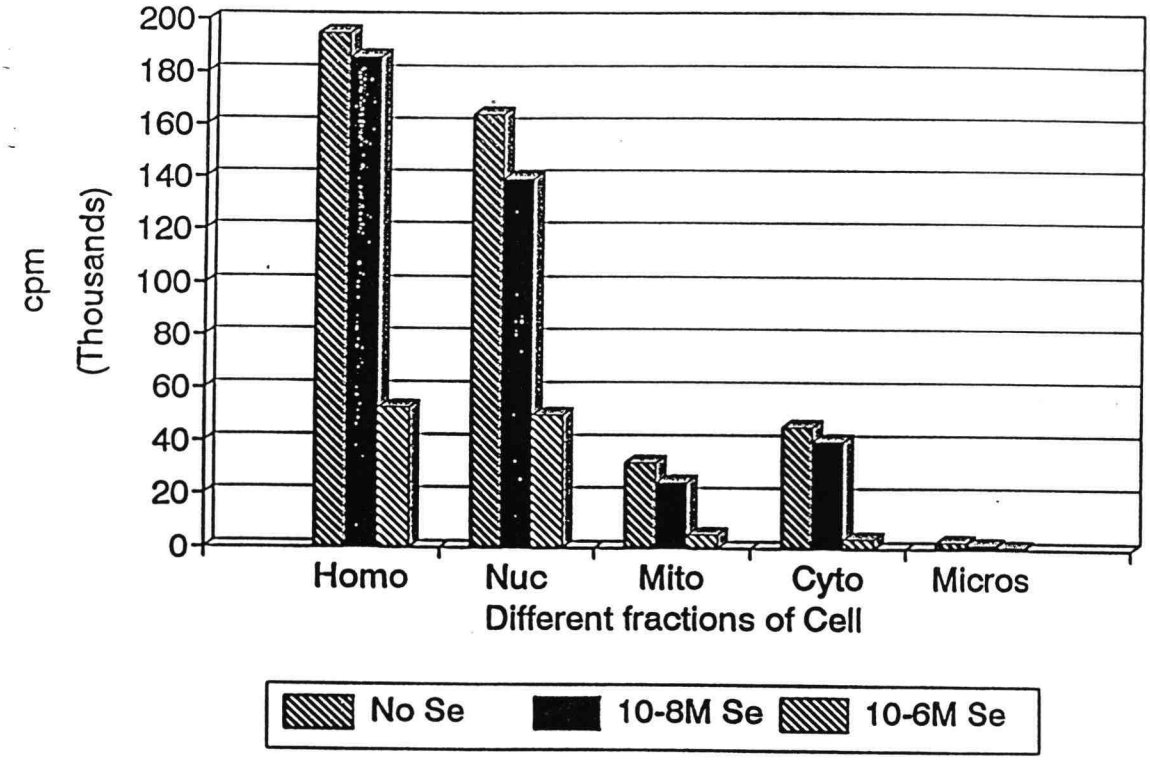


Figure 5. $^{75}Se$ uptake in different fraction of human U118MG glialblastoma cells.

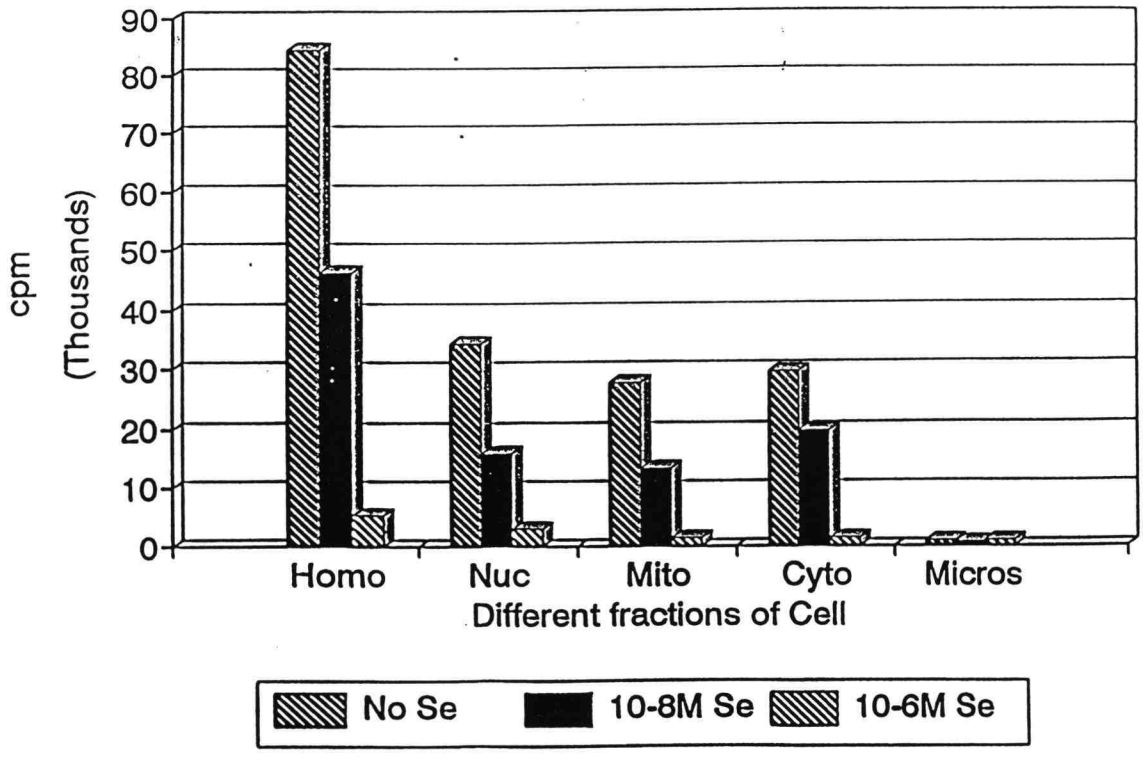


Figure 6. $^{75}Se$ uptake in different fraction of human IMR neuroblastoma cells.

## DISCUSSION

Autoradiography of selenium-containing protein abundance and tissue distribution has been widely used in vivo (Wayen et al., 1985; Davidson et al., 1993; Behne et al., 1996) and in vitro (Danielson and Medina, 1986). Selenium-containing proteins in muscle and brain cells were examined by autoradiography in this study. In rat L8 muscle, C6 glial and B104 neuroblastoma cells, ten plus selenium-containing proteins were found, but the major molecular weights are 58, 20, 18 and 14 kDa (figure 1). The pattern of selenium-containing protein in the three rat cell lines is very similar, especially for those major selenoproteins. The four selenium-containing protein found in this study is in agreement with the findings in mouse mammary epithelial cells (Danielson and Medina, 1986). The pattern of selenium-containing protein in human glialblastoma and neuroblastoma cells is very similar, but it is different from that of rat cells. The major selenium-containing proteins in human brain cells are molecular weights at 58, 20 and 16 kDa (figure 1). The 20 kDa protein in the three cell lines could correspond to glutathione peroxidase subunits. There are some weakly labeled selenium-containing protein bands, which suggests that they are present in only very low concentrations. Different pattern of selenium-containing proteins in human and rat cells provided evidence that there are species differences in selenoprotein. A 10 kDa selenium-containing protein was found in all three rat cell lines, but the signal of this protein is weak. Therefore, this selenium-containing protein is not a major selenoprotein in rat muscle and brain cells. A 10 kDa selenoprotein was not detected in human glialblastoma and neuroblastoma cells by autoradiography, even though this protein was detectable by western blotting.

$^{75}$Se uptake in different cells showed that it depended on the Se concentration in culture media. $^{75}$Se uptake was greater when the Se content in culture medium was low. This result is consistent with a previous report in sheep (Davidson and Kennedy, 1993). The distribution of $^{75}$Se in cell fractions indicated that the highest $^{75}$Se uptake occurred in the nuclear fraction, followed in decreasing order by mitochondria, cytosol and microsome.

# APPENDIX 2

## IMMUNOCYTOCHEMISTRY OF SELENOPROTEIN W IN MUSCLE, HEART AND BRAIN OF RAT AND SHEEP

Sun, Y, Fisher, K.*, Hedstrom, O. R.* and P. D. Whanger
Departments of Agricultural Chemistry and Veterinary Medicine*
Oregon State University, Corvallis, OR USA 97331

Western blots of rat and sheep tissues showed that selenoprotein W (SeW) is highest in muscle, heart and brain in Se adequate animals. When the diet is deficient in Se, SeW is undetectable in muscle and heart, but SeW levels in brain remain unchanged. Since the function of this selenoprotein is currently not clear, it is of interest to know the distribution of SeW in different tissue cells. The location of the SeW in cells may give a clue on its possible function.

Sheep and rats were anaesthetized with sodium pentobarbital. Muscle, heart and brain were removed and rinsed with phosphate-buffered saline (PBS). Seven millimeter sections of tissues were fixed in 10% neutral buffered formalin at 4°C for several days then changed to 20% sucrose. These specimens were processed on the tissue processor and embedded in paraffin which were cut and mounted on slides. Before immunostaining, slides were dewaxed and rehydrated. Endogenous horseradish peroxidase was removed by dipping the slides in 2% hydrogen peroxide in methanol for 15 min at room temperature (RT), then slides were blotted on an organic blotting pad, dipped in 70% ethanol and blotted on an aqueous blotting pad. Slides were then ready for blocking non-specific bindings. Slides were blocked with normal goat serum for 30 min at RT, then incubated

with straight rabbit antiSeW polyclonal antibody for 1 hour at RT. They were subsequently washed for 10 min in PBS. Next slides were incubated with 1:200 goat anti-rabbit IgG conjugated with biotin (Vector Laboratories, Inc. Burlingame, CA) for 1 hour at RT, and washed for 10 min in PBS. Slides were Incubated in 1:100 vector A and 1:100 vector B (Vectastain ABC Kit, Vector, Burlingame, CA) in PBS for 1 hour at RT, washed 10 min in PBS and incubated with 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co. St. Louis, MO) as the chromogen for 5 min. Color reaction was stopped by washing slides in tap water, and counterstain before cleaning and mounting.

Immunostaining showed that SeW is located in cytoplasm. In white muscle disease lamb muscle and heart, SeW is undetectable, but this protein was still present in the brain. It also showed that SeW in Purkenje cells tended to be higher than in other brain cells, but this result was not consistent. SeW in rat muscle, heart and brain showed the same pattern as in sheep tissues.

This study showed that SeW is evenly distributed in cytoplasm. However, efforts were unsuccessful to locate the protein to a particular organelle. SeW was detectable by immunostaining in both cortex and cerebellum.