AN ABSTRACT OF THE THESIS OF

Jan-Ying Yeh for the degree of Doctor of Philosophy in Animal Science presented on November 20, 1995.

Title: The Influence of Selenium on Selenoprotein W

Abstract approved: Redacted for Privacy
Philip D. Whanger

The objective of our study was to investigate the effect of selenium on selenoprotein W. Western blot analysis indicated that selenoprotein W is present in muscle, brain, testis and spleen of rat tissues. Tissue distribution of selenoprotein W was not altered in rats fed various selenium levels. Among muscle, brain, testis and spleen, selenoprotein W in muscle was most responsive to selenium status, but brain appeared to be the least responsive organ. Northern blot data indicated that selenoprotein W mRNA in rat muscle increased significantly as levels of selenium supplementation increased. Western blot data indicate that selenoprotein W in rat muscle is non-detectable until selenium supplementation increased to 0.06 ppm, and the level of selenoprotein W in muscle reached a plateau at 1 ppm selenium supplementation where no further increase occurred. Except brain, selenoprotein W was higher in all tissues in sheep fed a selenium supplemented diet than in those fed the deficient diet. However, selenoprotein W levels were not different in brains between selenium-deficient and selenium-supplemented sheep. In contrast to rats, selenoprotein W was as high in the heart as in the muscle of selenium-supplemented

sheep.

In the study with L8 muscle cells, selenoprotein W did not change significantly during cell differentiation, indicating that selenoprotein W was not affected by muscle cell differentiation. Selenite was the most effective form of selenium for selenoprotein W synthesis in these muscle cells. Selenoprotein W level reached a plateau when L8 myotubes were incubated with 10^{-7} M selenium, whereas selenoprotein W mRNA reached a plateau with 10^{-8} M selenium.

The Influence of Selenium on Selenoprotein W

by

Jan-Ying Yeh

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed November 20, 1995

Commencement June 1996

Doctor of Philosophy thesis of Jan-Ying Yeh presented on November 20, 1995

APPROVED:

Redacted for Privacy

Major Professor, representing Animal Science

Redacted for Privacy

Chairman of Department of Animal Sciences

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of thesis to any reader upon request.

Redacted for Privacy

Jan-Ying Yeh, Author

ACKNOWLEDGEMENTS

I would like to thank Dr. Philip D. Whanger, my mentor, for his guidance, encouragement, consideration and financial support. I thank Dr. Neil E. Forsberg for his friendship and for providing me free access to his lab. Thanks go to lab collegues, Ms. Judy A. Butler for her tender loving care and assistance in everything, to Dr. Susan C. Vendeland for her assistance in tissue assays, to Mr. Michael A. Beilstein for his assistance in producing antibodies, to Ms. Azizah Mohd for her assistance in experiments, and to Ms. Dawn L. Whanger for her friendship. Also, I would like to thank fellow graduate students, Qui-ping Gu, Calvin Nunn and Yu Sun, for their friendships and their assistance in the experiments and in slide preparations.

Special thanks go to the secretaries, Ms. Deloras Martin and Ms. Helen Chesbrough, in Department of Animal Sciences for their assistance during my study and their loving care, to the accountants in Department of Agricultural Chemistry, Ms. Noreen Rogers and Ms. Sheila Paxton for their assistance and friendships, to Dr. Janet S. Andrews for her friendship and her valuable assistance in generation of antibodies and to Ms. Julia Willis for her friendship and babysitting my two children.

In addition, I would like to thank my parents, Shian-juh Yeh and Choying Chen, for their total supports, especially my mother. She came to Corvallis to help taking care of my two children during the final stages of my study. Finally, a deep appreciation goes to my husband, Bor-rung Ou, and our children, Tingwei and Tingyoung, for their loves and sacrifice. Without their support, this degree would be impossible.

CONTRIBUTION OF AUTHORS

Dr. Philip D. Whanger was involved in designing experiments, interpretation of data and writing manuscripts. Cell culture was performed in Dr. Neil E. Forsberg's lab. Dr. Bor-Rung Ou assisted in Northern blot analysis. Ms. Judy A. Butler assisted in selenium analysis. Dr. Janet S. Andrews assisted in generation of antibodies and immunological experiments. Dr. Susan C. Vendeland, Ms. Azizah Mohd, Ms. Qui-ping Gu and Mr. Michael A. Beilstein assisted in the dissection of animals and experiments.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

THE INFLUENCE OF SELENIUM ON SELENOPROTEIN W

CHAPTER 1

INTRODUCTION

Until 1957, the only physiological significance of selenium was thought to be its toxicity. When selenium was shown to prevent dietary liver necrosis in rats (Schwarz and Foltz, 1957), this resulted in a reassessment of this element. Interestingly, that same year the properties of a newly discovered enzyme, glutathione peroxidase (GSH-Px) were reported (Mills, 1957). He found that this enzyme would protect hemoglobin against oxidative breakdown. However, it took a decade and a half to discover that GSH-Px had a relationship to selenium, namely that it is a selenoenzyme (Rotruck et al., 1973). This selenoenzyme, which was later termed cellular GSH-Px (cGSH-Px), was the first of a family of selenoenzymes to be discovered. It was more than 10 years later when it was realized that the plasma GSH-Px was a different gene product (Takahashi et al, 1987). About the same time, the third member of this family, phospholipid hydroperoxidase GSH-Px, was discovered (Ursini et al, 1985). The latest one of this family, GI-tract specific GSH-Px, was discovered about two years ago. It was found predominantly in the intestinal mucosa (Chu et al, 1993). The functions of these GSH-Pxs are to convert the damaging peroxides (organic and inorganic) to the less harmful alcohols.

The sole relationship of selenium to GSH-Pxs was broken down when evidence was presented that this element was required for the activity of type I iodothyronine

deiodinase (Arthur et al, 1990a). This was confirmed to be a selenoenzyme the following year (Berry et al, 1991). This provided a link between iodine and selenium metabolism. Recently, type III iodothyronine deiodinase was also recognized to be a selenoenzyme (Croteau et al., 1995).

In the early research with selenium, it was realized that there was a metabolic relationship of this element to vitamin E (Hoekstra, 1975). There was considerable debate on the essentiality of selenium in the 1960s because vitamin E appeared to fulfill most of the functions of selenium. However, with the demonstration of selenium as a requirement for the activity of enzymes, this left no doubt of its essentiality. Selenium deficiency results in white muscle disease in lambs and calves, liver necrosis in rats, hepatosis dietetica (also called mulberry disease) in pigs, exudative diathesis in chicks and myopathy of the gizzard in turkey poults (Ullrey et al, 1983). The selenium requirements for animals are about 0.1 mg per kg diet. However, some animals such as turkeys appear to have a higher requirement and 0.3 mg selenium per kg diet are recommended.

Selenium is also important in human health. A severe nutritional deficiency in discrete regions of China was found to be associated with an endemic juvenile cardiomyopathy disorder called Keshan disease (Chen et al, 1980). This problem was in villages where the residents grew all of their food. The soil in these areas is deficient in selenium and thus when vegetables are grown in these areas they do not contain enough selenium to protect against deficiency. In the United States it was shown that muscle weakness could be prevented by selenium supplementation in patients on long-term parenteral nutrition (Brown et al, 1986). Therefore, selenium is definitely essential for optimum human health.

Even though selenium was once thought to be a carcinogenic element, it is now recognized to have anticarcinogenic properties (Whanger, 1992). Interest grew on this relationship when epidemiological studies suggested an inverse relationship between the incidence of certain cancers in humans and the selenium content of soil in these areas (Combs and Combs, 1986). Subsequently, work with laboratory animals indicated that selenium would counteract to various degrees the carcinogenic effects of a number of chemicals. Now, there is intense interest in the relationship of selenium to cancers in humans, and the preliminary results look encouraging. After supplementation with selenium and vitamins A & E in Chinese subjects for 5 years, there has been a significant reduction in the incidence of stomach and throat cancer (Blot et al, 1993). There is currently a study in the United States where the influence of selenium on colon cancer is under investigation (Clark, University of Arizona). Although, none of the results have been published, preliminary information indicate a positive effect of selenium on this disorder in humans. Therefore, selenium is a very unique element in that it has come full circle in two aspects--from a toxic element to an essential one and secondly from a carcinogen to an anticarcinogen.

Another interesting characteristic of selenium is that it can counteract the toxicities of some heavy metals (Whanger, 1985; 1992). This rather unusual feature of an element such as selenium which is highly toxic itself at elevated levels counteracting the toxicity of heavy metals stimulated much interest in the relationship of this element to other elements. Selenium appears to be very effective in counteracting the toxicities of mercury, thallium and cadmium, but less effective against silver and lead.

Like any other essential element, selenium can be very toxic at elevated levels. The toxic nature of selenium compounds remained mostly a laboratory curiosity until the 1930s when it was discovered that selenium was the active principle in forages and grains that caused alkali disease in livestock raised in certain areas of the American great plains (Ullrey et al, 1983). Three distinct forms of selenium poisoning have been described in livestock. Acute poisoning is caused by the ingestion of a large quantity of highly seleniferous accumulator plants in a short period of time. The most common characteristic sign of acute selenosis is a "garlic breath" before sudden death. Chronic selenosis, of the blind staggers type, occurs in animals that consume a limited amount of selenium accumulator plants over a period of several weeks. The affected animals have impaired vision, wander, stumble and finally succumb to respiratory failure. Third, chronic selenosis, of the alkali-disease type, is the result of animals consuming high selenium feed over a period of many months. Signs of this disorder include liver necrosis, lameness, hoof malformations, loss of hair, and emaciation. Although they have been studied extensively there have not been any health problems identified with selenium in those individuals living in high selenium areas of the world, except for some residents living in certain areas of Enshi county, Hubei Province, China. The soil in this area of China is naturally high in this element and the consumption of food in these areas contain enough selenium to cause toxicity problems. The signs of selenosis in humans include hair loss, abnormal finger and toe nails, loss of nails and skin lesions (Yang and Zhou, 1994).

SELENOPROTEINS

Under physiological conditions, selenium can be incorporated into several proteins either specifically or non-specifically. A group of selenium-containing proteins has received much attention recently. This group of proteins, called selenoproteins, contain selenium in the form of selenocysteine in their active sites and thus is selenium-dependent. Selenium was first found in protein A component of glycine reductase complex of Clostridium sticklandii (Turner and Stadtman, 1973). Subsequently, several selenoproteins were identified in both prokaryotes and eukaryotes, including formate dehydrogenases (Jones and Stadtman, 1981; Zinoni et al., 1986) and hydrogenase (Yamazaki, 1982), the glutathione peroxidase family (Rotruck et al., 1973; Takahashi and Cohen, 1986; Schuckeil et al., 1991; Chu et al., 1993), type I iodothyronine 5'-deiodinase (Behne et al., 1990), type III iodothyronine 5-deiodinase (Croteau et al., 1995), selenoprotein P (Yang et al., 1987), selenoprotein W (Vendeland et al., 1993) and mitochondrial capsule selenoprotein (Karimpour et al., 1992). Although several selenoenzymes have been characterized, there are also some mammalian selenoproteins without a known function that have been studied. The three most studied ones are selenoprotein P, mitochondrial capsule selenoprotein, and selenoprotein W, the one studied in the Oregon laboratory.

Prokaryotic Selenoproteins

Selenoprotein A

Selenoprotein A in glycine reductase of Clostridium is a small, acidic, heat-stable glycoprotein which contains selenium in the form of a selenocysteine (Cone et al., 1976; Turner and Stadtman, 1973). This selenoprotein had been cloned, sequenced and expressed in bacteria. The gene encoding selenoprotein A contains an in-frame TGA codon which codes for selenocysteine. The oxidized form of this protein, which is a dimer, can be converted to biologically active form (reduced form) by treatment with dithiothreitol or borohybride. This selenoprotein may serve as the immediate reductant in the reduction of glycine to acetate and ammonia (Garcia and Stadtman, 1992; Stadtman, 1990).

Formate dehydrogenases

Formate dehydrogenases are present in various methane-producing bacteria that utilize formate as a sole fermentable substrate. The approximate molecular weight of selenocysteine-containing subunit in formate dehydrogenase from Methanococcus vannielii is 100 KD. Formate dehydrogenase is extremely sensitive to the molecular oxygen. It is involved in gas formation and delivery the electrons from formate to nitrate reductase (Jones and Stadtman, 1981).

Hydrogenases

A hydrogenase isolated from Methanococcus vannielii was the first one found that contains selenium as selenocysteine (Yamazaki, 1982). It is a 340 KD selenoenzyme that contains 3.8 gram atoms of selenium as selenocysteine residues in four β-subunits and is involved in transfer of molecular hydrogen. Some of the hydrogenases found in different bacteria also contain selenium in their amino acid sequences. However, the forms of selenium in these hydrogenases have not been identified yet.

Other prokaryotic selenoproteins

Nicotinic acid hydroxylase, xanthine dehydrogenase and thiolase are the other selenium-containing enzymes found in bacteria, but the form of selenium in these selenoenzymes is not selenocysteine (Axley and Stadtman, 1989).

Eukaryotic Selenoproteins

Glutathione peroxidase family. Glutathione peroxidase family includes at least four different peroxidases --- cellular glutathione peroxidase, plasma glutathione peroxidase, phospholipid hydroperoxidase glutathione peroxidase and GI-tract specific glutathione peroxidase.

Cellular glutathione peroxidase (cGSH-Px)

Cellular glutathione peroxidase (cGSH-Px) was the first mammalian selenoprotein found in animals (Rotruck et al., 1973). cGSH-Px is present in most tissues, espectially

high in liver and erythrocyte, but its tissue distribution varies from species to species. It is a tetramer composed of four identical 22-KD subunits and each subunit contains one selenocysteine. The function of cGSH-Px is to catalyze the reduction of hydrogen peroxide, organic hydroperoxide and fatty acid hydroperoxides using glutathione as a cofactor. It can protect animals from hemolysis.

Plasma glutathione peroxidase (eGSH-Px)

Plasma glutathione peroxidase (eGSH-Px) was purified from human plasma (Takahashi and Cohen, 1986). It is a glycoprotein and a tetramer composed of four identical 23-KD subunits and each subunit contains one selenocysteine. eGSH-Px has been found in human and rodent plasma, but its mRNA is not expressed in rodent liver. The exact function of eGSH-Px is still unclear. It may also metabolize hydrogen peroxide and fatty acid hydroperoxides as cGSH-Px does. Since the concentration of glutathione in extracellular fluid is very low, eGSH-Px may have functions other than a glutathione peroxidase.

Phospholipid hydroperoxidase glutathione peroxidase (phGSH-Px)

Phospholipid hydroperoxide glutathione peroxidase (phGSH-Px) was purified from porcine heart and liver (Schuckelt et al., 1991). It is a monomer with 20-KD molecular weight and contains one selenocysteine. phGSH-Px exists in several tissues but is highest in testis. The function of this GSH-Px is to reduce linoleic acid hydroperoxide and fatty acid hydroperoxides esterified to phospholipids, and therefore protects animals against lipid peroxidation.

GI-tract specific glutathione peroxidase (GSH-Px-GI)

The newest member of GSH-Px family is GI tract-specific glutathione peroxidase (GSH-Px-GI) which appears to be the major glutathione-dependent peroxidase in rodent GI tract (Chu et al., 1993). It is a cytosolic protein and a tetramer composed of four 22-KD subunits. Its mRNA also exists in human liver and colon, but only exists in rodent GI tract. The functions of GSH-Px-GI is assumed to be the same as those of cGSH-Px which catalyzes the reduction of hydrogen peroxide, linoleic acid hydroperoxide, tert-bytyl hydroperoxide and cumene hydroperoxide. This enzyme appears to protect animals from toxicity of ingested lipid hydroperoxides.

Type I iodothyronine 5'-deiodinase (5'-DI)

Type I iodothyronine 5'-deiodinase (5'-DI) is present mainly in the endoplasmic reticulum of liver and kidney (Behne et al., 1990). It is a homodimer composed of two 27-KD subunits and each subunit contains one selenocysteine which is required for maximal enzyme activity. The function of 5'-DI is to convert thyroxine (T_4) to the biologically active triiodothyronine (T_3) and to regulate thyroid hormones. Selenium deficiency results in the increase of plasma T_4 levels, due to the decrease of 5'-deiodinase activity. Since plasma T_3 only decreased slightly in selenium deficiency, selenium-deficient animals remained euthyroid (Beckett et al., 1987).

Type III iodothyronine 5-deiodinase

This enzyme was recently found to contain selenocysteine in its amino acid sequence. It acts as a 5-deiodinase to metabolize the active thyroid hormine, thyroxine

and 3, 5, 3'-triiodothyronine, to inactive metabolites, 3, 3', 5'-triiodothyronine (reverse T_3) and 3, 3'-diiodothyronine (T_2). Recently, a cDNA was found to encode this enzyme which contains an in-frame TGA codon that coded for selenocysteine at the active site (Croteau et al., 1995). This selenoenzyme is highly expressed in mammalian placenta and several fetal tissues. In addition, it is maintained at normal levels in brain, placenta and skin of selenium-deficient rats. The type III iodothyronine deiodinase plays a protective role in preventing over-exposure of tissues to excessive levels of active thyroid hormones, thus it is critical to thyroid hormone homeostasis in thyroid gland (St. Germain, 1994).

Selenoprotein P (Se-P)

Selenoprotein P (Se-P) is secreted by liver into plasma and turns over rapidly (Yang et al., 1987). In rats and humans, Se-P contains about 65% of plasma selenium. Se-P is a glycoprotein with 57-KD molecular weight and it contains multiple selenocysteine residues. mRNA of Se-P was detected in several tissues, suggesting that it might be synthesized in other tissues as well, but this protein was only found in plasma. The function of Se-P is unknown. Since a Se-P receptor was found in rats, Se-P was suspected to be a transport protein (Gomez and Tappel, 1989). However, due to the fast turnover rate, it is unlikely that Se-P serves as a transport protein. Cysteine and selenocysteine, which are the amino acids expected to have redox roles, are conserved to a very high degree from rat Se-P to human Se-P. Thus this selenoprotein may have an electron transfer function and also may function as a free radical scavenger (Hill et al., 1993). Selenoprotein P is the most studied selenoprotein without a known function and selenium status has been shown to affect its concentrations (Yang et al, 1989).

Selenoprotein W (Se-W)

Evidence for a new selenoprotein --- Selenoprotein W (Se-W) was found about twenty years ago, but had not been purified until recently (Vendeland et al., 1993). Se-W is a 10-KD cytosolic protein which contains 1 g atom Se/mol as selenocysteine at position 12 in the amino acid sequences. Glutamate, glycine, lysine, leucine and valine are the major amino acids in this protein. Tissue distribution of Se-W in rat indicates that it is present in muscle, brain, testis and spleen. This new selenoprotein appears to be selenium-dependent and selenium status of animals will affect its concentration in tissues (Vendeland et al, 1995). However, its characteristics and function remain unknown.

Mitochondrial capsule selenoprotein

Mitochondrial capsule selenoprotein (MCS) was found in the mid-piece region of mouse sperm in association with the mitochondrial helix (Calvin et al., 1981). It is a cysteine-rich protein and contains 3 selenocysteine per subunit. The cDNA encoding mouse MCS contains 3 in-frame UGA codons and the predicted molecular weight is 21.1 KD (Karimpour et al, 1992). However, no information is available on its function.

Other eukaryotic selenoproteins

A 14-KD fatty acid binding protein from mouse liver was found to be a selenium-binding protein, but the form of selenium in this protein remains unknown (Bansal et al., 1989). There are evidences presented for other eukaryotic selenoproteins. However, no information is available on their characteristics (Behne et al., 1995; Hawkes et al., 1985; Whanger, 1987).

CHARACTERISTICS OF SELENOPROTEINS

Form of Selenium in Selenoproteins

It has been reported that 92% of muscle selenium is protein-bound (Hawkes et al., 1985). Several forms of selenium have been found in protein. They are selenocysteine, selenomethionine, selenotrisulfides and selenium-heavy metal complexes (Burk and Hill, 1993). Selenocysteine-containing proteins are selenium-dependent and selenocysteine is specifically incorporated into proteins. Replacement of selenocysteine by cysteine or sulfur results in decrease or complete abolishment of enzymatic activity. Evidence suggests that animals can not distinguish between selenomethionine and methionine, therefore, selenomethionine can be incorporated into protein in place of methionine during protein synthesis. Incorporation of selenomethionine is non-specific and replacement of selenomethionine by methionine does not appear to affect protein activity. Selenotrisulfides are mostly an in-vitro phenomenon and represent non-specific incorporation of selenium, and thus selenotrisulfide-containing proteins are unlikely to be of physiological importance. Selenium can form complexes with heavy metals, especially mercury. These complexes may serve to decrease toxicity of heavy metals in animals. There are other forms of selenium in proteins, but they are not selenium-dependent and their biological significance remain uncertain.

More than 80% of whole body selenium was present as selenocysteine in protein when animals were given selenite, and less than 3% of whole body selenium was present as other forms of selenium in protein (Hawkes et al., 1985). Thus, selenocysteine appears

to be the most important form of selenium in protein. All the selenoproteins found so far contain selenium in the form of selenocysteine in their amino acid sequence.

Selenium-Dependent Regulation

Selenoproteins can be differentially regulated by selenium and the regulation of individual selenoproteins occurs at different levels of selenium. An increase in selenium concentration would increase selenoprotein P concentration first, followed by increases in eGSH-Px and cGSH-Px (Burk and Hill, 1993). In selenium-deficieny, cGSH-Px decreases faster than 5'-deiodinase (Arthur et al., 1990b). The formation of most other selenoproteins has priority over that of eGSH-Px and cGSH-Px (Behne et al., 1995). The regulation of selenoprotein gene expression by selenium occurs primarily at the transcriptional level in prokaryotes, while the regulation occurs at various levels in eukaryotes.

Novel In-Frame UGA Codon

UGA is one of the stop codons which is used in translation of mRNA. By comparing portions of genes encoding selenoproteins, it was found that the position of a TGA, which corresponds to UGA in mRNA, was occupied by a selenocysteine residue in amino acid sequence. Therefore, it implied that the UGA codon was not a normal nonsense codon but was involved in the insertion of selenocysteine (Böck et al., 1991b).

Berry and colleagues constructed different deletions and frame-shift mutants of type 1 5' deiodinase cDNA (Berry et al., 1991a). Plasmid DNA of these mutants was transcribed in vitro. The resulting RNA was injected into Xenopus oocytes and oocyte homogenates were assayed for their ability to deiodinate 3, 3', 5'-triiodothyronine (rT_3). They found that the frame-shift mutant and mutants deleted sequence downstream of TGA resulted in complete lose of deiodinase activity in oocytes. These results indicated that the UGA codon for selenocysteine insertion was an in-frame UGA codon and sequence downstream of TGA was required for deiodinase activity.

They also generated UGA codon mutants by site-directed mutagenesis and examined the deiodinase activities of these mutants in oocytes. When the UGA codon was mutated to UAA (stop codon) or UUA (codon for leucine), deiodinase activity in oocytes was completely lost. The UGA codon mutated to UGU (codon for cysteine) retained only 10 % of deiodinase activity in oocytes. In the study using formate dehydrogenase gene from E. coli (Zinoni et al., 1987), ^{75}Se incorporation was found to be completely abolished when the UGA codon was converted to UCA (codon for serine), but ^{75}Se incorporation was decreased to about 10 % when the UGA codon was converted to cysteine codon (UGC or UGU). These results indicated that the UGA codon directed the incorporation of selenium in the form of selenocysteine which was required for maximal enzyme activity of the deiodinase.

Comparison between the predicted secondary structures around the 5'-DI and GSH-Px UGA codons and in the coding region of Se-P shows no common features in UGA codon context. This suggests that there is no stringent requirement for a particular environment for UGA codon translation. To examine the role of UGA codon environment

or context on the efficiency of selenocysteine incorporation, site-directed mutagenesis was used to introduce UGA codons into the rat 5'-DI coding sequence (Berry et al., 1993). Several mutations were constructed by converting cysteine codon to UGA codon in the wild-type coding sequence and also converted the wild-type UGA codon to a cysteine codon and introduced a UGA in an 'out-of-context' position. These mutant constructs were used to transfect COS-7 cells and translation of UGA codons was assessed by affinity labelling of [^{125}I]BrAcT_3, which was a substrate analog of T_3, in COS-7 cell homogenates following transient expression. All constructs containing a single 'out-of-context' UGA codon produced BrAcT_3-labelled proteins of the same size as the wild-type protein, but constructs without SECIS (selenocysteine-insertion sequence) motif did not produce detectable BrAcT_3-labelled proteins. In addition, translation of constructs containing two UGA codons are more difficult than those containing a single UGA codon. Except translational efficiency, expression of deiodinase activity was also assayed in cell homogenates transfected with these mutants. Only the full-length UGA mutant construct produced deiodinase activity comparable to the wild-type enzyme, and the deiodinase activity produced by all other mutants was lower than that produced by the wild-type enzyme. These results indicate that a specific UGA codon context is not required for efficient selenocysteine incorporation in eukaryotes.

Selenium-Dependent Cotranslational Insertion of Selenocysteine

A UGA codon was demonstrated to direct the insertion of selenocysteine in selenoproteins. The selenium-dependency of this incorporation was demonstrated by

Zinoni and his coworkers by using the formate dehydrogenase gene from E. coli (Zinoni et al., 1987). They constructed several fusion plasmids with different N-terminal of formate dehydrogenase gene (fdhF) fused to ß-galactosidase gene (lacZ). E. coli were transfected with different fusion plasmids and tested for ß-galactosidase activity in the medium with or without selenium. They found that translation of hybrid gene containing UGA codon was selenium-dependent, while translation of hybrid gene without a UGA codon did not depend on selenium status. However, transcription did not require selenium in both types of hybrid genes. By site-directed mutagenesis, the UGA codon was converted to UGC or UGU (codon for cysteine), or UCA (codon for serine). Ability to incorporate ^{75}Se was examined in those mutant plasmids. When the UGA codon was converted to sense codons, the selenium-dependency of formate dehydrogenase mRNA was relieved. In addition, ^{75}Se incorporation can be inhibited by cycloheximide (Sunde and Hoekstra, 1980) and by nuclease treatment (Wilhelmsen et al., 1985). These results indicated that translation of UGA codon was selenium-dependent and this UGA codon cotranslationally directed the insertion of selenocysteine into formate dehydrogenase polypeptide chain.

Sunde and Evenson investigated the origin of the skeleton of selenocysteine moiety by perfusing isolated rat liver with ^{14}C- or ^{3}H-labeled amino acids (Sunde and Evenson, 1987). Glutathione peroxidase (GSH-Px) was purified and the specific activity of selenocysteine in GSH-Px determined. They found that ^{14}C-serine perfusion labeled serine, glycine and selenocysteine in purified GSH-Px, but ^{3}H-serine perfusion only labeled serine and selenocysteine. These results indicated that selenocysteine in GSH-Px

is derived from serine which provides the carbon skeleton for the selenocysteine moiety in selenoprotein synthesis.

Structures of Selenoprotein mRNAs

Stem-loop structure of mRNA in prokaryotes

The in-frame UGA codon of selenoprotein mRNA directs the incorporation of selenocysteine. Ability of how the ribosomal translation assembly can distinguish this special UGA codon of selenoprotein mRNA from the stop UGA codon in other mRNA species is still unknown. It seems most likely that the specific secondary structure of mRNA is involved.

By using computer analysis, it was reported that there was a putative stem-loop structure located immediately downstream of UGA in formate dehydrogenase mRNA (Zinoni et al., 1990). Zinoni and his coworkers introduced several different deletions from the 3' end approaching to TGA of formate dehydrogenase gene (fdhF). These different truncated 5' segments of fdhF gene were fused in-frame to the lacZ reporter gene. ß-galactosidase activity and ^{75}Se incorporation were measured in fusion genes. They found that at least 40 bases of fdhF mRNA downstream of UGA codon, which corresponded to the putative stem-loop structure, was required for both ß-galactosidase expression and ^{75}Se incorporation. Thus this stem-loop structure immediately downstream of formate dehydrogenase mRNA is required for decoding UGA and for incorporation of selenocysteine into formate dehydrogenase from E. coli. However, similar secondary structures can also be formed in mRNAs coding for other selenoproteins. In addition,

disruption of the stem or changes in single nucleotide on the loop abolish selenocysteine incorporation (Böck et al., 1991b). Thus, the stem-loop structure appears to interact with the ribosome directly or indirectly to mediate incorporation of selenocysteine.

3' UTR, Stem-loop structure and SECIS motif of mRNA in eukaryotes

In eukaryotes, a predicted stem-loop structure similar to the one in E. coli is not immediately adjacent to UGA codon but is located several hundreds of bases away from UGA codon in the 3' untranslated region (3' UTR) of type 1 5'-deiodinase (Berry et al., 1991b). Berry and collegues generated several deletion mutants of rat 5'-DI cDNA. By using site-directed mutagenesis, they also mutated the UGA codon (code for selenocysteine) into a cysteine codon and generated a mutant deleted of the 3' UTR of the cysteine mutant. These mutants were assayed for 5'-DI activity after transient transfection in COS-7 or JEG-3 cells. They found that mutants without 3' UTR did not express deiodinase activity and deletion of the same sequences did not affect the activity expressed by the cysteine mutants. This indicated that 3' UTR was required for selenocysteine incorporation only and not for cysteine. In order to identify which specific sequence is needed for selenocysteine incorporation, they generated different internal deletion mutants of 3' UTR and found that a specific 3' UTR segment of about 200 nucleotides (from nucleotide 1440 to 1615) was required for selenocysteine incorporation. In addition, two deletion mutants with reduced spacing between the coding region and the 3' UTR produced higher 5'-DI activity than the wild-type cDNA. This suggested that the spacing between 3' UTR and UGA codon influences the efficiency of selenocysteine incorporation. To confirm that this sequence is required only at translation, mutant

plasmids were transcribed in vitro and the resulting RNAs were injected into Xenopus oocytes. The relative 5'-DI activity produced was in parallel to that produced by transfection. Thus the reduced expression caused by 3' UTR mutants was due to impaired translation. This indicates that selenocysteine is cotranslationally incorporated into the polypeptide chain of selenoproteins.

A predicted stem-loop structure of 5'-DI mRNA was found to be located in this required 200 nucleotides of 3' UTR segment. Berry and collegues constructed a hybrid mutant with coding region of rat 5'-DI mRNA and 3' UTR of rat glutathione peroxidase mRNA, and measured deiodinase activity of this mutants. Although the primary sequences between the 3' UTR of rat 5'-DI mRNA and that of rat glutathione peroxidase are different, the 3' UTR of rat glutathione peroxidase mRNA can substitute for the 3' UTR of rat 5'-DI mRNA in directing selenocysteine incorporation. In addition, computer analysis predict similar stem-loop structures in the 3' UTR of both rat 5'-DI mRNA and rat glutathione peroxidase mRNA, and limited mutations in these structures could decrease or block the translation of these selenoproteins. These results suggest that secondary structure of mRNA may be involved in translational regulation of UGA codon in mRNAs of selenoproteins. Thus, Berry and collegues generated several deletion mutants within the predicted stem-loop region of rat 5'-DI mRNA and measured their deiodinase activities. They found that a short sequence in 3' UTR, SECIS motif, of rat 5'-DI mRNA was essential for successful translation of 5'-DI and possibly other eukaryotic selenoproteins both in vivo and in vitro.

Sequence analysis of human cellular glutathione peroxidase (cGSH-Px) mRNA predict two possible stem-loop structures around the in-frame UGA codon (Shen et al.,

1993). One putative stem-loop structure, which is similar to those found in formate dehydrogenase mRNA of E. coli and other prokaryotic selenoproteins, is located in the open reading frame and lies immediately downstream of UGA codon. The other putative stem-loop structure; which is similar to the one found in rat 5'-DI mRNA, is located in the 3' UTR. Several mutants, in which different segments from the potential in-frame stem-loop structure were deleted, were used to transfect ^{75}Se-labeled COS-1 cells and incorporation of selenocysteine into glutathione peroxidase was examined. None of the deletion mutants in in-frame stem-loop structure blocked the selenocysteine incorporation into glutathione peroxidase, but mutants with different deletions in potential stem-loop structure in 3' UTR completely blocked the selenocysteine incorporation into glutathione peroxidase. These results indicate that the stem-loop structure in open reading frame of cGSH-Px mRNA is not involved in selenocysteine incorporation, whereas the stem-loop structure in 3' UTR of cGSH-Px is required for selenocysteine incorporation.

The sequences of the 3' UTR of mRNAs of different eukaryotic selenoproteins are not conserved except for the limited consensus sequences. By comparing the 3' UTR of cGSH-Px with that of 5'-DI, two 4-nucleotide sequences within the loop (UAAA in the first loop and UGAU in the second loop) were identical to those at the same positions within the SECIS motif in the 5'-DI mRNA. Mutants with deletions of either one of the two 4-nt sequences completely abolished the incorporation of selenocysteine. These results indicate that SECIS motif and part of the 3' UTR sequence are both necessary and sufficient for translational insertion of selenocysteine at UGA codon in mRNAs of eukaryotic selenoproteins.

Selenoprotein P (Se-P) contains two functional SECIS motifs with each in one stem-loop structure in 3' UTR. Berry and collegues examined the two stem-loops of Se-P in combination and individually for their ability to confer 5'-DI expression by using the rat 5'-DI coding region as a reporter for translation of UGA as selenocysteine (Berry et al., 1993). They found that a segment consisting of both stem-loops and an additional 600 nucleotides in 3' UTR of Se-P produced four-fold higher deiodinase activity than the rat 5'-DI SECIS motif. Deletion of the last 600 nucleotides decreased deiodinase activity only slightly. The first Se-P stem-loop alone produced a three-fold higher deiodinase activity than the 5'-DI SECIS motif, while the second Se-P stem-loop alone produced comparable deiodinase activity to that of the rat 5'-DI. In addition, deletion of the 8 bp loop from the second Se-P SECIS motif eliminated translation. These results indicate that one stem-loop is sufficient for translation of multiple UGA codons and translation of multiple UGA codons does not require a Se-P SECIS motif, but can be directed by the 5'-DI SECIS motif. The relation of stem-loop and SECIS motif to each other is not the same in all mRNAs of eukaryotic selenoproteins, and this suggests that the mechanism by which the 3' UTR mediates selenocysteine incorporation by UGA codon is complex. Stop codon function at 'out-of-context' UGA codons is suppressed by the SECIS motif in 3' UTR of eukaryotic mRNA, and hence the presence of a SECIS motif in eukaryotic selenoprotein mRNA relieves the necessity for stem-loop structures in the coding region which is a required characteristic of prokaryotic selenoprotein mRNAs, and permits complete amino acid sequence flexibility in UGA codon position (Berry et al., 1993).

Comparison of stem-loop structures and sequences in 3' UTR of 5'-DI, GSH-Px and Se-P identified several conserved features: AAA in the loop region, an unpaired

AUGA in the 5' arm of the stem and an unpaired UGR in the 3' arm of the stem. In addition, the distance between these unpaired bases and the loops, which are 10-12 bases, is highly conserved. Site-directed mutagenesis of three conserved A residues, the 3' unpaired UGR and 5' unpaired AUGA indicate that the three conserved A residues in the stem-loop of SECIS motif are optimal for selenocysteine incorporation and the unpaired conserved nucleotides in the stem are critical for SECIS function. This indicates that multiple contact sites are required for SECIS function. In addition, the SECIS motif is predicted to form stem-loops with high negative free energy. Disruption of particular secondary structure in the rat 5'-DI SECIS motif results in loss of activity, while restoring the secondary structure in SECIS motif restores full activity. Thus, the secondary structure in SECIS motif is also important in selenocysteine incorporation (Berry et al., 1993).

Berry and collegues constructed several mutants containing 'out-of context' UGA codons in the rat 5'-DI coding sequence, and these mutant constructs were used to transfect COS-7 cells and measured deiodinase activity. They found that the deiodinase activity was significatly higher with the 5'-DI SECIS motif present than it was for the SECIS deletion constructs in 'out-of context' mutants, and substitution of the Se-P SECIS motif increased deiodinase activity up to 4-fold compared with the 5'-DI SECIS motif. In addition, they introduced the rat 5'-DI SECIS motif in different positions relative to the coding region and examined the effects of different SECIS positions on selenocysteine incorporation. When the coding regions were separated by inserting an additional 'spacer' DNA, expression of deiodinase activity was comparable to the wild-type enzyme. When the 5'-DI SECIS motif was in the 5' end of coding sequence or in

trans position, the expression efficiency was much lower than that of the wild-type and the expression of SECIS motif in trans was in a dose-dependent fashion. To prove that this is only involved in translation, corresponding RNAs were transcribed in vitro and injected into Xenopus oocytes followed by assay of deiodinase activity in oocyte homogenates. This result was in similar to that by transfection (Berry et al., 1993).

MECHANISM OF SELENOPROTEIN SYNTHESIS

Prokaryotic Selenoprotein Synthesis

Selenium-deficient E. coli mutants were used to study selenium incorporation into formate dehydrogenases. Four different genes, selA, selB, selC and selD, were identified to be required for selenoprotein synthesis and selenocysteine incorporation in prokaryotes (Leinfelder et al., 1988).

selA gene encodes a selenocysteine synthase which is a pyridoxyl 5-phosphate enzyme and a 600-KD homodimer composed of two 50-KD subunits. Selenocysteine synthase is responsible for the correct aminoacylation of $tRNA^{(Sec)}$ with selenocysteine and thus have a proofreading function.

selB gene encodes a elongation factor (SELB) whose function is similar to that of the elongation factor, EF-Tu, which serves for all other amino acid-tRNAs. SELB is a GTP-binding protein and is sensitive to the length of the aminoacyl-acceptor helix, which appears to be a major recognition determinant (Baron and Böck, 1991). It has a 43-KD region at the N-terminal which is similar to EF-Tu, and an additional 25-KD region which functions to recognize selenocysteyl-$tRNA^{(Sec)}$. SELB binds stoichiometrically to selenocysteyl-$tRNA^{(Sec)}$ but not seryl-$tRNA^{(Sec)}$, while EF-Tu shows very low affinity for $tRNA^{(Sec)}$, irrespective of whether it is charged with serine or selenocysteine. These results suggest that SELB has a function in the decoding process at the ribosome and specifically recognizes the aminoacyl residue of $tRNA^{(Sec)}$.

selC gene encodes a specific $tRNA^{(Sec)}$ which is required for selenoprotein synthesis in E. coli. This $tRNA^{(Sec)}$ differs from other tRNAs which are used for other amino acids in length (95 bases versus 76 bases), D-loop (22 bases versus 10 bases), amino acid acceptor stem (8 bp versus 7 bp) and several deviations from consensus nucleotides usually found in E. coli tRNAs. This special $tRNA^{(Sec)}$ carries the anticodon sequence (UCA) for UGA and can be charged by L-serine.

selD gene encodes an enzyme (SELD) which catalyzes the production of selenophosphate compound which is required for selenocysteine synthesis and for the conversion of the 5-methyl-aminomethyl-2-thio-uridine into 5-methylamino-methyl-2-seleno-uridine of $tRNA^{(Glu)}$ and $tRNA^{(Lys)}$ species (Leinfelder et al., 1990).

The mechanism responsible for selenoprotein synthesis is understood better in prokaryotic systems. Specific $tRNA^{(Sec)}$ encoded by selC gene is charged with L-serine, which is converted to seryl-$tRNA^{(Sec)}$ by seryl-tRNA ligase. Selenocysteine synthase encoded by selA gene can discriminate parts of this $tRNA^{(Sec)}$ molecule and recognize the seryl moiety of seryl-$tRNA^{(Sec)}$. After binding to seryl-$tRNA^{(Sec)}$, selenocysteine synthase converts seryl-$tRNA^{(Sec)}$ to selenocysteyl-$tRNA^{(Sec)}$ by catalyzing the 2,3-elimination of a H_2O molecule from the seryl-residue. This results in the formation of an aminoacrylyl-$tRNA^{(Sec)}$ intermediate which is bound to pyridoxal-phosphate via an aldimine linkage (Forchhammer and Böck, 1991). H_2Se is added subsequently to the double bond of the aminoacrylyl residue which results in the replacement of the side-chain oxygen in serine by selenium to yield selenocysteine. SELD, which is required for the addition of H_2Se to the aminoacrylyl double bond, catalyzes the production of selenophosphate compound from selenide. This step requires ATP which is hydrolyzed during this process. The

selenocysteyl-tRNA$^{(Sec)}$ is then released from selenocysteine synthase and becomes bound by SELB elongation factor which is encoded by selB gene. SELB forms a ternary complex with selenocysteyl-tRNA$^{(Sec)}$ and GTP, which in turn attaches to the stem-loop immediately downstream of UGA in the open reading frame of mRNA. This facilitates the incorporation of selenocysteine into polypeptide chain of selenoprotein, probably either by bringing the selenocysteyl-tRNA$^{(Sec)}$ closer to the UGA codon or by blocking the access of release factor 2 to A site on ribosome where it acts to terminate translation of mRNAs (Böck et al., 1991b). Although sequences upstream of UGA codon in prokaryotic selenoprotein mRNA are not required for specificity of selenocysteine incorporation, they may be involved in the modulation of the efficiency of this process.

Eukaryotic Selenoprotein Synthesis

The use of UGA as a selenocysteine codon occurs in both prokaryotes and eukaryotes, but the mechanism of recognition differs between these two types of organisms. Much less imformation is available on biochemistry of selenoprotein synthesis in eukaryotes. However, there are several features of eukaryotes which are similar to that of prokaryotic selenoprotein synthesis.

It has been reported that the incorporation of selenocysteine was mediated by a selenocysteine-specific tRNA (Hawkes et al., 1982). Hawkes and his coworkers studied the aminoacylation of rat liver tRNA with selenocysteine in tissue slices and in a cell-free system by using [^{75}Se]selenocysteine and [^{75}Se]selenite as substrates (Hawkes et al., 1982). They found that [^{75}Se]selenocysteine was specifically associated with selenocysteyl-

tRNA via an aminoacyl linkage and this specific aminoacylation was resistant to competition by excess of either unlabeled cysteine or a mixture of the other 19 amino acids in the cell-free selenocysteine aminoacylation system. This specific $tRNA^{(Sec)}$ can carry selenocysteine in vivo and is similar to $tRNA^{(Sec)}$ in E. coli. It carries an anticodon for UGA and can be charged with L-serine in a manner similar to that in E. coli $tRNA^{(Sec)}$. In addition, [^{75}Se]selenocysteyl-$tRNA^{(sec)}$ was purified from rat mammary tumor cells and used in E. coli ribosomal binding studies. Most of the ^{75}Se is attached to selenocysteyl-$tRNA^{(Sec)}$ which recognizes UGA codon only, but not any of the other codons. Thus, this selenocysteyl-$tRNA^{(Sec)}$ appears to function both as a carrier for selenocysteine synthesis and as selenocysteine donor in response to specific UGA codon (Lee et al., 1989).

In addition to the specific $tRNA^{(Sec)}$ for selenocysteine, the special in-frame UGA codon is used for coding selenocysteine in eukaryotic mRNAs. Stable stem-loop structures are located in 3' UTR of all eukaryotic selenoprotein mRNA for decoding UGA codon. SECIS motif, a short sequence in 3' UTR of eukaryotic selenoprotein mRNA is also required for directing selenocysteine insertion at UGA codon.

The mechanism responsible for selenocysteine synthesis and its incorporation in eukaryotes is only partial characterized. The specific $tRNA^{(Sec)}$,which carries the anticodon for UGA, is charged with serine and seryl-$tRNA^{(Sec)}$ is formed by seryl-tRNA ligase. The mechanism of conversion of seryl-$tRNA^{(Sec)}$ to selenocysteyl-$tRNA^{(Sec)}$ has not been established yet. Recent evidence suggests that enzymes analogous to selenocysteine synthase and SELD in prokaryotes also exist in mice (Mizutani et al., 1992). Therefore, these enzymes may catalyze the production of required seleno-compounds from selenium

presursors and the conversion of serine to selenocysteine, to form selenocysteyl-tRNA$^{(Sec)}$. In addition to seryl-tRNA$^{(Sec)}$ and selenocysteyl-tRNA$^{(Sec)}$, phosphoseryl-tRNA$^{(Sec)}$ has been isolated. Phosphoseryl-tRNA$^{(Sec)}$ can be converted to selenocysteyl-tRNA$^{(Sec)}$ in vitro, thus this tRNA species may compete with selenocysteine synthase and serve as an intermediate in selenoprotein synthesis (Lee et al., 1989). However, there is no evidence to indicate that seryl-tRNA$^{(Sec)}$ can be converted to phosphoseryl-tRNA$^{(Sec)}$. The mechanism of how the selenocysteyl-tRNA$^{(Sec)}$ interacts with mRNA of eukaryotic selenoprotein is still unknown. There may be a translational elongation factor similar to the one in prokaryotes which is involved in the association of selenocysteyl-tRNA$^{(Sec)}$ with mRNA. Furthermore, the stem-loop structures in 3' UTR of mRNA may interact with the translational machinery (selenocysteyl-tRNA$^{(Sec)}$, elongation factors and other factors) and possibly with RNA structures or the nascent protein. This complex would then allow translation of UGA codons in the adjacent coding region, without a strict requirement for the UGA codons to be in a particular environment, and specifically direct selenocysteine incorporation probably by pausing the ribosomes when they approch the UGA codon to allow time for binding of selenocysteyl-tRNA$^{(Sec)}$. In addition, the stem-loop structure of eukaryotic mRNA is in the 3' UTR which is different from that of prokaryotic mRNA. The reasons for the different mechanisms responsible for eukaryotic selenoprotein synthesis as compared to prokaryotes may be the different location of stem-loop structures in selenoprotein mRNAs.

Differences in Selenoprotein Synthesis Between Prokaryotes and Eukaryotes

The mechanisms responsible for prokaryotic and eukaryotic selenoprotein synthesis are not completely understood. However, several features required for selenocysteine incorporation are different between prokaryotes and eukaryotes. The required stem-loop structure for selenocysteine insertion is located in coding sequence immediately downstream of UGA codon in prokaryotes, while it is located in 3' UTR with several hundred bases away from UGA codon in eukaryotes. The sequences upstream of UGA codon are not involved in incorporation of selenocysteine in prokaryotes, whereas they are involved in selenocysteine incorporation in eukaryotes. In addition, SECIS (selenocysteine-insertion sequence) motifs are required for directing of selenocysteine insertion in eukaryotes, while this SECIS motif is not found in prokaryotes. Thus, the mechanism responsible for selenoprotein synthesis in eukaryotes may be different and more complex from that for selenoprotein synthesis in prokaryotes. These differences may account for the failure of attempts to express eukaryotic selenoprotein mRNA in prokaryotic systems.

GENERAL ASPECTS OF SELENOPROTEINS

There are three stop codons used for termination in mRNA translation, which are UGA, UAA and UAG. The rat 5'-DI coding sequence terminates with a UAG, while the human 5'-DI, rat GSH-Px, and rat and human selenoprotein P coding sequences all terminate with UAA. Therefore, it appears that the translational machinery of selenoprotein specifies UGA as a selenocysteine codon by using stop codons other than UGA. However, the mechanism for recognization of appropriate UGA codons and distinguision of them from the UGAs used as stop codons is unclear. Selenium is incorporated specifically in amino acid tRNAs. In certain anaerobic bacteria and E. coli, glutamate- and lysine-accepting tRNAs are the species that contain the most conspicuous amounts of selenium. Selenium occurs in the wobble position of the anticodons of these seleno-tRNA in the form of a 5-methylaminomethyl-2-selenouridine residue. The presence of selenium in the anticodons of glutamate- and lysine-accepting tRNAs appears to influence which codons prefer a particular amino acid. When the sulfur-modified uridine is present in the wobble position of the anticodons, the glutamate and lysine codons ending in A generally are preferred, but with the substitution of selenium, interaction with anticodons ending in G is facilitated. Such differential effects on codon-anticodon interactions may also be important in specifying synthesis of selenoproteins and in modulating rates of selenoprotein synthesis under various conditions (Stadtman, 1987). In addition, the UGA codon for selenocysteine has been conserved in all three lines of descent (Böck et al., 1991a). This suggests that it existed before bacteria, archaea and eukarya separated. Its higher susceptibility to oxidation or its extreme sensitivity to heavy

metal ions may be the reasons that selenocysteine was selected and restricted to a few special proteins (Böck et al., 1991b).

The translation of multiple UGA codons is more difficult than that of single UGA codon. Selenoprotein P mRNA contains 10 UGA codons, therefore the translation rate of this selenoprotein should be lower than that of other selenoproteins. However, the half-life of selenoprotein P is very short. The mechanisms responsible for balancing between high turn-over rate and low synthesis rate of this selenoprotein P are not understood. It is possible that certain UGA codons are translated more efficiently than others because the ribosomal pauses occur at some positions more than at others. Thus, UGA codons of selenoprotein P may be translated more efficiently than that of other selenoproteins. In addition, some specific proteins have been reported to be involved in recognizing certain nucleotide sequences. Regulation of ferritin and transferrin receptor expression by iron response element-binding proteins is a good example of protein recognition of both base-paired regions and specific unpaired nucleotides. Therefore, there may be some unidentified proteins or factors involved in eukaryotic selenoprotein synthesis. Probably some specific proteins or factors would specifically increase selenoprotein P synthesis but not other selenoproteins.

Certain selenoproteins have priority for selenium when it is limited. The differences in efficiency of stem-loop function may allow the cellular translation machinery to express some selenoproteins preferentially over others. In fact, the relative efficiencies of the SECIS motifs in Se-P, 5'-DI and GSH-Px parallel their order of appearance upon selenium repletion in selenium-depleted animals (Hill et al., 1991).

Selenoprotein P has multiple UGA codons in the coding region and two stem-loop structures in the 3' UTR of its mRNA. Thus, it can be used as a model protein to study the mechanism responsible for eukaryotic selenoprotein synthesis. However, the attempts to express selenoprotein P have not been successful so far. From the present analysis with selenoprotein P, it seems that a separate stem-loop structure is not required for translation of each UGA codon in mRNA.

Knowledge of the detailed mechanism responsible for selenoprotein synthesis can apply to design and construct selenoproteins through selenocysteine insertion at selected positions. The selenoprotein engineering has several uses: (1) It may help to determine the location of cysteine residues involved in disulfide bridge formation using NMR analysis of the protein in solution. (2) The replacement of a single cysteine residue by selenocysteine may make such a protein amenable to X-ray analysis where specific heavy metal derivatives are unavailable. Two principal strategies can achieve this targeted insertion: (1) Change the particular codon into a UGA codon and alter the mRNA context to create the downstream stem-loop structure that possesses the sequence in the loop which is required for selenocysteine insertion. This strategy has already been carried-out successfully (Heider and Böck, 1992). (2) Change the specific $tRNA^{(Sec)}$ structure in a way that it can be recognized by elongation factor EF-Tu. The mutant $tRNA^{(Sec)}$ variant with a 7-bp aminoacyl-acceptor helix should be an excellent candidate for the targeted insertion of selenocysteine (Böck et al., 1991). In addition, the ability to introduce a heavy metal into a specific site in proteins could prove to be a powerful aid to three-dimensional structure studies.

When animals are deficient in selenium, the first priority of body selenium is to supply selenium to the brain, and endocrine and reproductive organs, while the second priority is to supply selenium to heart, muscle and liver. However, in those tissues, selenium is mostly used to synthesize selenoproteins other than glutathione peroxidases (Behne et al., 1988). A study in rat tissues showed that only one-third of whole body selenium was used for synthesis of glutathione peroxidases and the remaining two-thirds of whole body selenuim was used for synthesis of other selenoproteins. Therefore, other selenoproteins may be critical for normal cellular metabolism (Hawk et al., 1985).

REFERENCES

Arthur, J. R., Nicol, F. and Beckett, G. J. (1990a) Hepatic iodothyronine 5'-deiodinase. Biochem. J.272: 537-540.

Arthur, J. R., Nicol, F., Hutchinson, A. R. and Beckett, G. J. (1990b) The effects of selenium depletion and repletion on the metabolism of thyroid hormones in the rat. J. Inorg. Chem. 39: 101-108.

Axley, M. J. and Stadtman, T. C. (1989) Selenium metabolism and selenium-dependent enzymes in microorganisms. Annu. Rev. Nutr. 9: 127-137.

Bansal, M. P., Cook, R. C., Danielsom, K. G. and Medina, D. (1989) A 14-kilodalton selenium-binding protein in mouse liver is fatty acid-binding protein. J. Bio. Chem. 264: 13780-13784.

Baron, C., Böck, A. (1991) The length of the aminoacyl-acceptor stem of the selenocysteine-specific $tRNA^{Sec}$ of Escherichia coli is the determinant for binding to elogation factors SELB ot Tu. J. Biol. Chem. 266:20375-20379.

Beckett, G. T., Beddows, S. E., Morrice, P. C., Nicol, F. and Arthur, J. R. (1987) Inhibition of hepatic deiodination of thyroxine is caused by selenium deficiency in rats. Biochem. J. 248: 443-447.

Behne, D., Hilmert, H., Scheid, S., Gessner, H., Elger, W. (1988) Evidence for specific selenium target tissues and new biologically important selenoproteins. Biochim Biophys. Acta 966:12-21.

Behne, D., Kyriakopoulos, A., Meinhold, H., Köhrle, J. (1990) Identification of type 1 iodothyronine 5'-deiodinase as a selenoenzyme. Biochem. Biophys. Res. Commun. 173:1143-1149.

Behne, D., Weiss-Nowak, C., Kalcklosch, M., Westphal, C., Gessner, H. and Kyriakopoulos, A. (1995) Studies on the distribution and characteristics of new mammalian selenium-containing proteins. Analyst 120: 823-825.

Berry, M. J., Banu, L., Larsen, P. R. (1991a) Type 1 iodothyronine deiodinase is a selenocysteine-containing enzyme. Nature 349:438-440.

Berry, M. J., Banu, L., Chen, Y., Mandel, S. J., Kieffer, J. D., Harney, J. W., Larsen, P. R. (1991b) Recognition of UGA as a selenocysteine codon in Type 1 deiodinase requires sequences in the 3' untranslated region. Nature 353:273-276.

Berry, M. J., Banu, L., Harney, J. W., Larsen, P. R. (1993) Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. EMBO J. 12:3315-3322.

Blot W. J. et al. (1993) Nutrition intervention trials in Linxian, China: Supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. J. Natl. Cancer Inst. 85: 1483-1498.

Böck, A., Forchhammer, K., Heider, L., Leinfelder, W., Sawers, G., Veprek, B., Zinoni, F. (1991a) Selenocysteine: the 21st amino acid. Mol. Microbiol. 5:515-520.

Böck, A., Forchhammer, K., Heider, J., Baron, C. (1991b) Selenoprotein synthesis: an expansion of the genetic code. Trends Biochem. Sci. 16:463-467.

Brown, M. R., Cohen, H. J., Lyons, J. M., Curtis, T. W., Thunberg, B., Cochran, W. J. and Klish, W. J. (1986) Proximal muscle weakness and selenium deficiency associated with long term parenteral nutrition. Amer. J. Clin. Nutr. 43: 549-554.

Burk, R. F., Hill, K. E. (1993) Regulation of selenoproteins. Annu. Rev. Nutr. 13:65-81.

Chen, X. S., Yang, G. Q., Chen, J. S., Chen, X. C., Wen, Z. M. and Ge, K. Y. (1980) Studies on the relations of selenium and Keshan disease. Biol. Trace Elem. Res. 2: 91.

Chu, F. F., Dorooshow, J. H., Esworthy, R. S. (1993) Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. J. Biol. Chem. 268:2571-2576.

Combs, G. F. and Combs, S. B. (1986) The role of selenium in nutrition. Academic Press, New York. pp. 413-461.

Cone, J. E., del Rio, M. R. Davis, J. N. and Stadtman, T. C. (1976) Chemical characterization of the selenoprotein component of clostridial glycine reductase: identification of selenocysteine as the organoselenium moiety. Proc. Natl. Acad. Sci. USA 73: 2659-2663.

Croteau, W., Whittemore, S. L., Schneider, M. J. and St. Bermain, D. L. (1995) Cloning and expression of a cDNA for a mammalian type III iodothyronine deiodinase. J. Biol. Chem. 270:16569-16575.

Forchhammer, K., Böck, A. (1991) Biology and biochemistry of selenium. Naturwissenschaften 78:497-504.

Garcia, G. E. and Stadtman, T. C. (1992) *Clostridium sticklandii* glycine reductase selenoprotein A gene: cloning, sequencing and expression in *Escherichia coli*. J. Bacteriol. 174: 7080-7089.

Gomez, B. Jr., Tappel, A. L. (1989) Selenoprotein P receptor from rat. Biochim. Biophys. Acta 979:20-26.

Hawkes, W. C., Lyons, D. E., Tappel, A. L. (1982) Identification of a selenocystein-specific aminoacyl transfer RNA from rat liver. Biochim. Biophys. Acta 699:183-191.

Hawkes, W. C., Wilhelmsen, E. C., Tappel, A. L. (1985) Abundance and tissue distribution of selenocysteine-containing proteins in the rat. J. Inorg. Biochem. 23:77-92.

Heider, J. and Bock, A. (1992) Targeted insertion of selenocysteine into the α-subunit of formate dehydrogenase from *Methanobacterium formicicum*. J. Bacteriol. 174: 659-663.

Hill, K. E., Lloyd, R. S., Yang, J. G., Read, A., Burk, R. F. (1991) The cDNA for rat selenoprotein P contains 10 TGA codons in the open reading frame. J. Biol. Chem. 266:10050-10053.

Hill, K. E., Lloyd, R. S., Burk, R. F. (1993) Conserved nucleotide sequences in the open reading frame and 3' untranslated region of selenoprotein P mRNA. Proc. Natl. Acad. Sci. USA 90:537-541.

Hoekstra, W. G. (1975) Biochemical function of selenium and its relation to vitamin E. Fed. Proc. 34: 2083.

Jones, J. B. and Stadtman, T. C. (1981) Selenium-dependent and selenium-independent formate dehydrogenases of *Methanococcus vannielii*. Separation of the two forms and characterization of the purified selenium-independent form. J. Biol. Chem. 256: 656-663.

Karimpour, I., Cutler, M., Shih, D., Smith, J., Keene, K. C. (1992) Sequence of the gene encoding the mitochonidrial capsule selenoprotein of mouse sperm: identification of three in-phase TGA selenocysteine codons. DNA Cell Biol. 11:692-699.

Lee, B. J., Worland, P. J., Davis, J. N., Stadtman, T. C., Hatfield, D. L. (1989) Identification of a selenocysteyl-tRNA^{scr} in mammalian cells that recognizes the nonsense codon, UGA. J. Biol. Chem. 264:9724-9727.

Leinfelder, W., Forchhammer, K., Zinoni, F., Sawers, G., Mandrand-Berthelot, M.-A., Böck, A. (1988) Escherichia coli genes whose products are involved in selenium metabolism. J. Bacteriol. 170:540-546.

Leinfelder, W., Forchhammer, K., Veprek, B., Zehelein, E., Böck, A. (1990) In vitro synthesis of selenocysteinyl-tRNA$_{UCA}$ from seryl-tRNA$_{UCA}$: Involvement and characterization of the selD gene product. Proc. Natl. Acad. Sci. USA 87:543-547.

Mills, G. C. (1957) Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J. Biol. Chem. 229:189.

Mizutani, T., Kurata, H., Yamada, K., Totsuka, T. (1992) Some properties of murine selenocysteine synthase. Biochem. J. 284:827-834.

Read, R., Bellew, T., Yang, J.-G., Hill, K. E., Palmer, I. S., Burk, R. F. (1990) Selenium and amino acid composition of selenoprotein P, the major selenoprotein in rat serum. J. Biol. Chem. 265:17899-17905.

Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G. and Hoekstra, W. G. (1973) Selenium: Biochemical role as a component of glutathione peroxidase. Science 179: 588-590.

Schuber, A. P., Orr, E. C., Recny, M. A., Schendel, P. F., May, H. D., Schauer, N. L., Ferry, J. G. (1986) Cloning, expression, and nucleotide sequence of the formate dehydrogenase genes from Methanobacterium formicicum. J. Biol. Chem. 261:12942-12947.

Schuckelt, R., Brigelius-Flohè, R., Maiorino, M., Roveri, A., Reumkens, J., StraßBurger, W., Ursini, F., Wolf, B., Flohè, L. (1991) Phospholipid hydroperoxide glutathione peroxidase is a selenoenzyme distinct from the classical glutathione peroxidase as evident from cDNA and amino acid sequencing. Free Rad. Res. Comms. 14:343-361.

Schwarz, K. and Foltz, C. (1957) Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J. Amer. Chem. Soc. 79:3292.

Shen, Q., Chu, F. F., Newburger, P. E. (1993) Sequences in the 3'-untranslated region of the human cellular glutathione peroxidase gene are necessary and sufficient for selenocysteine incorporation at the UGA codon. J. Biol. Chem. 268:11463-11469.

St. Germain, D. L. (1994) In: Thyroid hormone metabolism: molecular biology and alternative pathway. (Wu, S-Y. and Visser, T. J. eds.) CRC Press, Ann Arbor, MI. pp. 45-66.

Stadtman, T. C. (1987) Specific occurence of selenium in enzymes and amino acid tRNAs. FASEB J. 1:375-379.

Sunde, R. A., Evenson, J. K. (1987) Serine incorporation into the selenocysteine moiety of glutathione peroxidase. J. Biol. Chem. 262:933-937.

Sunde, R. A., Hoekstra, W. G. (1980) Incorportaion of selenium into liver glutathione peroxidase in the Se-adequate and Se-deficient rat. Proc. Soc. Exp. Biol. Med. 165:291-297.

Takahashi, K., Avissar, N., Whitin, J. and Cohen, H. (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch. Biochem. Biophys. 256: 677-686.

Takahashi, K., Cohen, H. J. (1986) Selenium-dependent glutathione peroxidase protein and activity: Immunological investigations on cellular and plasma enzymes. Blood 68:640-645.

Turner, D. C., Stadtman, T. C. (1973) Purification of protein components of the clostridial glycine reductase system and characterization of protein A as a selenoprotein. Arch. Biochem. Biophys. 154:366-381.

Ullrey, D. E., Combs, J. F., Conrad, H. R., Hoekstra, W. G., Jenkins, K. J. W., Levander, O. A. and Whanger, P. D. (1983) Selenium in nutrition. National Academy Press, Washington, D. C.

Ursini, F., Maiorino, M. and Gregolin, C. (1985) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim. Biophys. Acta 839: 62-70.

Vendeland, S. C., Beilstein, M. A., Chen, C. L., Jensen, O. N., Barofsky, E., Whanger, P. D. (1993) Purification and properties of selenoprotein W from rat muscle. J. Biol. Chem. 268:17103-17107.

Vendeland, S. C. Beilstein, M. A., Yeh, J-Y., Ream, L. W. and Whanger, P. D. (1995) Rat skeletal muscle selenoprotein W: cDNA clone and mRNA modulation by dietary selenium. Proc. Natl. Acad. Sci., USA, 92:8749-8753.

Whanger, P. D. (1985) Metabolic interactions of selenium with cadmium, mercury and silver. In: Advances in nutritional research, H. H. Draper, ed, Plenum Publishing corp. 7:221-250.

Whanger, P. D. (1987) Evidence for non-glutathione peroxidase selenium-containing proteins in mammalian tissues. In Selenium in Biology and Medicine, Third International Symp., Beijing, PRC (Combs, G. F., et al, eds), Avi Book, van Nostrand Reinhold Comp., New York. pp. 197-212.

Whanger, P. D. (1992) Selenium in the treatment of heavy metal poisoning and chemical carcinogenesis. J. Trace Elem. Electrolytes Health Dis. 6: 209-221.

Wilhelmsen, E. C., Hawkes, W. C., Tappel, A. L. (1985) Substitution of selenocysteine for cysteine in a reticulocyte lysate protein synthesis system. Biol. Trace Elem. Res. 7:141-151.

Yamazaki, S. (1982) A selenium-containing hydrogenase from *Methanococcus vannielii* : identification of the selnium moiety as a selenocysteine residue. J. Biol. Chem. 257: 7926-7929.

Yang, J-G., Hill, K. E. and Burk, R. R. (1989) Dietary selenium intake controls rat plasma selenoprotein P concentration. J. Nutr. 119:1010-1012.

Yang, J-G., Morrison-Plummer, J. and Burk, R. F. (1987) Purification of quantitation of a rat plasma selenoprotein distinct from glutathione peroxidase using monoclonal antibodies. J. Biol. Chem. 262: 13372-13375.

Yang, G. and Zhou, R. (1994) Further observations on the human maximum safe dietary selenium intake in a seleniferous area of China. J. Trace Elem. Electrolytes Health Dis. 8: 159-165.

Zinoni, F., Birkmann, A., Leinfelder, W., Böck, A. (1987) Cotranslational insertion of selenocysteine into formate dehydrogenase from Escherichia coli directed by a UGA codon. Proc. Natl. Acad. Sci. USA 84:3156-3160.

Zinoni, F., Birkmann, A., Stadtman, T. C., Böck, A. (1986) Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from Escherichia coli. Proc. Natl. Acad. Sci. USA 83:4650-4654.

Zinoni, F., Heider, J., Böck, A. (1990) Features of the formate dehydrogenase mRNA necessary for decoding of the UGA codon as selenocysteine. Proc. Natl. Acad. Sci. USA 87:4660-4664.

CHAPTER 2

TISSUE DISTRIBUTION AND INFLUENCE OF SELENIUM STATUS ON LEVELS OF SELENOPROTEIN W*

Jan-Ying Yeh, Michael A. Beilstein, Janet S. Andrews and Philip D. Whanger

Department of Agricultural Chemistry, Department of Animal Sciences
and Center for Gene Research and Biotechnology
Oregon State University,
Corvallis, OR 97331

Shortened title: Selenoprotein W in tissues

Contact person: P. D. Whanger at the above address
Phone: 503-737-1803
Fax: 503-737-0497
email: Whangerp@bcc.orst.edu

*Published with the approval of the Oregon State Agricultural Experiment Station as Technical Paper number 10,481. This research was supported by Public Health Service Research Grant number DK 38306 from the National Institute of Diabetes and Digestive and Kidney Diseases.

ABSTRACT

Rabbits were immunized with two synthetic peptides based on hydrophilic regions of selenoprotein W from rat muscle. The resulting polyclonal antibodies were used in Western blots to determine the compartmentation and tissue distribution of selenoprotein W, and to determine the influence of selenium on the levels of this selenoprotein in rat muscle. Selenoprotein W exists mainly in cytosol but very small amounts were associated with membranes. Western blots revealed selenoprotein W in muscle, spleen, testis and brain of rats. Rats were fed diets of either no addition of selenium (0 ppm Se) or additions of 0.1 and 4.0 micrograms selenium per gram (0.1 ppm Se and 4.0 ppm Se) diet for 6 weeks. Selenoprotein W was undetectable in skeletal muscle of rats fed the basal (selenium-deficient) diet, detectable in those fed 0.1 ppm selenium in the diet, and were much higher in muscle from rats fed 4 ppm selenium diet. In a species comparison, Western blots indicated the presence of selenoprotein W in muscle of rabbits, sheep and cattle.

INTRODUCTION

At present there are only two known mammalian selenoenzymes. One is the selenium dependent glutathione peroxidase (GSH-Px) family in which at least four members have been identified. The first recognized some 20 years ago was the cellular GSHPx (1). It represented the only known biochemical function of selenium for many years and was used extensively to assess selenium nutritional status. This selenoenzyme (GSHPx-1) contains 4 g atoms of selenium per mole of protein (2, 3). A GSHPx was purified from plasma (GSHPx-p) and shown to be immunochemically distinct from the cellular GSHPx (4). Although it shares some sequence identity with GSHPx-1, it is clearly a separate gene product (5). Like GSHPx-1 the GSHPx-p consists of four identical subunits each of which contains one selenocysteine. A third GSHPx has been characterized in recent years (6). It is a monomer which is similar to the subunits of the previous two GSHPxs and contains one selenocysteine. It is capable of reducing fatty acid hydroperoxides esterified to phospholipids (7) and thus is identified as PHGPX. Evidence was obtained for a fourth GSHPx found predominantly in the gastrointestinal tract (8), designated as GSHPx-GI. The other known mammalian selenoenzyme is Type I iodothyronine 5'-deiodinase, which contains 1 g atom of selenium per mole protein (9, 10). It converts thyroxine to triiodothyronine and thus provides a metabolic link between selenium and iodine.

Selenoprotein P is the most studied selenoprotein without a known function. Its existence has been known for over 15 years and the majority of the selenium in plasma of rats (11) and humans (12) has been shown to be associated with this protein. The

cDNA for selenoprotein P has been cloned and sequenced (13). There are ten TGAs in the open reading frame, indicating that the protein contains ten selenocysteine residues in its primary structure. The levels of this protein in the plasma are affected by the selenium status of the animal (14). The only other selenoprotein known to possess more than one selenocysteine per subunit is mitochondrial capsule selenoprotein (MCS) from mouse sperm which interestingly contains all three selenocysteines in the first 35 amino acids of a total of 197 residues (15). Although evidence for a number of other selenoproteins has been presented (16, 17, 18), the properties of a small molecular weight protein, called selenoprotein W, from muscle were recently presented (19). The present report presents information on the tissue distribution and the effects of selenium status on tissue levels of this protein, an area where there is now no information.

MATERIALS AND METHODS

From the amino acid sequence of selenoprotein W (20) the hydrophilicity was calculated as described by Kyte and Doolittle (21). The first hydrophilic center was located between amino acid residues 13 and 31 (see figure 2-1). A peptide based on these residues (NH_2Cys-Gly-Tyr-Lys-Pro-Lys-Tyr-Leu-Glu-Leu-Lys-Glu-Lys-Leu-Glu-His-Glu-Phe-Pro-Gly-C00H) was synthesized using Fmoc chemistry (22) by the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. The amino terminal cysteinyl residue, which is not part of the selenoprotein sequence, was included for conjugation of the peptide to a carrier protein. The peptide was conjugated through the cysteinyl sulfhydryl to maleimide activated keyhole limpet hemocyanin (22). Rabbits were immunized by injecting 250 μg conjugated peptide in Freund's adjuvant (complete adjuvant for the first injection and incomplete adjuvant for later injections) of this peptide at three week intervals for 9 weeks. Blood sample was taken one week after the last injection and the serum obtained by centrifugation at 1,000 x g for 15 minutes. Polyclonal antibodies were purified from serum by sulfolink affinity columns (Pierce) coupled with the synthetic peptides. Antibodies were concentrated approximately ten fold relative to serum during purification and these preparations were used in the Western blot assays.

Western blot assays were used to survey tissues from a male rat fed commercial rodent chow. The presence of selenoprotein W was tested in muscle, heart, plasma, erythrocytes, liver, kidney, spleen, lungs, intestinal mucosa, intestinal muscle, testis and brain. To determine the effect of selenium status on selenoprotein W levels, weanling

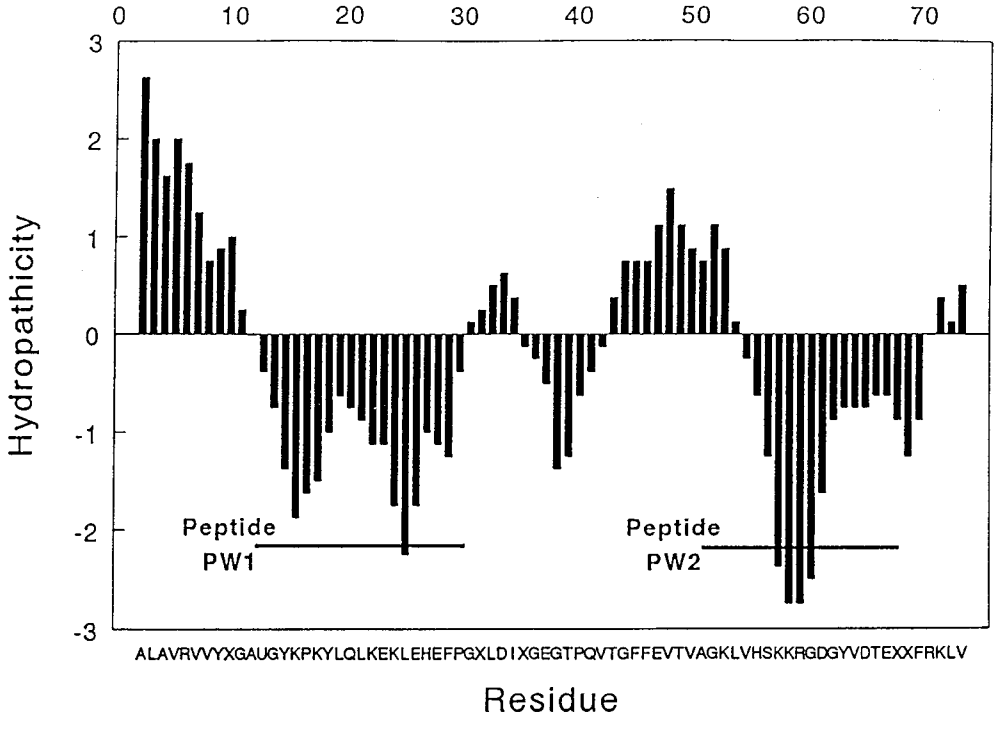

Figure 2-1. Graph of the calculation of the hydropathicity of selenoprotein W.

male rats (4 weeks of age) were fed either the basal diet or this diet with additions of either 0.1 or 4.0 ppm selenium as selenite for 6 weeks. The basal diet was composed of 30% torula yeast (Rhinelander Paper Co., Rhinelander, WI, USA), 51% sucrose, 9% purified cellulose (Solka Floc, Brown Co., Berlin, NH, USA), 5% corn oil, 3.5% AIN-76 mineral mix without selenium and 1% AIN-76 vitamin mix (23), 0.3% DL-methionine, and 0.2% choline citrate. In all studies animals were anesthetized with sodium pentobarbital (80 mg/kg i.p.) and blood was taken via cardiac puncture. After the rats were decapitated while under anesthesia, the tissues were removed and frozen immediately afterwards at -70 °C. In the species survey, muscle samples were taken from adult female rabbits, sheep (wether) and cattle (steer) at the time of slaughter. These animals had been fed commercial diets. The tissues were frozen at -70 °C until used.

Cytosol and membrane fractions of rat muscle were separated according to the method of Farese et al (24). Muscle samples were homogenized in 6 volumes of buffer A (20 mM Tris, pH 7.5, 0.25 M sucrose, 1 mM EGTA, 5 mM EDTA, 1 mM PMSF, 50 mM ß-mercaptoethanol and 25 µg/ml leupeptin). The homogenate was centrifuged at 100,000 x g for 20 min at 4 °C. After the supernatant (cytosol) was collected, the pellet was resuspended in buffer A containing 1% Trition X-100 and placed on ice for 30 min The pellet suspension was centrifuged at 100,000 x g for 20 min at 4 °C and the supernatant (membrane-bound proteins) was collected. The other tissues were homogenized in buffer A at ratio of 1 to 6. Supernatants of 17,000 x g 10 min centrifugation were used in the assays. Protein content of cytosols and membrane-bound protein fractions (muscle) were determined by the Bradford dye-binding assay (25) using bovine serum albumin as standard.

For Western Blot Analysis, samples (100 or 200 μg protein) were electrophoretically separated on 7.5 to 15% gradient SDS-polyacrylamide gels as described by Laemmli (26). Proteins in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 4°C were transferred onto nitrocellulose membrane (0.2 μm ; BA-83, Schleicher & Schuell) according to methods of Towbin et al (27). After transfer, membranes were incubated with blocking solution (5% non-fat dried milk in TTBS [0.05% Tween 20 in TBS]) at 25°C for 1 hr, then incubated with rabbit anti-peptide 1 polyclonal antibody (1:500 dilution) at 25°C for 1.5 hr. Following three washes, membranes were incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad, Richmond, CA). Membranes were then washed four times with TTBS and specific bonding of anti-selenoprotein W antibody onto membrane was detected by ECL detection system (Amersham, Arlington Height, IL). The relative amounts of selenoprotein W present in the various samples were estimated by densitometric scanning of the X-ray film using a Model 1650 transmittance/reflectance scanning densitometer (Bio-Rad) and the Hoefer GS 350 Data System (Hoefer Scientific Instruments, San Francisco, CA).

RESULTS

As shown in Figure 2-1, two major hydrophilic regions in selenoprotein W are predicted by the Kyte and Doolittle algorithm. These are from amino acids 13 to 31 for peptide one, and from 51 to 69 for peptide two. Rabbits were immunized with peptides based on both regions, however, only antibodies to the first peptide (13 to 31) were used for the present studies because they were found to have higher titers and resulted in less background than those produced with antibodies to peptide two.

Western blots of rat tissue preparations revealed the presence of selenoprotein W only in muscle, spleen, testis and brain (Figure 2-2). Based on densitometer readings, this protein was highest in the muscle and brain, and lower in spleen and testis. It was not detected in liver, kidney, intestinal mucosa, intestinal muscle, lungs, heart, plasma and erythrocytes.

Diets supplemented with 0.1 ppm selenium significantly increased the levels of selenoprotein W in muscle as compared to rats fed the deficient diet (Figure 2-3). The bands were undetectable in Western blots of 100 μg muscle cytosol protein from rats fed the 0.1 ppm selenium diet (data not shown). The muscle preparations from rats fed the 4 ppm selenium diet contained much more selenoprotein W than muscle samples from the other two groups of rats. Using a standard curve of purified selenoprotein W, the mean concentration of selenoprotein W in rats fed 0.1 and 4 ppm selenium diets was calculated to be respectively 1.2 and 10.3 ng/mg protein. GSHPx activity and selenium content were low in muscle of the rats fed the deficient diet, confirming their low selenium status (data not shown).

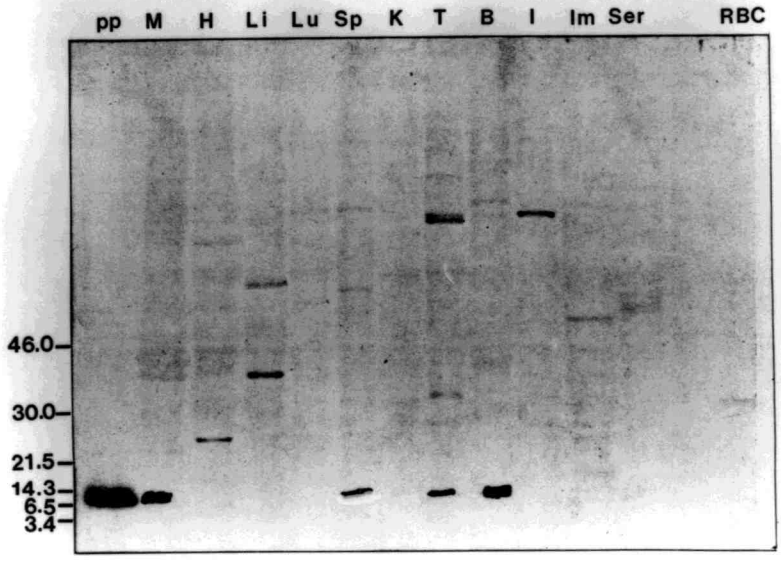

Figure 2-2. Western blot of the tissues from rats fed commercial rat chow. The preparations represent from left to right pure protein (10 ng) from rat muscle (PP), muscle (M), heart (H), liver (Li), lungs (Lu), spleen (Sp), kidney (K), testis (T), brain (B), intestine (I), intestinal mucosa (Im), serum (Ser) and erythrocytes (RBC). Levels of 100 μg protein were used for each tissue. Ovalbumin (46 KD), carbonic anhydrase (30 KD), trypsin inhibitor (21.5 KD), lysozyme (14.3 KD), aprotinin (6.5 KD) and insulin b chain (3.4 KD) were used as the molecular weight markers.

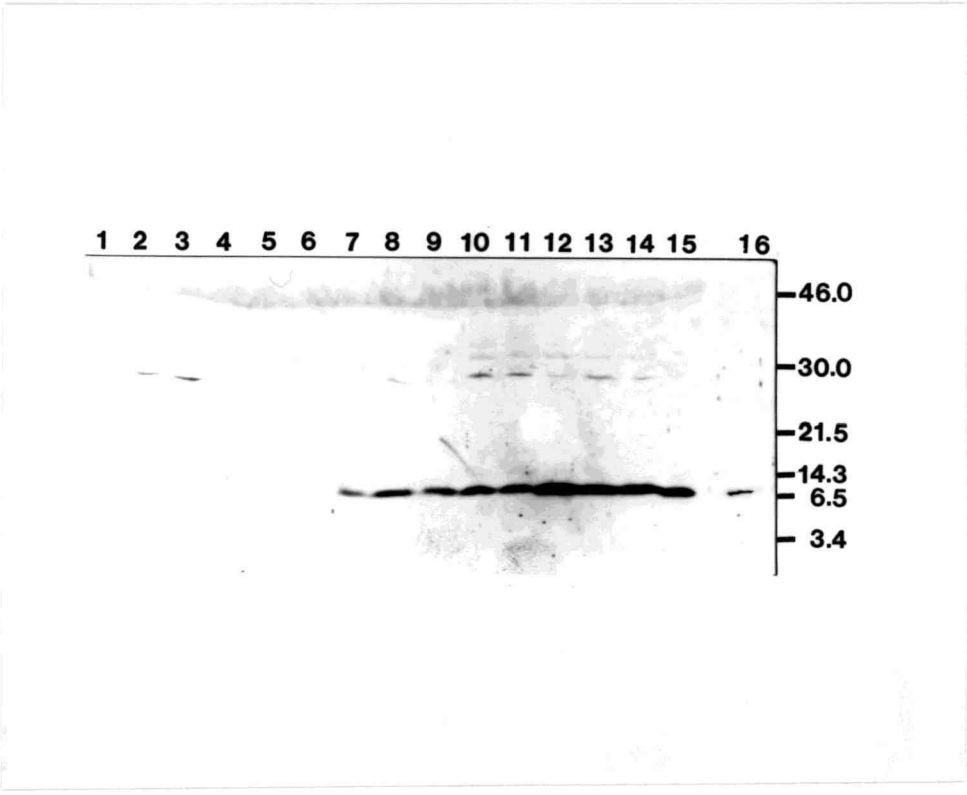

Figure 2-3. Effects of different levels of selenium on selenoprotein W in rat muscle. Lanes 1-6 are of muscle from rats fed diet with no added selenium; lanes 7-11 are of muscle from rats fed diet with 0.1 ppm Se and lanes 12-15 are of muscle from rats fed the diet with 4 ppm Se. An amount of 200 μg protein were used for all samples. Lane 16 is 5 ng of purified selenoprotein W from rat muscle. The same molecular weight standards as noted in figure 2-2 were used.

The antibody was shown to be specific for selenoprotein W by Western blot method (Figure 2-4). Although proteins of higher molecular weight produced a signal in the Western blot analysis, they did not interfere in the selenoprotein assay. The high molecular weight signals were artifacts produced by the secondary antibody. Omission of the primary antibody still produced the high molecular weight signal, but eliminated the selenoprotein W signal. Addition of synthetic peptide to the primary antibody solution before membrane incubation eliminated the selenoprotein W signal, but the high molecular weight signal was unaffected. As would be expected, the extraneous bands were not affected by the selenium status of the animal.

Selenoprotein W was present mainly in the cytosol of the muscle (Figure 2-5A, lanes 1 and 2) but very little was associated with the membranes (Figure 2-5A, lane 3). Muscle samples from rabbit, sheep and cattle indicated the presence of this protein in these tissues (Figure 2-5B). Based on the densitometric reading, it appears to be present at higher concentrations in muscle from sheep, cattle and rabbits than in this tissue from the rat.

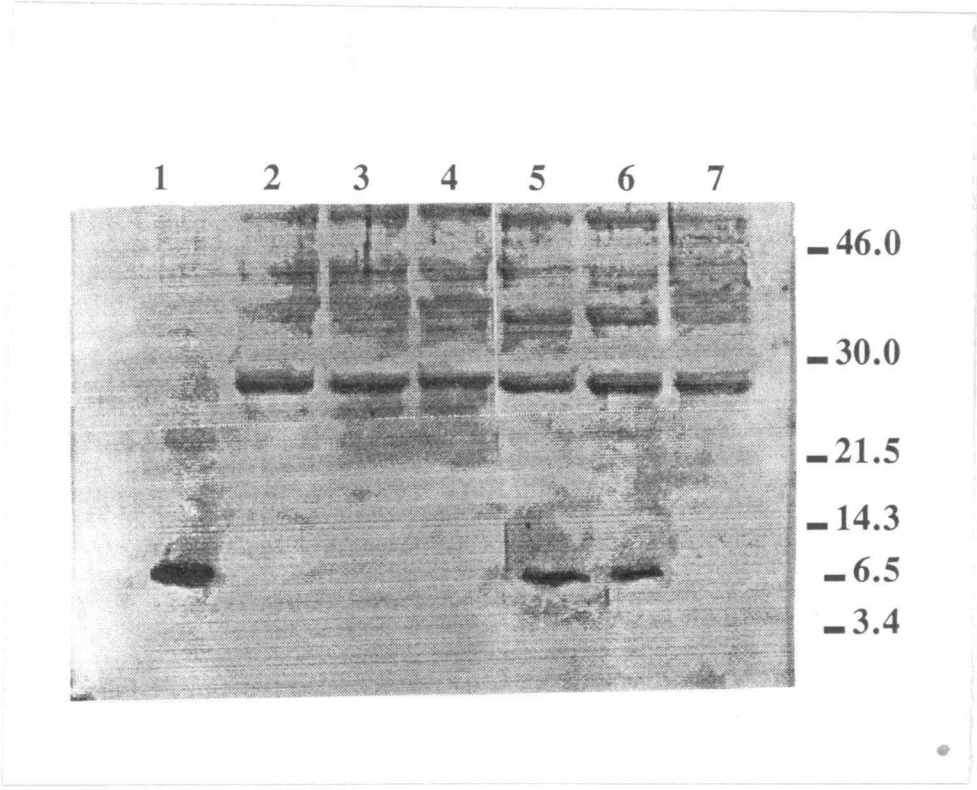

Figure 2-4. The effects of primary and secondary antibodies on the signals in the Western blots. Lane 1 is the standard purified selenoprotein W (10 ng) from rat muscle; lane 2 is where the primary antibody (anti-peptide antibody) was omitted but the secondary antibody (goat anti-rabbit IgG antibody) was included; lane 3 is where the muscle cytosol was incubated with the primary antibody before application to the nitrocellulose membrane; lane 4 is where the primary antibody was incubated with the peptide before application to the nitrocellulose membrane; lane 5 is where both primary and secondary antibodies were included (the usual procedure); lane 6 is cytosol from the selenium supplemented rat; and lane 7 is the muscle cytosol from selenium deficient rat.

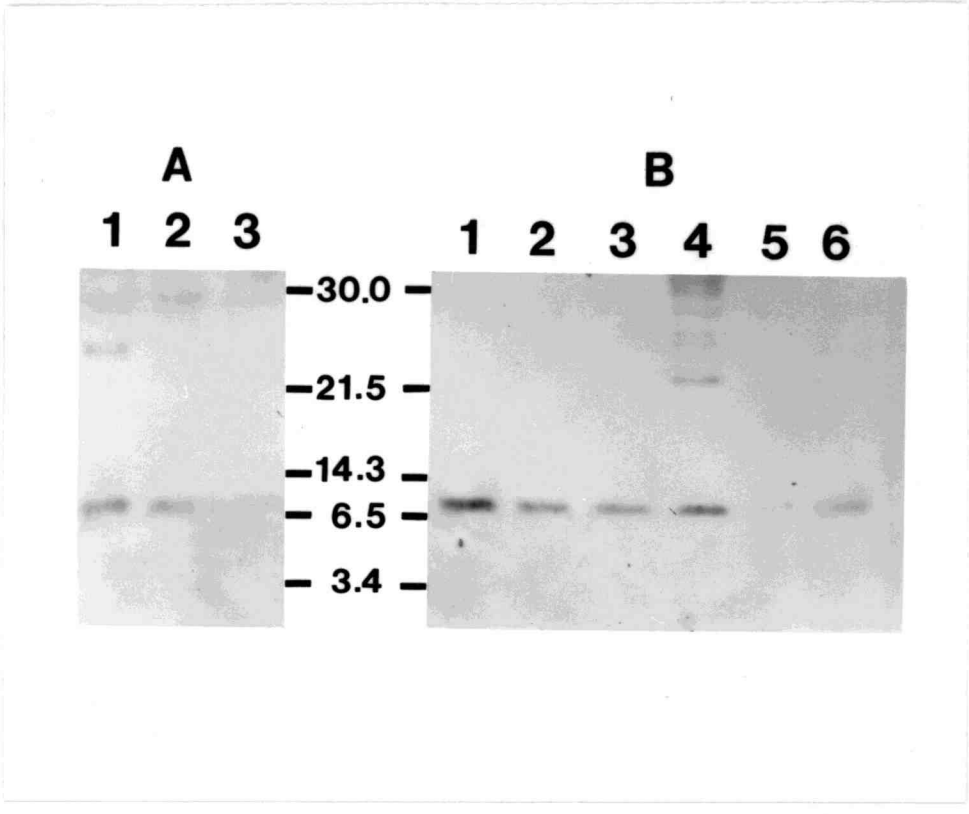

Figure 2-5. Western blots of muscle fractions from rats and of muscle cytosols from rabbits, sheep, cattle and rats. A: compartmentation of selenoprotein W in rat muscle. Lane 1: 100 μg protein from supernatant of muscle homogenate centrifuged at 17,000 xg for 10 mins. Lane 2: 100 μg protein from cytosol fraction of muscle homogenate centrifuged at 100,000 xg for 20 mins. Lane 3: 100 μg protein from membrane fraction of muscle homogenate. B: Western blot of selenoprotein W in muscle from different species. Lane 1: partial pure selenoprotein W. Lanes 2, 3, 4, 5 and 6 are 100 μg cytosolic protein from muscles of sheep, cattle, rabbit, male rat and female rat, respectively.

DISCUSSION

The tissue distribution of selenoprotein W is peculiar and does not offer obvious suggestions regarding its function. The presence in muscle, spleen, testis and brain is intriguing but it is not obvious why just these tissues would contain this protein. The tissue distribution may differ between species. For example, based on ^{75}Se incorporation selenoprotein W is assumed to be present in hearts of sheep (28). After injection with radioactive selenium, gel filtration patterns of sheep heart cytosol indicated equal incorporation of selenium into selenoprotein W and GSHPx. This was confirmed by recent Western blots of heart preparations from sheep fed high levels of selenium in the diet. Selenoprotein W was almost as high in the heart as in muscle. The absence of this protein in rat heart was somewhat surprising but this may relate to the difference in selenium deficiency syndromes between species. Selenium deficiency results in cardiac damage in sheep (29) but not in rats (30). Recent work with guinea pigs and sheep indicates the presence of selenoprotein W in a number of tissues, suggesting species differences on the tissue distribution of this selenoprotein.

We have recently cloned cDNA for selenoprotein W. The unknown amino acids identified by Xs in Figure 2-1 are now known to be cysteine at positions 9, 32 and 36, serine at position 70 and lysine at position 71. Selenocysteine is at position number 12 as identified by U in this figure. The final 10 amino acids (77-87) are threonine, alanine, isoleucine, lysine, alanine, alanine, leucine, alanine, glycine, cysteine and glycine. Examination of the amino acid sequence around the selenocysteine residues of GSH-Px-1 from rat liver (31) or bovine erythrocytes (32), GSH-Px-P from human plasma (5) or rat

plasma (33), PHGPX (6), GSH-Px-GI (8), type I iodothyronine 5'-deiodinase (34), MCS (15) and selenoprotein P (13) revealed no homology among them. All except selenoprotein P have the selenocyteine residues in the early part of the molecule, which is similar to selenoprotein W. GSH-Px-1, GSH-Px-P, PHGPX and GSH-Px-GI have valine, alanine, and serine as the second, third and fourth amino acid before the selenocysteine residue, but this is not the case for the other selenoenzymes or selenoproteins. A unique feature of MCS is the presence of a cysteine residue adjacent to all three selenocyteine residues (15). The unique features of selenoprotein P are that most of the selenocysteine residues are near the carboxyl end of the protein and there are seven histidines in a row in this protein (13). Selenoprotein W (Figure 2-1) is similar to MCS (15) in that there is a proline (third and fourth respective for MCS and selenoprotein W) near the selenocysteine residues. It should be noted that in the earlier work tryptophan was mistakenly indicated to be present in selenoprotein W (19). Instead, this should have been tyrosine.

Selenium status can affect tissue levels of this protein (Figure 2-3). This is consistent with earlier studies in which radioactive selenium was used. Gel filtration showed a lower molecular weight radioactive selenium containing peak in tissue preparations from sheep, rats and cattle (28, 35, 36). In sheep, the level of the low molecular weight selenoprotein appeared to be affected by selenium status (28). Antibodies to a peptide from selenoprotein W detected the selenoprotein in muscle from sheep, rabbit and cattle (figure 2-5B). The immunological cross-reactivity implies that this protein may have highly homologous amino acid sequence among these species. In addition, the higher densitometric readings in sheep, cattle and rabbit suggest either that

higher concentrations of homologous proteins exist in these species or that antibody affinity is higher against the homologous proteins.

Selenium deficiency results in a muscular and cardiac degeneration in lambs and calves called White Muscle Disease (29). Likewise, a severe nutritional selenium deficiency in discrete regions of China was associated with an endemic juvenile cardiomyopathy disorder called Keshan disease (37). Muscle weakness has been shown to be prevented by selenium supplementation in patients on long term parenteral nutrition (38, 39). Therefore, there is sufficient evidence to indicate that selenium is important for normal muscle metabolism. Whether selenoprotein W has any significant role in muscle metabolism cannot be determined from the present work, but since it is present in muscle at higher concentration than other tissues this could be suggestive of a critical role in this tissue. The ability to determine tissue levels of selenoprotein W by an immunological assay as shown in the present report should assist in the determination of its role in normal muscle metabolism and possibly how it is involved in the prevention of nutritionally induced myopathies.

The reason we used peptides with the most hydrophilic character is because work by others indicated that the hydrophilic peptides are the most antigenic (40). The first region of the protein contains two prolines, which are very antigenic. We have also made polyclonal antibodies to the second hydrophilic region (amino acids 51-64), which we have called peptide 2. In addition, monoclonal antibodies have been made to both peptides. Unfortunately, neither of the two monoclonal antibodies would function in the Western blots with tissue cytosols.

REFERENCES

1. Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. and Hoekstra, W. G. (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179, 588-90.

2. Oh, S-H, Ganther, H. E. and Hoekstra, W. G. (1974) Selenium as a component of glutathione peroxidase isolated from ovine erythrocytes. Biochemistry 13, 1825-1829.

3. Flohe, L. Gunzler, W. A. and Schock, H. H. (1973) Glutathione peroxidase: a selenoenzyme. FEBS Lett. 32, 132-134.

4. Takahashi, K., Avissar, N., Whitin, J. and Cohen, H. (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch. Biochem. Biophys. 256, 677-86.

5. Takahashi, K., Akasaka, M., Yamamoto, Y., Koyayashi, C., Mizoguchi, J. and Koyama, J. (1990) Primary structure of human plasma glutathione peroxidase deduced from cDNA sequences. J. Biochem. 108, 145-48.

6. Schuckelt, R., Brigelius-Flohe, R., Maiorino, M., Roveri, A. and Reumkens, J. (1991) Phospholipid hydroperoxide glutathione peroxidase is a selenoenzyme distinct from the classical glutathione peroxidase as evident from cDNA and amino acid sequencing. Free Radical Res. Commun. 14, 343-61.

7. Ursini, F., Maiorino, M. and Gregolin, C. (1985) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim. Biophys. Acta 839, 62-70.

8. Chu, F-F., Doroshow, J. H. and Esworthy, R. S. (1993) Expression, characterization and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. J. Biol. Chem. 268, 2571-2576.

9. Behne, D., Kyriakopoulos, A., Meinhold, H. and Kohrie, J. (1990) Identification of type I iodothyronine 5'-deiodinase as a selenoenzyme. Biochem. Biophys. Res. Commun. 173, 1143-49.

10. Arthur, J. R., Nicol, F. and Beckett, G. J. (1990) Hepatic iodothyronine 5'-deiodinase. Biochem. J. 272, 537-40.

11. Read, R., Bellew, T., Yang, J-G., Hill, K. E., Plamer, I. S. and Burk, R. F. (1990) Selenium and amino acid composition of selenoprotein P, the major selenoprotein in rat serum. 265, 17899-17905.

12. Deagen, J. T., Butler, J. A., Zachara, B. A. and Whanger, P. D. (1993) Determination of the distribution of selenium between glutathione peroxidase, selenoprotein P and albumin in plasma. Anal. Biochem. 208, 176-184.

13. Hill, K. E., Lloyd, R. S., Yang, J-G, Read, R. and Burk, R. F. (1991) The cDNA for rat selenoprotein P contains 10 TGA codons in the open reading frame. J. Biol. Chem. 266, 10050-53.

14. Yang, J-G, Hill, K. E. and Burk, R. F. (1989) Dietary selenium intake controls rat plasma selenoprotein P concentration. J. Nutr. 119,1010-12.

15. Karimpour, I., Cutler, M., Shih, D., Smith, J. and Keene, K. C. (1992) Sequence of the gene encoding the mitochondrial capsule selenoprotein of mouse sperm: identification of three in-phase TGA selenocysteine codons. DNA Cell Biol. 11, 692-699.

16. Behne, D., Hilmert, H., Scheid, S., Gessner, H. and Elger, W. (1988) Evidence for specific selenium target tissues and new biologically important selenoproteins. Biochim. Biophys. Acta. 966, 12-21.

17. Hawkes, W. C., Wilhelmsen, E. C. and Tappel, A. L. (1985) Abundance and tissue distribution of selenocysteine-containing proteins in the rat. J. Inorg. Biochem. 23, 77-92.

18. Whanger, P. D. (1987) Evidence for non-glutathione peroxidase selenium-containing proteins in mammalian tissues. In: Selenium in Biology and Medicine, Third International Symp., Beijing, PRC (Combs, G. F. et al, eds) Avi Book, van Nostrand Reinhold Comp., New York, pp.197-212.

19. Vendeland, S. C., Beilstein, M. A., Chen, C. L., Jensen, O. N., Barofsky, E. and Whanger, P. D. (1993) Purification and properties of selenoprotein W from rat muscle. J. Biol. Chem. 268, 17103-17107.

20. Beilstein, M. A., Vendeland, S. C., Andrews, J. and Whanger, P. D. (1993) Immunization with a synthetic peptide to produce antibodies to a rat muscle selenoprotein. The FASEB J. 7, A289.

21. Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105-132.

22. Fields, C. G., LLoyd, D. H., MacDonald, R. L., Otteron, K. M. and Noble, R. L. (1991) HBTV activation for automated Fmoc solid-phase peptide synthesis. Peptide Research 4, 95-101.

23. American Institute of Nutrition. (1977) Report of the AIN Ad Hoc Committee on standards for nutritional studies J. Nutr. 107, 1340-1348.

24. Farese, R. V., Standaert, M. L., Francois, A. J., Ways, K., Arnold, T. P., Hernandez, H. and Cooper, D. R. (1992) Effects of insulin and phorbol esters on subcellular distribution of protein kinase C isoforms in rat adipocytes. Biochem. J. 288, 319-323.

25. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72, 248-254.

26. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

27. Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci., U. S. A. 76, 4350-4354.

28. Whanger, P. D. (1993) Some biochemical properties of selenoprotein W. TEMA-8 (Anke, M. et al, eds) Verlag Media Touristik, Gersdorf, Germany, pp.119-126.

29. Schubert, J. R., Muth, O. H., Oldfield, J. E. and Remmert, L. F. (1961) Experimental results with selenium in white muscle disease of lambs and calves. Fed Proc 20, 689-692.

30. Schwarz, K and Foltz, C. M. (1957) Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J. Amer. Chem. Soc. 79, 3292-3293.

31. Ho, Y-S., Howard, A. J. and Crapo, J. D. (1988) Nucleotide sequence of a rat glutathione peroxidase cDNA. Nucleic Acids Res. 16:5207-5210.

32. Gunzler, W. A., Steffens, G. J., Grossmann, A., Kim, S. M. A., Otting, F., Wendel, A. and Flohe, L. (1984) The amino acid sequence of bovine glutathione peroxidase. Hoppe-Zeyler's Z. Physiol. CHem. 365, 195-212.

33. Yoshimura, S., Wantanabe, K., Shemizu, H., Onozawa, T., Mizoguchi, J., Tosuda, K. Hatta, H. and Morinchi, T. (1991) Tissue specific expression of the plasma glutathione peroxidase gene in rat kidney. J. Biochem. 109,918-923.

34. Berry, M. J., Banu, L. and Larsen, P. R. (1991) Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. Nature 349,438-440.

35. Beilstein, M. A. and Whanger, P. D. (1984) Effects of cyanide on selenium metabolism in rats. J. Nutr. 114, 929-937.

36. Pond, F. R., Tripp, M. J., Wu, A. S. H., Whanger, P. D. and Schmitz, J. A. (1983) Incorporation of selenium-75 into semen and reproductive tissues of bulls and rams. J. Reprod. Fert. 69, 411-418.

37. Chen, X. S., Yang, G. Q., Chen, J. S., Chen, X. C., Wen, Z. M., and Ge, K. Y. (1980) Studies on the relations of selenium and Keshen disease. Biol. Trace Elem. Res. 2,91.

38. Brown, M. R., Cohen, H. J., Lyons, J. M., Curtis, T. W. Thunberg, B., Cochran, W. J. and Klish, W.J. (1986) Proximal muscle weakness and selenium deficiency associated with long term parenteral nutrition. Am. J. Clin. Nutr. 43, 549-554.

39. van Rij, A. M., McKenzie, J. M. Thomson, C.D. and Robinson, M. F. (1981) Selenium supplementation in total parenteral nutrition. JPEN 5,120-124.

40. Brodt, P. (1983) Tumor immunology--three decades in review. Ann. Rev. Microbiol. 37, 447-476.

CHAPTER 3

EFFECT OF DIETARY SELENIUM UPON SELENOPROTEIN W LEVELS IN RAT TISSUES*

Jan-Ying Yeh, Susan C. Vendeland, Azizah Mohd, Qui-ping Gu,
Judy A. Butler, Bor-Rung Ou and Philip D. Whanger

Department of Agricultural Chemistry and Animal Sciences,
Oregon State University,
Corvallis, OR 97331

Shortened title: Tissue selenoprotein W

*Published with the approval of the Oregon State Agricultural Experiment Station as Technical Paper_____. This research was supported by Public Health Service Research Grant number DK 38306 from the National Institute of Diabetes and Digestive and Kidney Diseases.

ABSTRACT

Two experiments were conducted to evaluate the influence of dietary selenium on tissue levels of selenoprotein W. Glutathione peroxidase (GPX) activity and selenium levels were also determined for comparative measurements. In the first experiment rats were fed a basal deficient diet or this diet supplemented with either 0.1 or 4.0 mg selenium per kg diet. Selenoprotein W levels were significantly higher in muscle, brain, spleen and testes of rats fed the diet with 0.1 mg selenium per kg as compared to controls, but those fed the diet with 4.0 mg selenium per kg had even higher levels than those fed the diet with 0.1 mg selenium per kg. There was a corresponding increase of mRNA encoding selenoprotein W with each increase of dietary selenium. In the second experiment rats were fed the basal diet or this plus 0.03, 0.05, 0.1, 0.5, 1.0, 2.0 or 4.0 mg selenium per kg diet. The levels of selenoprotein W in muscle started to increase with 0.06 mg selenium per kg and then increased markedly with 1.0 mg selenium per kg diet where a plateau was reached. There was a linear increase of selenoprotein W in brain and spleen with the selenium content in the diet, except a plateau was reached at 1.0 mg selenium per kg diet in the spleen. The testes showed a different pattern in that a very marked increase, as compared to controls, occurred with 0.01 mg selenium per kg diet where a plateau was reached. Except for the muscle, GPX activities reached a plateau in all tissues from 0.06 to 0.1 mg selenium per kg diet. The selenium content in these tissues increased at a linear rate with the selenium content in the diets. The results indicate that the regulation of selenoprotein W by selenium is different in various tissues and also differs from that for GPX.

INTRODUCTION

At present there are four known members of the family of glutathione peroxidases (GPX). The first to be recognized was the cellular GPX (cGPX, Rotruck et al, 1973). A GPX was purified from plasma (GPX-p) and shown to be immunochemically distinct from the cGPX (Takahashi et al, 1987). A third GPX was discovered to be capable of reducing fatty acid hydroperoxides esterified to phospholipids (Ursini et al, 1985) and has been identified as PHGPX. Evidence was obtained for a fourth GPX found predominantly in the gastrointestinal tract (Chu et al, 1993) and is designated as GPX-GI.

The use of graded levels of dietary selenium allows investigators to determine the selenium response curves for various parameters. PHGPX and cGPX have been shown to be differentially regulated by selenium status (Lei et al, 1995). Liver cGPX activity was highly regulated by selenium status with virtually no activity in rats fed a selenium deficient diet. Relative plateau levels of activity was reached with 0.1 mg selenium per kg diet (Hafeman et al, 1974; Knight and Sunde, 1987). In contrast, liver PHGPX was reduced only 40% of plateau with the deficient diet but reached relative plateau levels with dietary selenium levels less than 0.1 mg selenium per kg (Lei et al, 1995). Therefore, since the two GPXs are differentially regulated, other selenoproteins are likely to be affected differently by selenium.

A low molecular weight selenoprotein, selenoprotein W, was recently purified and characterized (Vendeland et al, 1993). In rats fed a regular diet, Western blots indicated that this selenoprotein is detectable in muscle, brain, spleen and testes (Yeh et al, 1995). This selenoprotein was found to be undetectable in muscle of deficient rats but was

present in this tissue of rats given selenium, suggesting that selenium status will affect the level of this selenoprotein. This is supported by information showing that mRNA levels for selenoprotein W are affected by selenium status (Vendeland et al, 1995). However, no information is available on the regulation of this selenoprotein by selenium and this is the purpose of this study. The response of this selenoprotein with respect to selenium status was investigated and is reported in this communication.

MATERIALS AND METHODS

Animal Treatment

Two experiments were conducted to evaluate the influence of selenium on selenoprotein W levels in tissues. In the first experiment, male weanling rats (4 weeks of age) were fed either the deficient diet (no selenium added) or this diet supplemented with either 0.1 or 4.0 ppm selenium for 6 weeks. In the second experiment, the rats were fed the deficient diet or this diet supplemented with either 0.01, 0.03, 0.06, 0.1, 1.0, 2.0 and 4.0 ppm selenium for 8 weeks. Selenium was added in the form of sodium selenite. The basal diet was composed of 30% torula yeast (Rhinelander paper Co, Rhinelander, Wis.), 51% 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 basal diet in both experiments was shown by analysis to contain about 0.03 mg selenium per kg diet. In both studies, the animals were anesthetized with sodium pentobarbital (80 mg/kg i.p) and blood was taken via cardiac puncture. After the rats were decapitated while under anesthesia, the tissues were removed and frozen immediately afterward at -70°C. The tissues were kept frozen at this temperature until used. Selenoprotein W levels and GPX activities were measured on all tissues examined.

Western Blot Analysis

Tissues were homogenized in 6 volumes of buffer A (20 mM Tris [pH 7.5], 0.25 M sucrose, 1 mM EGTA, 5 mM EDTA, 1 mM PMSF, 50 mM ß-mercaptoethanol and 25 ug/ml leupeptin), and were centrifuged at 17,000 xg for 10 min at 4°C. Protein content was measured in the supernatants by the dye-binding assay of Bradford (1976) using bovine serum albumin (Bio-Rad, Richmond, CA) as standards. Samples (200 µg protein each) were electrophoretically separated on SDS-polyacrylamide 7.5 to 15% gradient gels as described by Laemmli (1970). Proteins were transferred onto nitrocellulose membranes (0.2 µm, BA-S83; Schleicher & Schuell, Keene, NH) in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 4°C according to method of Towbin et al. (1979). After transfer, membranes were incubated with blocking solution (5% non-fat dried milk in TTBS [0.05% Tween 20 in TBS]) at 25°C for 1 hr, then incubated with rabbit anti-selenoprotein W polyclonal antibody at 25°C for 1.5 hr. Following three washes, membranes were incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad, Richmond, CA). Membranes were then washed four times with TTBS and specific bonding of anti-selenoprotein W antibody onto membrane was 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 (Molecular Dynamics, Sunnyvale, CA) analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA).

Total RNA Extraction

Extraction of total RNA has been described previously (Chomczynski and Sacchi, 1987). Briefly, tissue was homogenized in 2 ml of solution A (4 M guanidium isothionate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl and 10 mM ß-mercaptoethanol) and then transferred to a 15 ml polypropylene tube. To this, 0.2 ml of 2 M sodium acetate (pH 4.0), 2 ml of phenol and 0.4 ml of a chloroform-isoamyl alcohol mixture (49:1) were added sequentially and mixed by inversion after each addition. After brief vortexing, the mixture was kept on ice for 15 min and was separated by centrifugation (12,000 g) for 30 min at 4°C. The upper aqueous phase was transferred to a new tube and then an equal volume of ice-cold isopropanol was added. The samples were mixed and stored at -20°C overnight. RNA was collected by centrifugation (12,000 g, 20 min at 4°C). The supernatant was discarded and the RNA pellet was resuspended in 0.4 ml of solution A. One volume of isopropanol was added, and the mixture was stored at -20°C overnight. The RNA pellet was recovered by centrifugation at 12,000 g for 15 min at 4°C, washed twice with 70% ethanol and dried under a vacuum. The dried RNA pellet was then dissolved in diethylpyrocarbonate (DEPC)-treated water and was quantitated by spectrophotometry at a wavelength of 260 nm.

Northern Blot Analysis

Total RNA samples (25 µg) were denatured at 55°C for 15 min, applied to a 1.2% agarose gel containing 2.2 M formaldehyde, then electrophoresed at 30 V for 12 h. RNA

was transferred onto nylon membranes (GeneScreenPlus, DuPont/NEN) and immobilized by baking at 70°C for 40 min. Selenoprotein W cDNA and 16 S ribosomal RNA oligonucleotides (Giovannoni, 1991) were labeled by using DIG Labeling Kit (Boehringer Mannheim Biochemica, Indianapolis, IN). The RNA membrane was hybridized and washed according to the procedures described in DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim Biochemica, Indianapolis, IN). Briefly, membranes were prehybridized at 42°C for 1 hr in hybridization buffer (50% formamide, 5 X SSC, 0.02% SDS, 0.1 % N-lauroylsarcosine and 2% Blocking Reagent). After prehybridization, heat-denatured DIG labeled selenoprotein W probe was added to hybridization buffer and membrane was hybridized at 42°C overnight. Next day, the membrane was washed in 0.5 X SSC with 0.1% SDS at room temperature. After washing briefly in Buffer 1 containing 0.3% Tween 20, the membrane was incubated in Buffer 2 for 30 min. Anti-digoxigenin-AP conjugated antibody was diluted in Buffer 2 (1:5000) and the membrane was incubated in this buffer for 30 min. The unbound antibody conjugate was removed by washing the membrane in Buffer 1. The membrane was then equilibrated in Buffer 3 for 2 min and substrate solution was added. After incubation at 37°C for 5 min, the excess substrate was removed and the membrane was exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY). Developed films were scanned with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA). Before hybridization to the internal control probe, the membrane was rinsed thoroughly in water and then incubated in stripping buffer (50% formamide, 50 mM Tris-HCl [pH 8.0], and 1% SDS) at 68°C for 1 hr to remove the probe. After rinsing with water and in 2 X SSC,

the membranes were used for hybridization of internal control probe (16 S rRNA oligonucleotides).

Glutathione Peroxidase Activity and Selenium Content

cGPX activity was measured by an enzyme coupled method using glutathione reductase, utilizing hydrogen peroxide (0.171 mM) as substrate (Pagli and Valentine, 1967) by a DU Series 60 Spectrophotometer (Beckman Instrument, Fullerton, CA). After digestion of the tissue fractions with nitric and perchloric acids, selenium was determined by the semiautomated fluorimetric method (Brown and Watkinson, 1977) using an Alpchem II system (Alpchem. Corp., Milwaukie, OR).

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 for comparing groups (Steel and Torrie, 1980). A significance level of 5% was adopted for all comparisons.

RESULTS

Experiment 1.

There were no differences in the food consumption of the rats in this study. However, the rats fed the deficient diet gained (181 ± 5 gms) significantly less ($P < 0.05$) than those fed the other two diets (197 ± 5 for those fed 0.1 mg selenium per kg diet or 211 ± 4 for those fed 4.0 mg selenium per kg diet). There were no differences in the weight gains between rats fed the 0.1 mg selenium per kg diet versus those fed the 4.0 mg selenium per kg diet.

Except for the brain, there was a corresponding increase of tissue selenium with each increase of dietary selenium (Table 3-1). There was not a statistical increase of the selenium content of the brain. Except for the testis, the selenium content was higher in tissues from rats fed the 0.1 mg selenium per kg diet than those fed the basal diet, and those fed the 4.0 mg selenium per kg diet were higher than in tissues from those fed the 0.1 mg selenium per kg diet. The selenium content in the testes from rats fed the 0.1 mg selenium per kg diet was higher than in this organ from rats fed the basal diet but this content in testes was not significantly higher in rats fed the 4.0 mg selenium per kg diet as compared to those fed the 0.1 mg selenium per kg diet.

The cGPX and GPX-p activities were significantly higher as compared to the respective lower level of selenium fed in plasma, testes and spleen (Table 3-2). However, even though this activity was higher in muscle in rats fed the 0.1 mg selenium per kg diet than muscle from control rats, there was not further significant increases when

Table 3-1. Tissue selenium content of rats fed Torula yeast diets supplemented with 0, 0.1 or 4.0 mg selenium as selenite per kg.

Tissues	0 ppm Se	0.1 ppm Se	4.0 ppm Se
plasma	0.03 ± 0.001^{a}	0.38 ± 0.02^{b}	0.45 ± 0.02^{c}
muscle	0.14 ± 0.01^{a}	0.41 ± 0.01^{b}	0.71 ± 0.10^{c}
brain	0.52 ± 0.02	0.56 ± 0.09	0.71 ± 0.06
testis	5.08 ± 0.11^{a}	6.92 ± 0.45^{b}	7.75 ± 0.18^{b}
spleen	0.63 ± 0.03^{a}	1.70 ± 0.04^{b}	3.02 ± 0.26^{c}
liver	0.07 ± 0.02^{a}	1.67 ± 0.04^{b}	3.15 ± 0.28^{c}

* Values are means of each treatments ± SE. Different superscripts denote significance ($p < 0.05$).

** Tissue selenium contents are expressed as μg Se/ g dry weight.

Table 3-2. GPX activities of rats fed Torula yeast diets supplemented with 0, 0.1 or 4 mg selenium as selenite per kg.

Tissues	0 ppm Se	0.1 ppm Se	4.0 ppm Se
plasma	3.4±0.2[a]	47.9±2.5[b]	58.8±1.7[c]
Muscle	36.7±2.4[a]	146.9±13.4[b]	179.4±21.8[b]
Brain	83.4±2.6[a]	82.2±1.9[a]	103.4±6.0[b]
Testis	55.6±1.6[a]	103.3±1.0[b]	118.2±4.3[c]
Spleen	249.5±17.7[a]	594.1±14.0[b]	685.8±4.4[c]

* Values are means of each treatments ± SE. Different superscripts denote significance ($p < 0.05$).

** GPX activity expressed as nmoles NADPH oxidized/min/mg protein.

4.0 mg selenium was present in the diet. The brain showed a different pattern in that this activity was not significantly higher in rats fed the 0.1 mg selenium diet versus those fed the basal diet, but was significantly higher in this tissue of rats fed the 4.0 mg selenium per kg diet.

Western blots for selenoprotein W in brain, testes and spleen are shown in figure 3-1. These blots for the muscle are shown in an earlier publication (Yeh et al, 1995). The density of the bands were used to determine the concentration of selenoprotein W in tissues.

Selenoprotein W was not detectable in muscle of deficient rats, but was present in muscle of rats fed the diet with 0.1 mg selenium per kg (Table 3-3). Those fed 4.0 mg selenium per kg had significantly higher ($P < 0.05$) levels in the muscle than in muscle from those fed the 0.1 mg selenium per kg diet.

Northern blots of tissues are shown in figure 3-2. The intensities of the bands were greatest for testes, muscle and brain. This information was used to calculate the relative levels of mRNA in tissues.

The northern blots of the muscle from rats fed the three levels of selenium are shown in figure 3-3. The intensities of the bands increased with each increase of the dietary selenium levels.

The information in the Northern blots was used to determine the relative mRNA levels in rats fed the three levels of dietary selenium (Table 3-4). The mRNA levels when expressed as the ratio to the internal standard increased significantly ($P < 0.05$) with each increase of dietary selenium.

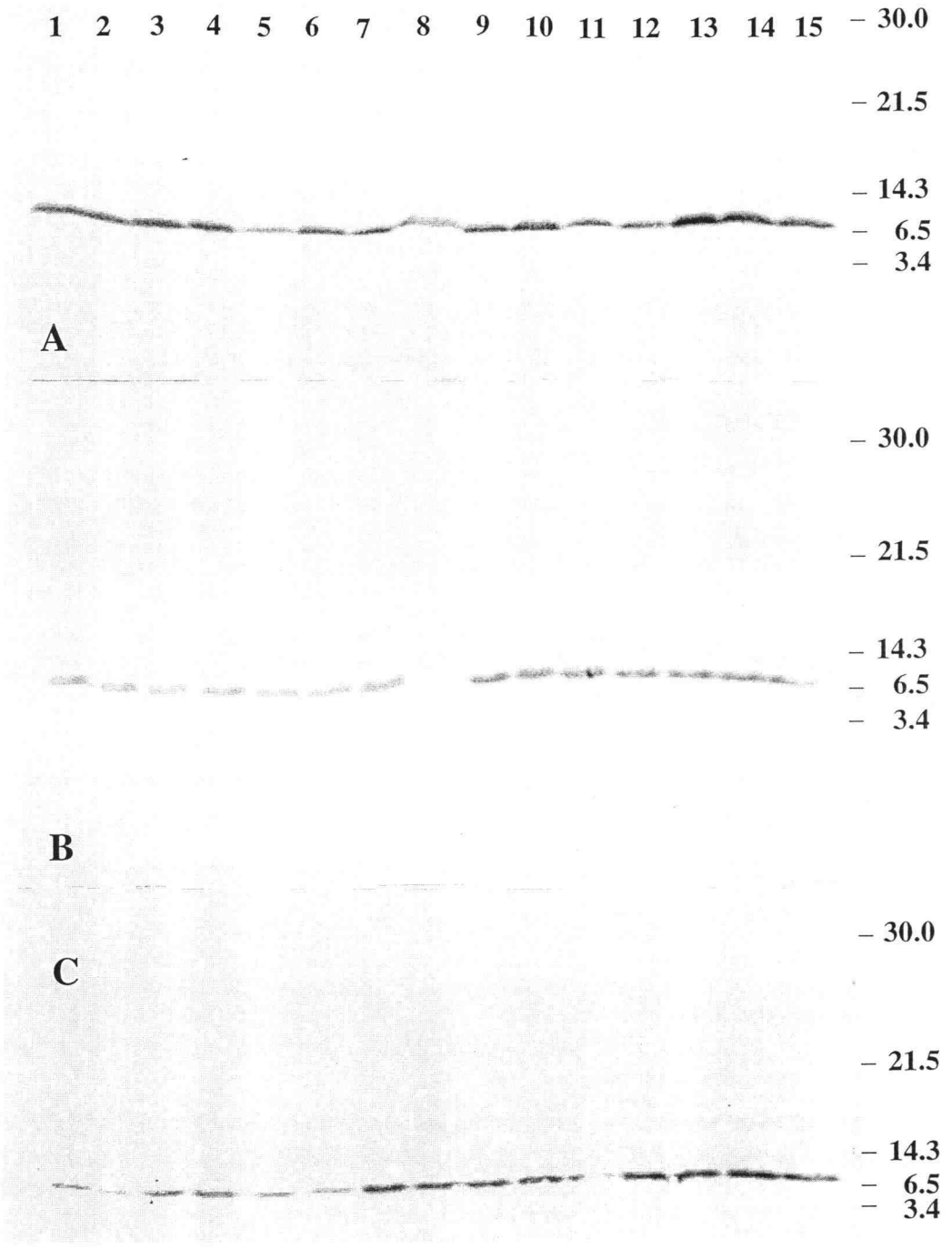

Figure 3-1. Western blots of selenoprotein W in different tissues of rats fed Torula yeast diets with various selenium supplementation. (A) Brain, (B) Testis and (C) Spleen. Lanes 1-6 are from rats fed basal diet. Lanes 7-11 are from rats fed 0.1 ppm Se diet. Lanes 12-15 are from rats fed 4.0 ppm Se diet.

Table 3-3. Effects of selenium status on selenoprotein W of rats fed Torula yeast diets supplemented with 0, 0.1 or 4 mg selenium as selenite per kg.

Tissue	0 ppm Se	0.1 ppm Se	4.0 ppm Se
Muscle	N.D.	893±181[a]	2235±383[b]
Brain	722±59[a]	728±67[a]	1066±182[b]
Testis	359±6[a]	638±56[b]	613±31[b]
Spleen	242±34[a]	599±40[b]	829±45[c]

* Values are means of each treatments ± SE. Different superscripts denote significantly different ($p < 0.05$). The values are given in scan units.

** N.D. = non-detectable.

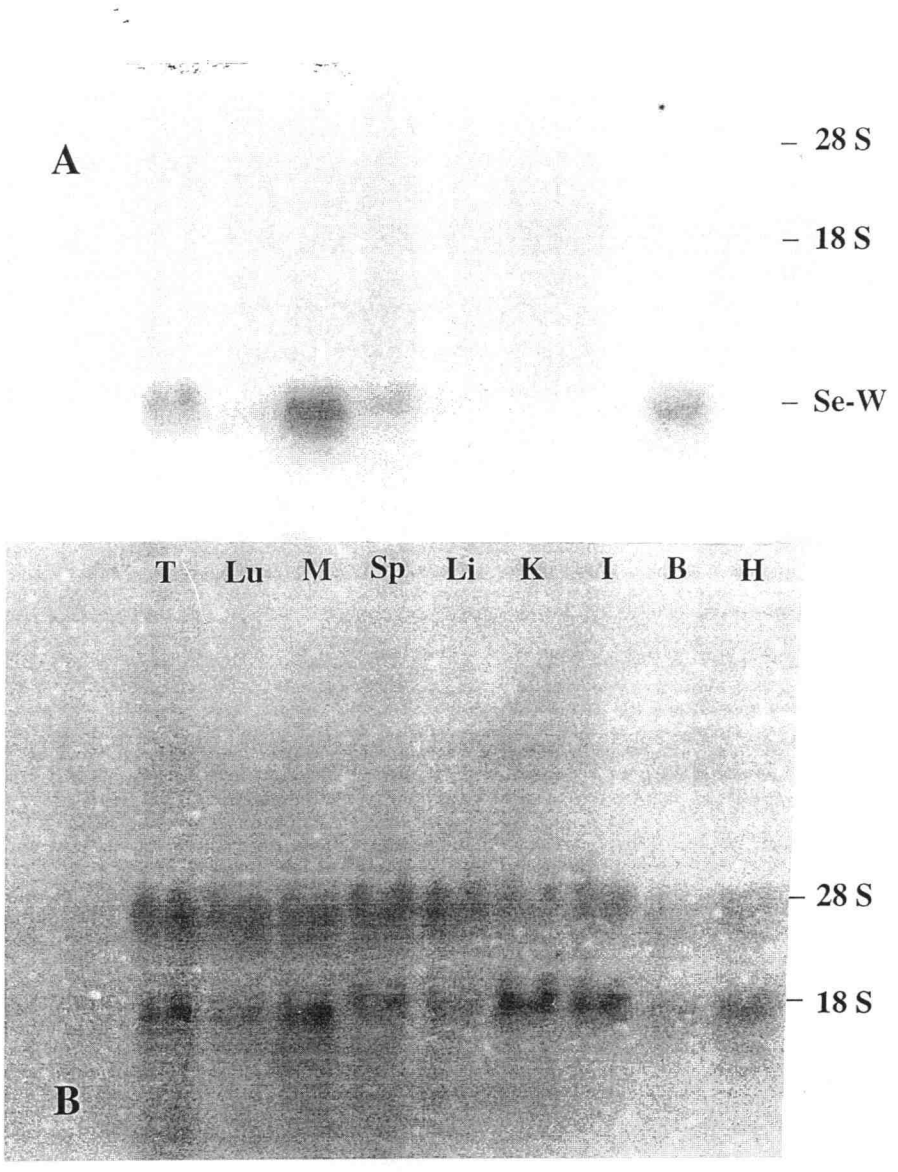

Figure 3-2. Tissue distribution of selenoprotein W mRNA from rat fed 4 ppm Se diet. (A) Hybridization to selenoprotein W cDNA probe (B) Hybridization to internal control probe (18 S rRNA). T: testis, Lu: lung, M: muscle, Sp: spleen, Li: liver, K: kidney, I: intestine, B: brain and H: heart.

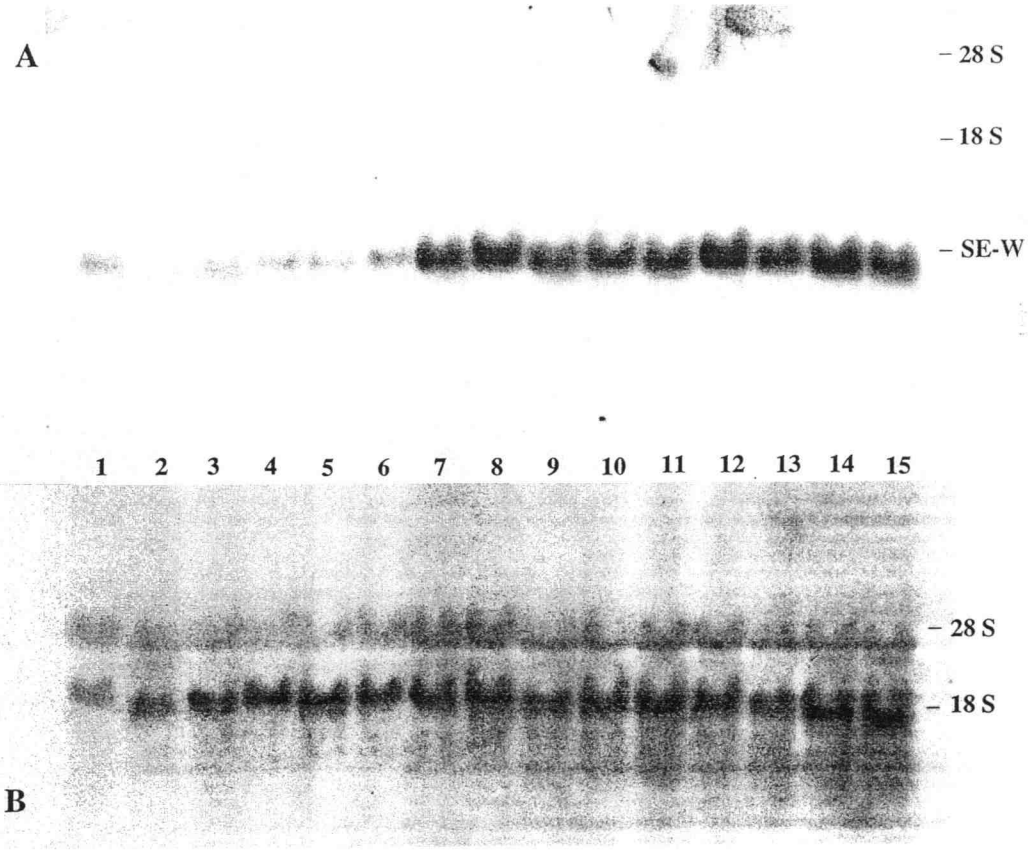

Figure 3-3. Northern blots of selenoprotein W mRNA in muscles from rats fed Torula yeast diets with additions of 0, 0.1 and 4.0 ppm Se. (A) Hybridization to selenoprotein W cDNA probe (B) Hybridization to internal control probe (18 S rRNA). Lanes 1-6 are from rats fed basal diet. Lanes 7-11 are from rats fed 0.1 ppm Se diet. Lanes 12-15 are from rats fed 4.0 ppm Se diet.

Table 3-4. Northern blot data of selenoprotein W (Se-W) mRNA and internal control (18 S rRNA) mRNA in muscles of rats fed Torula yeast diets supplemented with 0, 0.1 and 4 mg selenium as selenite per kg.

mRNA	0 ppm Se	0.1 ppm Se	4.0 ppm Se
Se-W	1287±289[a]	4982±226[b]	5891±451[b]
18 S rRNA	4638±442	3798±411	3493±415
Ratio	0.3±0.1[a]	1.4±0.1[b]	1.7±0.2[c]

* Values are means of each treatments ± SE. Different superscripts denote significantly different ($p < 0.05$).

** Data are expressed as scan units. Ratio = Se-W/ 18 S rRNA.

Experiment 2.

The weight gain and feed consumption of the rats in this experiment are shown in Table 3-5. There were no differences in feed consumption between the different dietary groups. However, there were some differences in weight gain. Those fed the diet with 0.03 mg selenium per kg gained significantly ($P < 0.05$) more than those fed the diets with the two lower levels of this element. The weight gain of those rats fed the diet with 4 mg selenium per kg were not significantly different from controls or those fed the diet with 0.01 mg selenium per kg.

The whole blood and hepatic selenium levels were determined as a measure of the selenium status of the animals (figure 3-4). A nearly linear increase of selenium content occurred in both whole blood and liver with increased levels of this element in the diet.

Western blots of selenoprotein W in muscles from rats fed various dietary selenium was shown in figure 3-5. The selenium content, GPX activity and selenoprotein W levels in muscle followed different patterns with respect to selenium intake (figure 3-6). Increase of selenium levels occurred with increased dietary selenium intake. GPX activity also increased with increased selenium intake but reached a plateau at 2.0 mg selenium per kg diet. The first detectable level of selenoprotein W was in muscle from rats fed the diet with 0.06 mg selenium per kg. A very marked increase of selenoprotein W occurred with the two next highest levels of selenium where a plateau was reached at 1.0 mg selenium per kg diet.

In contrast to the muscle, similar patterns of increase occurred for cGPX activity, and selenium and selenoprotein W contents in brain with respect to the selenium content in the diet (figure 3-7).

The pattern of selenoprotein W in the testes was different from that seen in the other tissues (figure 3-8). The selenoprotein W content increased significantly ($P < 0.05$) in rats fed the diet with 0.01 mg selenium per kg as compared to the controls. There were only slight increases of this selenoprotein with higher levels of selenium. This is in contrast to the selenium content and cGPX activity where plateaus were reached at about 0.06 mg selenium per kg diet.

A fairly linear increase of selenoprotein W occurred in the spleen with the increase of dietary selenium to 1.0 mg per kg, where a plateau was reached (figure 3-9). The cGPX activity reached a plateau at 0.06 mg selenium per kg diet but the selenium content continued to increase with increases of dietary selenium.

Table 3-5. Average food intake and total weight gain for rats fed Torula yeast diets supplemented with 0, 0.01, 0.03, 0.06, 0.1, 1, 2 or 4 mg selenium as selenite per kg diet.

Diet (mg Se kg)	Weight gain	Food intake
0	196±3[a]	14.9±0.2
0.01	203±7[a,b]	15.4±0.3
0.03	231±5[c]	16.6±0.5
0.06	207±4[a,b]	15.9±0.3
0.1	220±7[b,c]	15.9±0.6
1	216±4[b,c]	16.2±0.2
2	211±10[a,b]	15.5±0.2
4	196±7[a]	16.0±0.3

* Values are means of each treatments ± SE. Different superscripts denote significantly different ($p < 0.05$).

** Weight gain is given as grams per rat for the entire study. Food consumed is given as grams per rat per day.

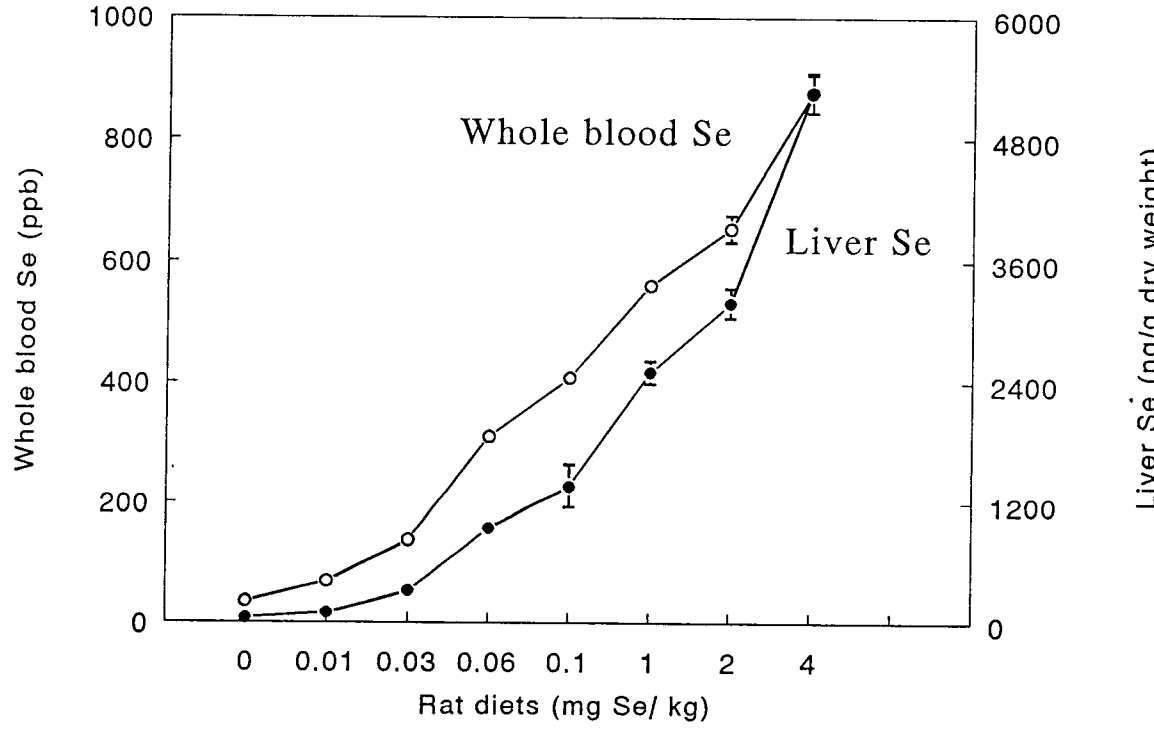

Figure 3-4. Selenium content of whole blood and liver tissue from rats fed various levels of dietary selenium. The whole blood selenium are expressed as ng/ml and the selenium for liver are expressed as ng/ g dry weight.

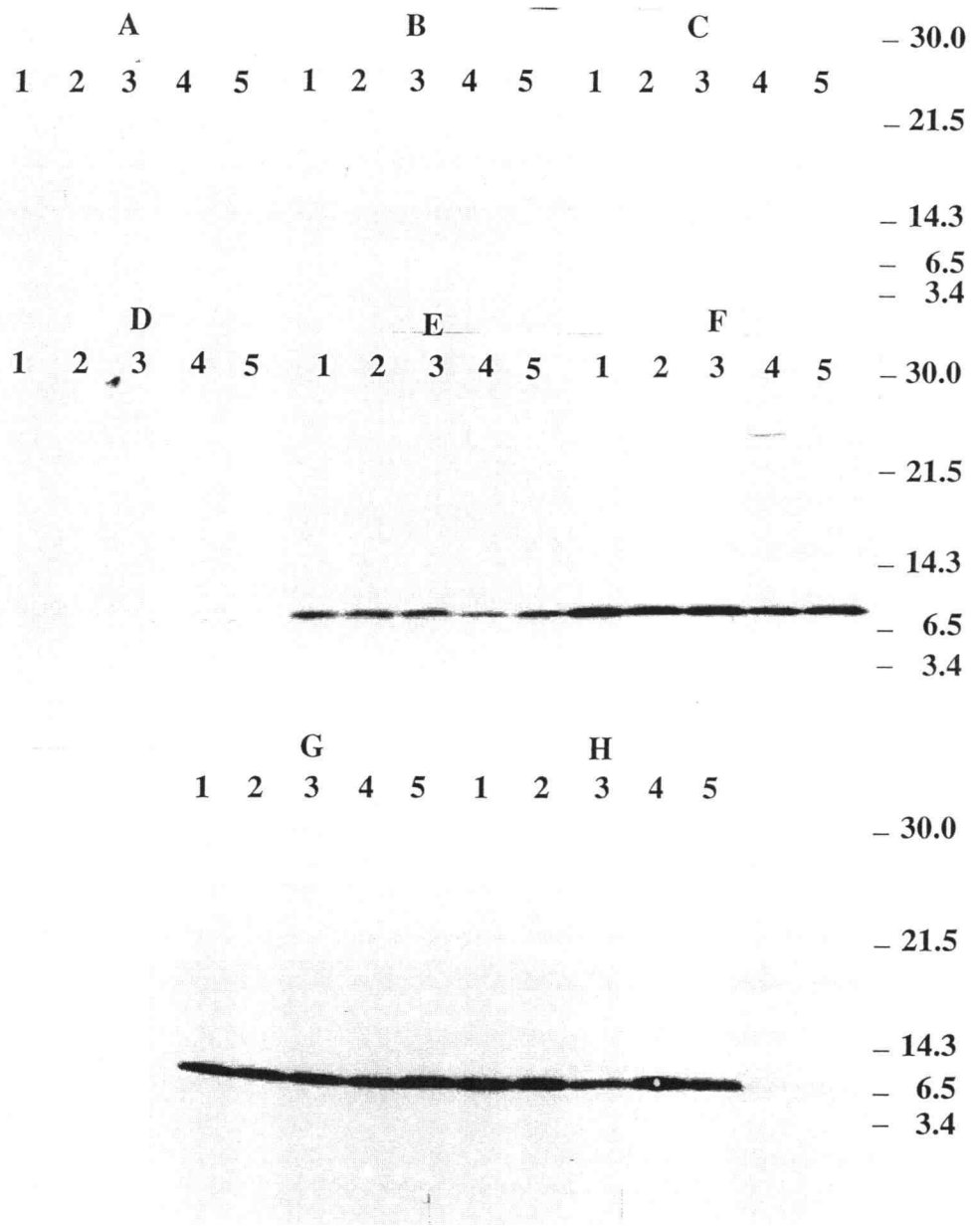

Figure 3-5. Western blot analysis of selenoprotein W in muscles from rats fed various levels of dietary selenium. Lanes A1-5 are from rats fed basal diet. Lanes B1-5 are from rats fed 0.01 ppm Se diet. Lanes C1-5 are from rats fed 0.03 ppm Se diet. Lanes D1-5 are from rats fed 0.06 ppm Se diet. Lanes E1-5 are from rats fed 0.1 ppm Se diet. Lanes F1-5 are from rats fed 1.0 ppm Se diet. Lanes G1-5 are from rats fed 2.0 ppm Se diet. Lanes H1-5 are from rats fed 4.0 ppm Se diets.

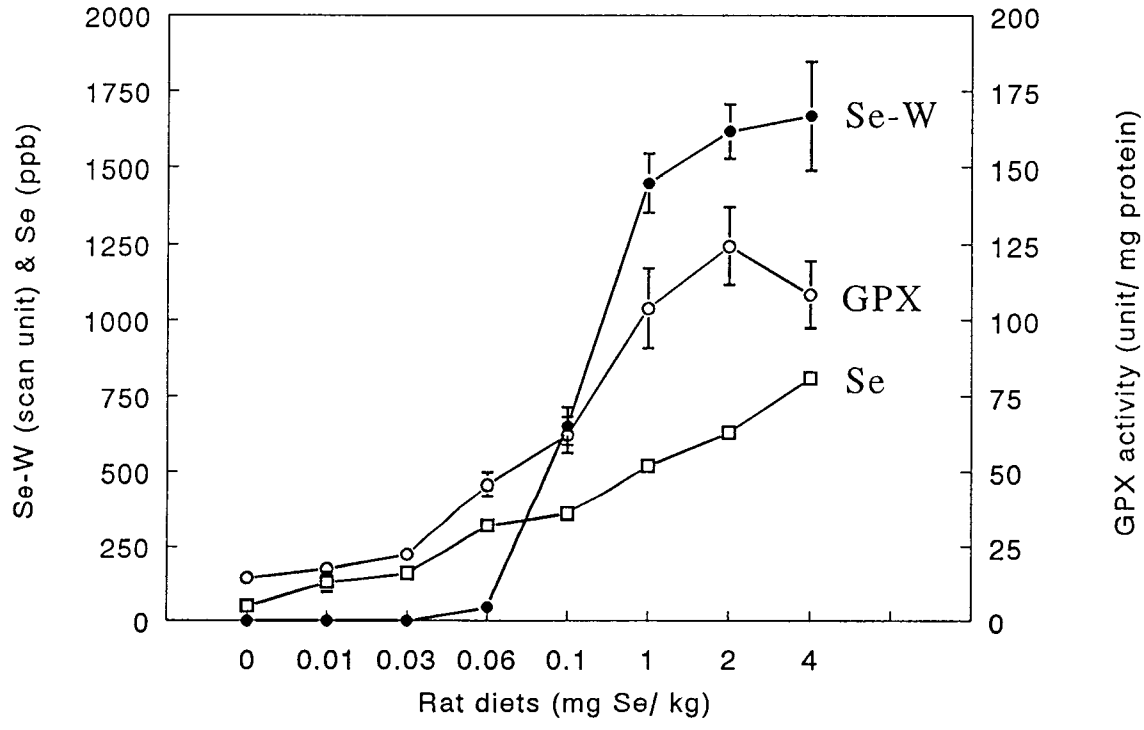

Figure 3-6. Selenoprotein W level (scan unit), GPX activity (nmol NADPH oxidized/ min/ mg protein) and tissue selenium content (ng/ g dry weight) in muscle from rats fed various levels of dietary selenium.

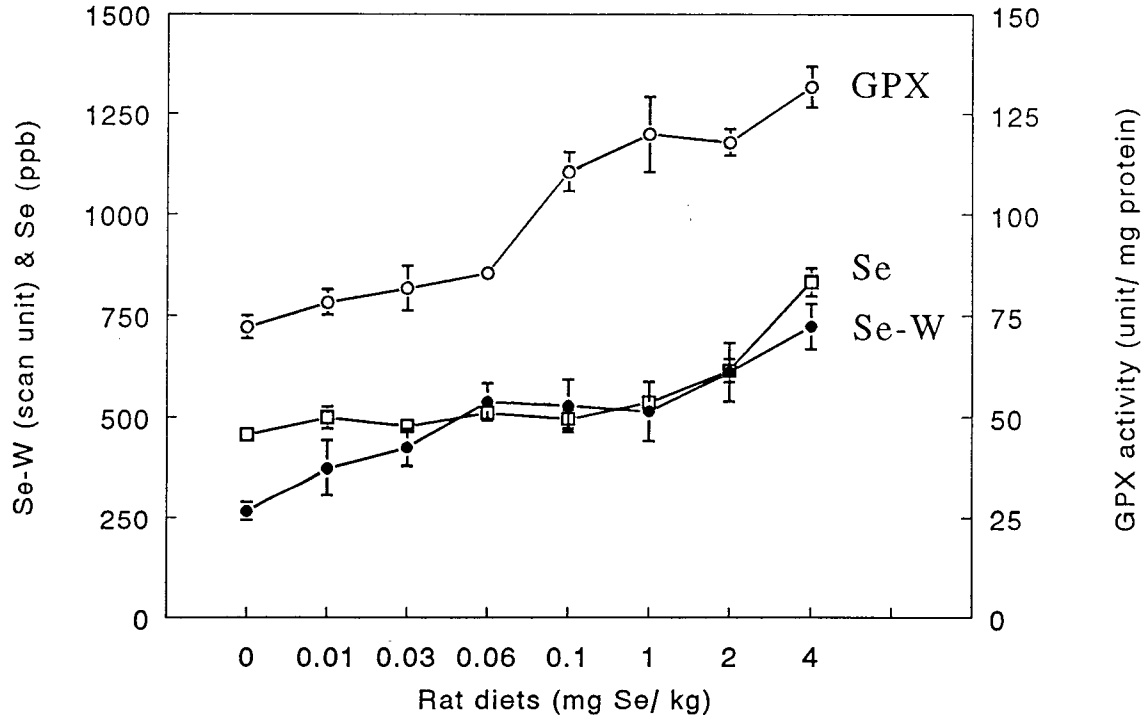

Figure 3-7. Selenoprotein W level (scan unit), GPX activity (nmol NADPH oxidized/ min/ mg protein) and tissue selenium content (ng/ g dry weight) in brain from rats fed various levels of dietary selenium.

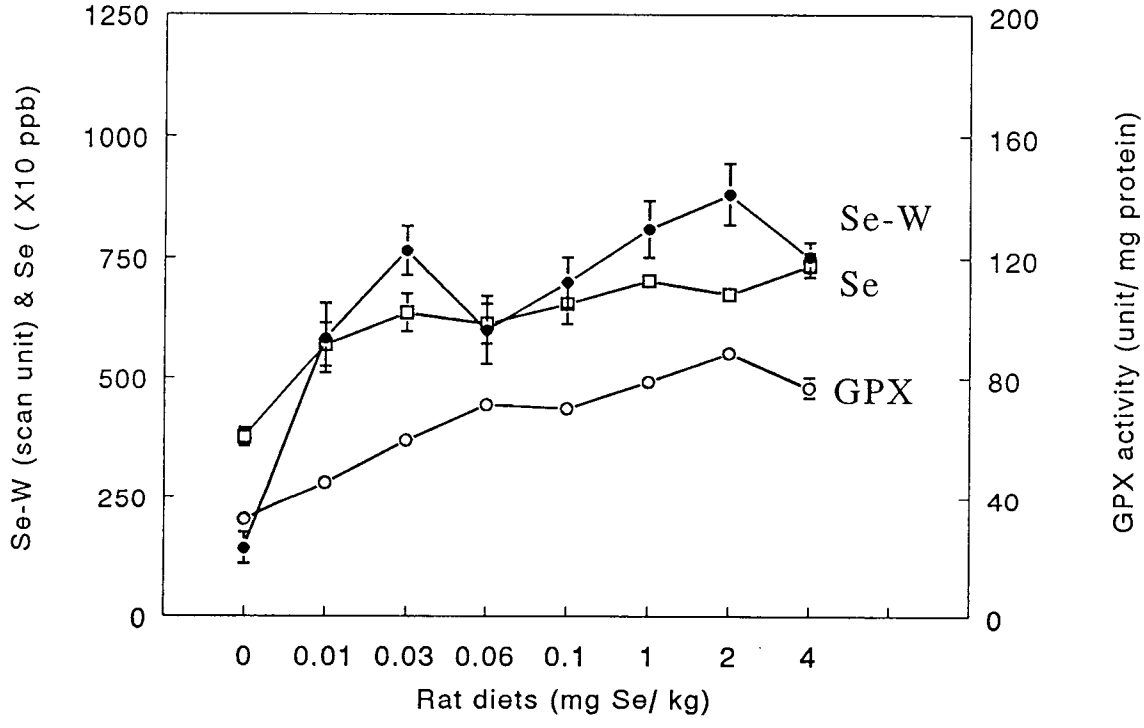

Figure 3-8. Selenoprotein W level (scan unit), GPX activity (nmol NADPH oxidized/ min/ mg protein) and tissue selenium content (ng/ g dry weight) in testis from rats fed various levels of dietary selenium.

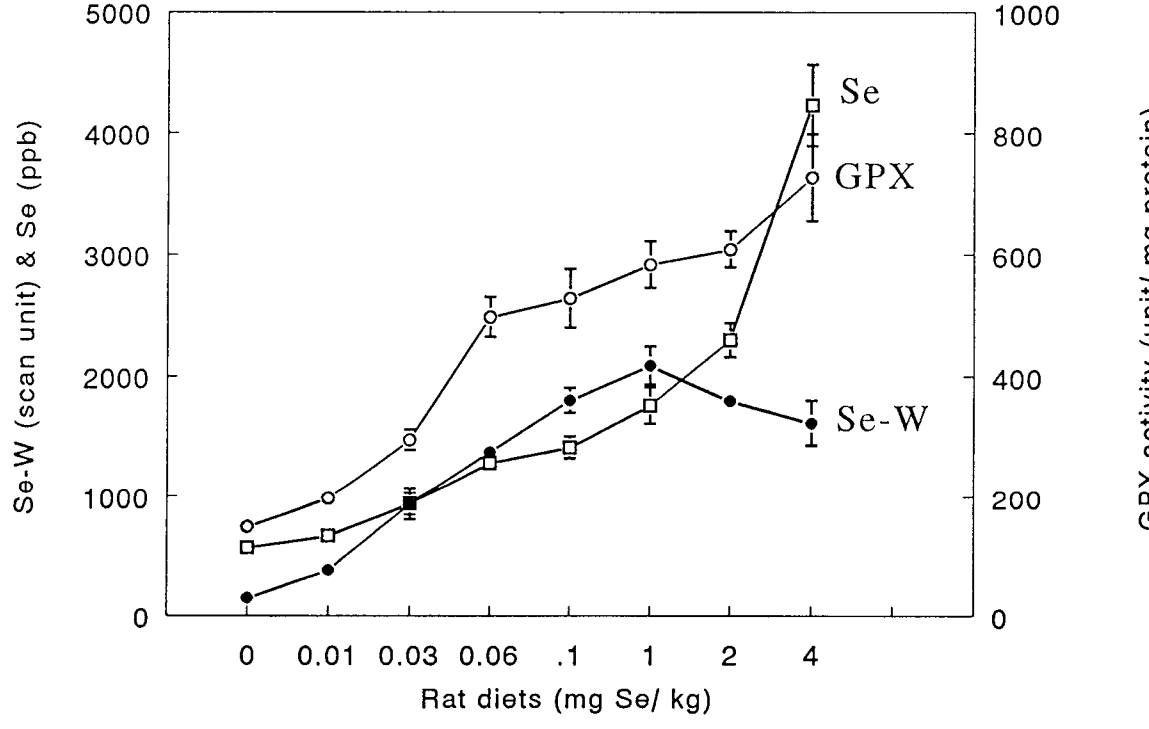

Figure 3-9. Selenoprotein W level (scan unit), GPX activity (nmol NADPH oxidized/ min/ mg protein) and tissue selenium content (ng/ g dry weight) in spleen from rats fed various levels of dietary selenium.

DISCUSSION

Various levels of dietary selenium indicated that this element affects selenoprotein W differently in the tissues studied. The results of the first experiment indicated that selenoprotein W responded to selenium intake, but those of the second experiment gave more precise information on the regulation of selenoprotein W by selenium. It is obvious that the regulation of selenoprotein W by selenium is different between tissues and also differs from that for cGPX. A very marked increase of selenoprotein W was found in muscle when levels of selenium greater than 0.06 mg selenium per kg were fed to the rats (figure 3-5 and 3-6). This is in contrast to the testes where a plateau was reached with only 0.01 mg selenium per kg diet (figure 3-8). Presumably this means that the selenium responsive turnover elements are more sensitive in the testes than the muscle. The patterns in the brain (figure 3-7) and spleen (figure 3-9) were more linear with the selenium intake. However, a plateau was reached in the spleen at 1.0 mg selenium per kg, which is similar to that found in the muscle.

The regulation of cGPX by selenium is different from selenoprotein W. The plateau for cGPX was reached at 0.1 mg selenium per kg diet in the brain (figure 3-7), at 0.06 mg selenium per kg diet in both the testes (figure 3-8) and spleen (figure 3-9) but this plateau in muscle was not reached until 1.0 mg selenium per kg diet was fed (figure 3-5 and 3-6). As far as we know these results with the muscle have not been reported previously. The regulation of cGPX and PHGPX activities were studied in liver, heart, kidney, lung and testes with rats (Lei et al, 1995). Plateaus in activities for both isoenzymes of GPX was reached from 75 to 130 micrograms selenium per kg diet.

Except for the muscle, the present results with the various tissues are consistent with those obtained by other investigators who indicated this plateau is reached around 0.1 mg selenium per kg diet (Hafeman et al, 1974; Knight and Sunde, 1987).

The rapid response of selenoprotein W to selenium in the testes is similar to that reported for PHGPX. The levels of selenoprotein W reached a plateau at only 0.01 mg selenium per kg (figure 3-8) whereas the plateau for PHGPX was reported to be reached at 0.025 mg selenium in the testes (Lei et al, 1995). This may suggest that these two selenoproteins have similar response elements in testes.

The question arises as to the regulation of selenoprotein W by selenium. Table 3-4 indicates that the mRNA levels for selenoproteins are sensitive to selenium. However, additional information is needed to evaluate this regulation. In other work, nuclear run-off transcription assays show that selenium status has no effect on the transcription of cGPX mRNA levels (Christensen and Burgener, 1992, Toyoda et al, 1990), suggesting that selenium status influences the stability of cGPX mRNA. Experiments are presently planned to evaluate the mechanism of regulation of selenoprotein W by selenium as to whether this is through transcription or through stability of mRNAs. The reason for different responses between cGPX and selenoprotein W in various tissues are not known. One hypothesis could be that there exist rapid turnover elements in cGPX that are not present in selenoprotein W mRNA. Reduced selenium status would elicit degradation of cGPX mRNA but not in selenoprotein W mRNA or mRNA for other selenoenzymes. An alternative hypothesis is that selenocysteine insertion sequences in the mRNA which are necessary for selenium insertion (Berry et al, 1993) may also affect stability. Berry et al (1993) found different

levels of deiodinase activity in cells transfected with recombinant deiodinase fusion genes, depending on which selenoprotein provided the selenocysteine insertion sequence.

Prior work indicated that selenoprotein W was present in the muscle, brain, testes and spleen of rats (Yeh et al, 1995). However, there are some species differences in the tissue distribution of this selenoprotein. When selenium is given to sheep, the selenoprotein W content is just as high in the heart as in the muscle (Yeh et al, 1996). Although selenoprotein W was not detectable in hearts from rats, there is mRNA present in this organ (unpublished data), suggesting that mRNA levels are not correlated with selenoprotein W content in all tissues. Experiments on the influence of selenium on transcription and on stability should help to unravel these unknown aspects.

There is some information to suggest different affinities of various tissues for selenoprotein W. When sheep were fed a selenium deficient diet for about three months, selenoprotein W was significantly lower in all tissues examined except for the brain in comparison to selenium supplemented sheep (Yeh et al, 1996). However, the selenium content in the brain was 50% lower in the deficient sheep versus those getting selenium. This suggests preferential retention of selenoprotein W in the brain as compared to selenium.

The metabolic function of selenoprotein W is not known. Selenoprotein P and mitochondrial capsule selenoprotein (MCS, Karimpour et al, 1992) are the other two most studied selenoproteins without a known function. Selenoprotein P is present in plasma of rats (Read et al, 1990) and humans (Deagen et al, 1993) and has been shown to contain 10 selenocysteines (Hill et al, 1991). Like selenoprotein W, the levels of selenoprotein P are affected by the selenium status of the animal (Yang et al, 1989).

MCS is the only other known selenoprotein which contains more than one selenocysteine per subunit (Karimpour et al, 1992).

Selenium deficiency results in a muscular and cardiac degeneration, White Muscle Disease, in lambs and calves (Schubert et al, 1961). Likewise, a severe nutritional selenium deficiency in discrete regions of China was associated with an endemic juvenile cardiomyopathy disorder called Keshan disease (Chen et al, 1980). Muscle weakness has been shown to be prevented by selenium supplementation in patients on long-term parenteral nutrition (Brown et al, 1986). Therefore, there is sufficient evidence to indicate that selenium is important for normal muscle metabolism. Whether selenoprotein W has any significant role in muscle metabolism cannot be determined from the present work, but it is predicted that the higher level in the muscle is not a coincident.

REFERENCES

American Institute of Nutrition (1977) Report of the AIN ad hoc committee on standards for nutritional studies. J. Nutr. 107:1340-1348.

Berry, M. J., Banu, L., Harney, J. W. and Larsen, P. R. (1993) Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. EMBO J. 12:3315-3322.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72: 248-254.

Brown, M. R. Cohen, J. H., Lyons, J. M., Curtis, T. W., Thunberg, B., Cochran, W. J. and Klish, W. J. (1986) Proximal muscle weakness and selenium deficiency associated with long term parenteral nutrition. Amer. J. Clin. Nutr. 43: 549-554.

Brown, M. W. and Watkinson, J. H. (1977) An automated fluorimetric method for the determination of nanogram quantities of selenium. Anal. Chem. Acta 89: 29-35.

Chen, X. S., Yang, G. Q., Chen, J. S., Chen, X. C., Wen, S. M. and Ge, K. Y. (1980) Studies on the reactions of selenium and Keshan disease. Biol. Trace Elem. Res. 2:91.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Christensen, J. J. & Burgener, K. W. (1992) Dietary selenium stabilizes glutathione peroxidase mRNA in rat liver. J. Nutr. 122: 1620-1626.

Chu, F-F, Doroshow, J. H. and Esworthy, R. S. (1993) Expression, characterization and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPX-GI. J. Biol. Chem. 268: 2571-2576.

Deagen, J. T., Butler, J. A. Zachara, B. A. and Whanger, P. D. (1993) Determination of the distribution of selenium between glutathione peroxidase, selenoprotein P and albumin in plasma. Anal. Biochem. 208: 176-184.

Giovannoni, S. J. (1991) The polymerase chain reaction. In: Sequencing and hybrid techniques in bacterial systematics. (Stackebrandt, E. and Goodfellow, M. eds.) John Wiley and Sons, New York. pp. 176-201.

Hafeman, D. G., Sunde, R. A. and Hoekstra, W. G. (1974) Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. J. Nutr. 104: 580-587.

Hill, K. E., Lloyd, R. S., Yang, J-G., Read, R. and Burk, R. F. (1991) The cDNA for rat selenoprotein P contains 10 TGA codons in the open reading frame. J. Biol. Chem. 266: 10050-10053

Karimpour, J., Cutler, M., Shih, D., Smith, J. and Keene, K. C. (1992). Sequence of the gene encoding the mitochondrial capsule selenoprotein of mouse sperm: identification of three in phase TGA selenocysteine codons. DNA Cell. Biol. 11: 692-699.

Knight, S. A. B. and Sunde, R. A. (1987) The effect of progressive selenium deficiency on anti-glutathione peroxidase antibody reactive protein in rat liver. J. Nutr. 117: 732-738.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 68-685.

Lei, X. G., Evenson, J. K., Thompson, K. M. and Sunde, R. A. (1995) Glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase are differentially regulated in rats by dietary selenium. J. Nutr. 125: 1438-1446.

Paglia, D. E. and Valentine, W. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70: 158-169.

Read, R., Bellew, T., Yang, J-G., Hill, K. E. Palmer, I. S.,and Burk, R. F. (1990) Selenium and amino acid composition of selenoprotein P, the major selenoprotein in rat serum. J. Biol. Chem. 265: 17899-17905.

Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. and Hoekstra, W. G. (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179: 588-590.

Schubert, J. R., Muth, O. H., Oldfield, J. E. and Remmert, L. F. (1961) Experimental results with selenium in white muscle disease of lambs and calves. Federation Proc. 20: 689-694.

Steel, R. G. D. and L. H. Torrie. 1980. Principles and Procedures of Statistics, 2nd ed. Mcgraw-Hill Book Co., Inc., New York. pp. 186-190.

Takahashi, K., Avissar, N., Whitin, J. and Cohen, H. (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch. Biochem. Biophys. 256: 677-686.

Towbin, H., T. Staehelin and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamine gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci., U. S. A. 76:4350-4354.

Toyoda, H., Himeno, S. & Imura, N. (1990) Regulation of glutathione peroxidase mRNA level by dietary selenium manipulation. Biochim. Biophys. Acta. 1049: 213-215.

Ursini, F., Maiorino, M. and Gregolin, C. (1985) The selenoenzyme phospholipid hydroperoxide peroxidase. Biochim. Biophys. Acta 839: 62-70.

Vendeland, S. C., Beilstein, M. A., Chen, C. L., Jensen, O. N., Barofsky, E. and Whanger, P. D. (1993) Purification and properties of selenoprotein W from rat muscle. J. Biol. Chem. 268: 17103-17107.

Vendeland, S. C., Beilstein, M. A., Yeh, J-Y., Ream, W. and Whanger, P. D. (1995) Rat skeletal muscle selenoprotein W: cDNA clone and mRNA modulation by dietary selenium. Proc. Natl. Acad. Sci. USA 92: 8749-8753.

Yang, J-G., Hill, K. E. and Burk, R. F. (1989) Dietary selenium intake controls rat plasma selenoprotein P concentration. J. Nutr. 119: 1010-1012.

Yeh, J-Y., Beilstein, M. A., Andrews, J. S. and Whanger, P. D. (1995) Tissue distribution and influence of selenium status on levels of selenoprotein W. The FASEB J. 9: 392-396.

Yeh, J-Y, Gu, Q-P, Beilstein, M. A., Forsberg, N. E. and Whanger, P. D. (1996) Selenium supplementation results in increased levels of selenoprotein W in sheep tissues. J. Nutr. (to be submitted) and Chapter 5.

CHAPTER 4

EFFECTS OF SELENIUM AND SERUM ON SELENOPROTEIN W IN CULTURED L8 MUSCLE CELLS*

Jan-Ying Yeh, Bor-Rung Ou, Neil E. Forsberg and Philip D. Whanger
Departments of Agricultural Chemistry and Animal Sciences,
Oregon State University,Corvallis, OR 97330

Shorten title: Selenoprotein W in tissue culture

*Published with the approval of Oregon State University Experiment Station as paper number _____. This research was supported by Public Health Service Research Grant number DK 38306 from the National Institutes of Diabetes and Digestive and Kidney Diseases.

Contact person: P. D. Whanger, Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331, Phone, 503-737-1803; fax, 503-737-0497; email, whangerp@bcc.orst.edu.

ABSTRACT

Rat L8 muscle cells were cultured to examine the effects of selenium and serum concentration on selenoprotein W levels and glutathione peroxidase (GPX) activities. The data from various differentiation stages of L8 muscle cells indicated that there is no significant difference ($p < .05$) in selenoprotein W levels and GPX activities during differentiation. Selenoprotein W levels and GPX activities increased in L8 myotubes cultured in media supplemented with 3 different forms of selenium. Selenite was utilized more efficiently than selenocysteine for both selenoprotein W and GPX activity. Selenium as selenomethionine appeared to be the least available form for L8 cell usage in the synthesis of selenoprotein W and GPX. However, cysteine and methionine did not affect the selenoprotein W levels and GPX activities in L8 myotubes. The selenium content of the media was shown to affect both the protein and mRNA levels for selenoprotein W. The selenoprotein W levels and GPX activities increased in L8 muscle cells incubated with selenium for 48 hr. Selenium concentrations of 10^{-6} M and 10^{-7} M gave similar responses of selenoprotein W content in cultured L8 myotubes. Northern blot data indicated that the expression of selenoprotein W mRNA increased significantly when L8 myotubes were cultured with selenium ($p < .05$). These results imply that in addition to co-translational regulation, the regulation of selenoprotein W synthesis by selenium is, as least in part, at the transcriptional level. Selenoprotein W levels and GPX activities of L8 myotubes were both lower ($p < .05$) in media with low serum concentration (2%CS) than in media with high serum concentration (10%CS). However, there is no significant difference in selenoprotein W levels and GPX activities of L8 myotubes cultured in

10%CS versus 2%CS with addition of 10^{-8} M selenite ($p > .05$). These results suggest that the increase of selenoprotein W level in L8 myotubes cultured with higher serum concentration (10%CS) is due to the higher selenium concentration in media, rather than serum itself.

INTRODUCTION

Several selenoproteins have been found in mammalian tissues, but only two of these have known functions. There is a family of selenium-dependent glutathione peroxidases and to date there has been four members identified. The cellular glutathione peroxidase (cGPX) was the first one of this family discovered (1). Subsequently, the plasma GPX (pGPX, ref 2), phospholipid hydroperoxidase GPX (PHGPX, ref. 3) and gastrointestinal tract specific GPX (GPX-GI, ref. 4) have been identified. The other known mammalian selenoenzymes are type I (5) and type III (6) iodothyronine deiodinases. The type I enzyme converts thyroxine to triiodothyronine and type III converts thyroxine to the inactive metabolite called reverse T_3.

There are three mammalian selenoproteins identified in which there is no known function. The most studied one is selenoprotein P. It contains more selenium than any known protein. The cDNA for selenoprotein P has been cloned and sequenced (7) and contains 10 TGAs in the open reading frame. Thus it contains 10 selenocysteine residues in its primary structure. The only other selenoprotein known to possess more than one selenocysteine per subunit is mitochondrial capsule selenoprotein (MCS), which contains all three selenocysteines in the first 35 amino acids of a total of 197 residues (8). A third selenoprotein was recently characterized from our laboratory (9) and contains only 87 amino acid residues (10). Evidence has been presented for a number of other selenium containing proteins, but none of these has been characterized (11).

Cell cultures have been extremely useful in studying selenium metabolism. This was used to demonstrate that selenite was more effective than selenomethione (Semet) for

increasing GPX activity (12, 13). The conversion of Semet to selenocysteine was shown to be influenced by the methionine content of the media (13). Various selenium compounds have been studied in transsulfuration defective cells (14), and again higher levels of Semet were required to induce the same amount of GPX activity than either selenite or selenocysteine. Recognition of UGA as a selenocysteine codon in type I iodothyronine deiodinase was demonstrated in vitro (15), further demonstrating the value of this technique. Hence, cell cultures were used in the present investigation to study the influences of selenium and serum on selenoprotein W levels.

The L8 muscle cell line was originally established in 1969 by serial passage of myoblasts isolated from primary rat skeletal muscle cultures prepared from newborn Wistar rats (16). Of the tissues examined, selenoprotein W was highest in muscle from both rats (17) and sheep (18). Thus, experiments with rat muscle appears to be the most desirable tissue for studying the influences of selenium status and serum concentration on selenoprotein W levels. Therefore, rat L8 skeletal muscle cells were used in the present investigations.

MATERIALS AND METHODS

Materials

L8 rat myoblast cells were obtained from American Type Culture Collection (Rockville, MD). Calf serum (CS) was purchased from Hyclone (Logan, UT). Dulbecco's modified Eagle's media (DMEM), 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). Horse-radish 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 membrane was purchased from Schleicher & Schuell (Keene, NH). GeneScreenPlus nylon membrane was purchased from DuPont/NEN.

Cell Culture

Undifferentiated L8 myoblasts were cultured in basal media (DMEM, 100 units penicillin/ml, 100 μg streptomycin/ml and 44 mM sodium bicarbonate, pH 7.4) supplemented with 10% CS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The medium was changed every two days until cells reached confluence. Then medium was replaced with differentiation media (basal media with 2% CS) to induce differentiation. Microscopic examination was used to monitor cell differentiation.

Cell Treatments

Experiment 1.

This experiment was conducted to evaluate the influences of differentiation and selenium on selenoprotein W levels. Basal media supplemented with 10% CS was used to culture and differentiate L8 muscle cells for 6 days. Cells were collected every 2 days during differentiation to examine the effect of differentiation on selenoprotein W. In addition, cells were cultured in basal media supplemented with 10% CS to reach confluence, then myoblasts were cultured in differentiation media with various selenium concentration (10^{-6} M, 10^{-7} M or 10^{-8} M Se as selenite) for 6 days to examine the long term effects of selenium on selenoprotein W.

Experiment 2.

Effects of different forms of selenium on selenoprotein W levels and GPX activities were examined. Four-day differentiated myotubes were incubated in differentiation media supplemented with various concentrations (10^{-6}M, 10^{-7}M or 10^{-8}M) of selenium as selenite, L-selenomethionine or L-selenocystine for 0, 16, 24 and 48 hr. L8 myotubes were also incubated in differentiation media supplemented with 10^{-6} M selenium as L-cysteine and L-methionine.

Experiment 3.

The effect of serum on selenoprotein W levels and GPX activities were investigated. Four-day differentiated myotubes were incubated with either basal media

(without serum) or differentiation media. Each type of media were supplemented with various concentrations of selenium (10^{-6}M, 10^{-7}M and 10^{-8}M) as selenite for 0, 16, 24 and 48 hr.

After treatments, each plate of cells were washed with ice-cold 1X PBS (pH 7.4) three times, then homogenate buffer (20 mM Tris [pH 7.5], 0.25 M sucrose, 1 mM EGTA, 5 mM EDTA, 1 mM PMSF, 50 mM ß-mercaptoethanol and 25 ug/ml leupeptin) was added to each plate and cells were scraped into tubes. After sonicated for 10 seconds, the mixtures were centrifuged at 17,000 xg for 10 min at 4°C. Protein content was measured on the supernatants by the dye-binding assay of Bradford (19) using bovine serum albumin (Bio-Rad, Richmond, CA) as standards. Then these cell supernatants were used to measure selenoprotein W level by Western blotting technique and GPX activities by a coupled enzyme procedure.

Selenium content was determined in the media before use to ensure the correct selenium concentration.

Western Blot Assay

This assay was conducted as described previously (17). Briefly, the cell supernatants (150 µg protein each) were electrophoretically separated on SDS-polyacrylamide 7.5 to 15% gradient gels as described by Laemmli (20). Proteins were transferred onto a nitrocellulose membrane (0.2 µm; BA-S83, Schleicher & Schuell, Keene, NH) in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 4°C according to method of Towbin et al. (21). After transfer, membranes were incubated

with blocking solution (5% non-fat dried milk in TTBS [0.05% Tween 20 in TBS; pH 7.5]) at 25°C for 1 hr, then incubated with rabbit anti-selenoprotein W polyclonal antibody at 25 °C for 1.5 hr. Following three washes, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Bio-Rad, Richmond, CA). Membranes were then washed four times with TTBS and specific bonding of anti-selenoprotein W antibody onto membrane was detected by ECL detection system (Amersham, Arlington, IL). The relative amounts of selenoprotein W present in the various samples were estimated by densitometric scanning of the X-ray film using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA).

Glutathione Peroxidase Activity and Selenium Content

GPX activity was measured by an enzyme coupled method with glutathione reductase utilizing hydroperoxide as substrate (22) by a DU Series 60 Spectrophotometer (Beckman Instruments, Fullerton, CA). After digestion of media with nitric acid and perchloric acid, the selenium content was determined fluorometrically by a semi-automated method (23) using an Alpchem II system (Alpchem. Corp., Milwaukee, OR).

Total RNA Extraction

Extraction of total RNA has been described previously (24). Briefly, myotubes grown in 10-cm diameter dishes were washed three times with ice-cold phosphate

buffered saline (PBS) and lysed directly on the dishes using 2 ml of solution A (4 M guanidium isothionate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 10 mM ß-mercaptoethanol). The monolayer was scraped with a rubber policeman to ensure that all cells were released from the dishes. The lysate was transferred to a 15 ml polypropylene tube. To this, 0.2 ml of 2 M sodium acetate (pH 4.0), 2 ml of phenol and 0.4 ml of a chloroform-isoamyl alcohol mixture (49:1) were added sequentially. The lysate was mixed by inversion after each addition. After brief vortexing, the mixture was kept on ice for 15 min and was separated by centrifugation (12,000 g) for 30 min at 4°C. The upper aqueous phase was transferred to a new tube and an equal volume of ice-cold isopropanol was added. The samples were mixed and stored at -20°C overnight. RNA was collected by centrifugation (12,000 g, 20 min at 4°C). The supernatant was discarded and the RNA pellet was resuspended in 0.4 ml of solution A. One volume of isopropanol was added, and the mixture was stored at -20°C overnight. The RNA pellet was recovered by centrifugation at 12,000 g for 15 min at 4°C, washed twice with 70% ethanol and dried under a vacuum. The dried RNA pellet was then dissolved in diethylpyrocarbonate (DEPC)-treated water and was quantitated by spectrophotometry at a wavelength of 260 nm.

Northern Blot Analysis

Total RNA samples (25 µg) were denatured at 55°C for 15 min, applied to a 1.2% agarose gel containing 2.2 M formaldehyde, then electrophoresed at 30 V for 12 h. RNA was transferred onto nylon membranes (GeneScreenPlus, DuPont/NEN) and immobilized

by baking at 70°C for 40 min. Selenoprotein W cDNA and 16 S ribosomal RNA oligonucleotides (33) were labeled by using DIG Labeling Kit (Boehringer Mannheim Biochemica, Indianapolis, IN). The RNA membrane was hybridized and washed according to the procedures described in DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim Biochemica, Indianapolis, IN). Briefly, the membrane was prehybridized at 42 °C for 1 hr in hybridization buffer (50% formamide, 5 X SSC, 0.02% SDS, 0.1 % N-lauroylsarcosine and 2% Blocking Reagent). After prehybridization, heat-denatured DIG labeled selenoprotein W cDNA probe was added to hybridization buffer and membrane was hybridized at 42°C overnight. Next day, the membrane was washed in 0.5 X SSC with 0.1% SDS at room temperature. After washing briefly in Buffer 1 containing 0.3% Tween 20, the membrane was incubated in Buffer 2 for 30 min. Then anti-digoxigenin-AP conjugated antibody was diluted in Buffer 2 (1:5000) and the membrane was incubated in this buffer for 30 min. The unbound antibody conjugate was removed by washing the membrane in Buffer 1. The membrane was then equlibrated in Buffer 3 for 2 min and substrate solution was added. After incubation at 37°C for 5 min, the excess substrate was removed and the membrane was exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY). Developed films were scanned with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA). Before hybridization to the internal control probe, the membrane was rinsed thoroughly in water and then incubated in stripping buffer (50% formamide, 50 mM Tris-HCl [pH 8.0], and 1% SDS) at 68°C for 1 hr to remove the probe. After rinsing with water and then in 2

X SSC, the membrane was used for hybridization of internal control probe (16 S rRNA oligonucleotides).

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 for comparing groups (25). A significance level of 5% was adopted for all comparisons.

RESULTS

In the preliminary experiment, L8 muscle cells were cultured in basal media with 10%CS to reach confluence and then serum concentration of media was reduced to 2% to induce the differentiation of myotubes. Some of the 4-day differentiated myotubes were incubated with either 10^{-6} M (Fig. 4-1, lane 14) or 10^{-7} M (Fig. 4-1, lane 13) selenium as selenite for 3 days. These data showed that selenoprotein W levels and GPX activities decreased gradually during differentiation (Fig. 4-1 and Table 4-1). When selenium was added to the media in the 4-day differentiated myotubes, the selenoprotein W levels and GPX activities increased after 3 day incubation in response to selenium supplementation (Fig. 4-1 and Table 4-1).

Figure 4-2 showed the specific binding of antibody to selenoprotein W in L8 muscle cells. The effect of differentiation on selenoprotein W levels and GPX activities is shown in Table 4-2. These results indicated that selenoprotein W levels remained unchanged during cell differentiation ($p > .05$).

To further examine the long term effect of different selenium concentrations on selenoprotein W, L8 muscle cells which had differentiated in media were incubated with various concentrations of selenium as selenite for 6 days (Fig. 4-3). Both selenoprotein W level and GPX activity reached the plateau in media where 10^{-7} M Se as selenite was added (Table 4-3). These data showed that selenoprotein W increased as selenium concentration in media was increased ($p < .05$).

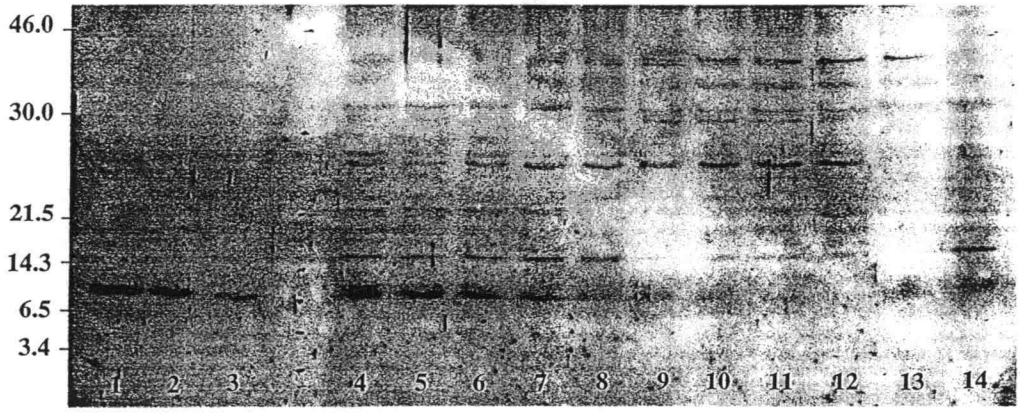

Figure 4-1. Western blot of selenoprotein W levels at various differentiation stages of rat L8 muscle cells. Lane 1-3: Pure selenoprotein W (15 ng, 10 ng, 5 ng). Lane 4 is myoblast in basal media + 10% CS. Lane 5-11 are 1-day, 2-day, 3-day, 4-day, 5-day, 6-day and 7-day differentiated myotubes in basal media + 2% HS, respectively. Lane 12-14 are 4-day differentiated myotubes incubated with different media (basal media + 2% HS, basal media + 2% HS $+10^{-7}$M selenite, basal media + 2% HS + 10^{-6}M selenite, respectively) for 3 days.

Table 4-1. Selenoprotein W (Se-W) levels and GPX activities in various differentiation stages of L8 muscle cells, as shown in Fig. 4-1.

Lane #	Se-W level	GPX activity
4	91.6	95.0
5	69.5	105.8
6	47.8	89.9
7	29.6	72.8
8	4.7	54.2
9	4.3	43.3
10	N.D.	38.4
11	N.D.	32.2
12	N.D.	41.5
13	N.D.	64.2
14	6.8	60.3

* ND = non-detectable

** Selenoprotein level is expressed as ng/mg protein whereas GPX activity is expressed as nmol NADPH oxidized/min/mg protein.

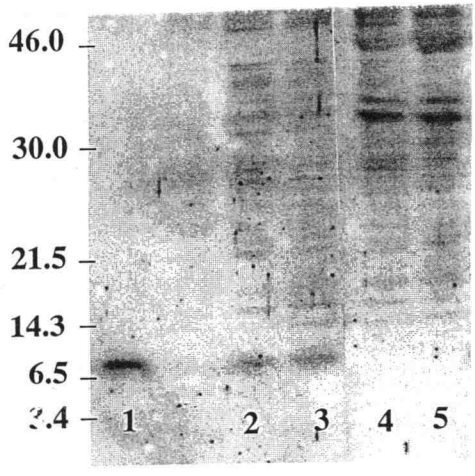

Figure 4-2. Western blot of selenoprotein W in L8 muscle cells. Rabbit polyclonal antibody against rat selenoprotein W peptide sequence was used in Western blot analysis. Lane 1 is pure selenoprotein W (5 ng) from rat muscle. Lanes 2 and 4 are L8 myoblast. Lanes 3 and 5 are 1-day differentiated muscle cells. For Western blot assay, lanes 2 and 3 were incubated with primary antibody whereas lanes 4 and 5 were incubated with antibody which was preabsorbed (10 ug peptide/ ml antibody solution) with synthetic peptide of selenoprotein W.

Table 4-2. Effect of differentiation on selenoprotein W level and GPX activity in L8 muscle cells.

Differentiated stage	Se-W level	GPX activity
Day 0	69±20	81.9±3.1[a]
Day 2	120±20	104.3±1.9[b]
Day 4	82±16	84.5±1.5[a,c]
Day 6	111±18	90.4±2.3[c]

* Values are expressed as mean ± S.E. Day 0 = beginning of differentiation. Day 6 = 6 day differentiation. Different superscripts denote significant difference ($p < .05$). Selenoprotein W level is expressed as scan unit whereas GPX activity is expressed as nmol NADPH oxidized/min/mg protein.

** L8 muscle cells were cultured and differentiated in basal media with 10%CS.

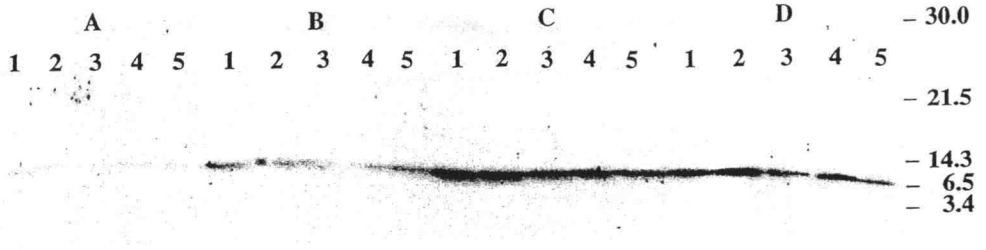

Figure 4-3. Western blot of selenoprotein W L8 myotubes cultured in differentiation media supplemented with various selenium concentration as selenite for 6 days. Rat L8 myoblasts were grown to reach confluence in basal media + 10% CS, different media were used to differentiate myoblasts to myotubes. Lanes A1-5: differentiation media (basal media + 2%CS). Lanes B1-5: differentiation media + 10^{-8}M Se. Lanes C1-5: differentiation media + 10^{-7}M Se. Lanes D1-5: differentiation media + 10^{-6} M Se.

Table 4-3. Effect of various selenium concentrations on selenoprotein W level and GPX activity in 6-day differentiated myotubes, as shown in figure 4-3.

Media	Se-W level	GPX activity
Differentiation media (DM)	20 ± 5^{a}	83.3 ± 7.8^{a}
DM + 10^{-8}M Se	151 ± 27^{b}	109.9 ± 5.3^{b}
DM + 10^{-7}M Se	714 ± 109^{c}	149.4 ± 7.3^{c}
DM + 10^{-6}M Se	411 ± 94^{d}	143.7 ± 8.8^{c}

* Values are expressed as mean ± S.E. Different superscripts denote significant difference ($p < .05$). Selenoprotein W level is expressed as scan unit whereas GPX activity is expressed as nmol NADPH oxidized/min/mg protein.

** L8 muscle cells were cultured in basal media with 10% CS to reach confluence, then incubated in various media to differentiate for 6 days.

*** Differentiation media = basal media + 2%CS.

Figure 4-4 gives the GPX activities of rat L8 muscle cell incubated with differentiation media (basal media with 2% CS) with additions of 10^{-6} M, 10^{-7} M or 10^{-8} M selenium (Fig. 4-4A, 4-4B and 4-4C, respectively) as either selenite, selenocystine (SeCys) or selenomethionine (SeMet) for 16, 24 and 48 hr. Similar patterns were noted when the myotubes were incubated with either 10^{-6} M (Fig. 4-4A) or 10^{-7} M (Fig. 4-4B) selenium as these three sources. A greater lag in GPX activity was observed when myotubes were cultured with 10^{-8} M selenium as the three sources (Fig. 4C). GPX activity at 16 hr incubation did not differ from the initial values ($p > .05$). However, GPX activity increased to the greatest extent at 48 hr incubation with selenite ($p < .05$), followed by selenocysteine and selenomethionine. These results indicated that the availability of different selenium forms for GPX synthesis is sodium selenite $\geq$ selenocystine $>$ selenomethionine ($p < .05$).

Effects of serum and selenium concentrations on GPX activity at various times are shown in Fig. 4-5. The patterns of GPX activities in myotubes in response to selenium concentrations for 10^{-6} M (Fig. 4-5A), 10^{-7} M (Fig. 4-5B) and 10^{-8} M (Fig. 4-5C) were similar. L8 myotubes cultured with basal media remained constant, but there were increases, although erratic, when serum was added to basal media ($p < .05$). When selenium was added to the incubation media, GPX activity increased dramatically to the greatest extent at 48 hr of incubation. There were no significant difference in GPX activities between myotubes incubated in basal media with serum and differentiation media with selenium ($p > .05$).

Figure 4-6 shows the effects of different forms of selenium at 10^{-6} M on selenoprotein W level during various incubation times. There were no significant

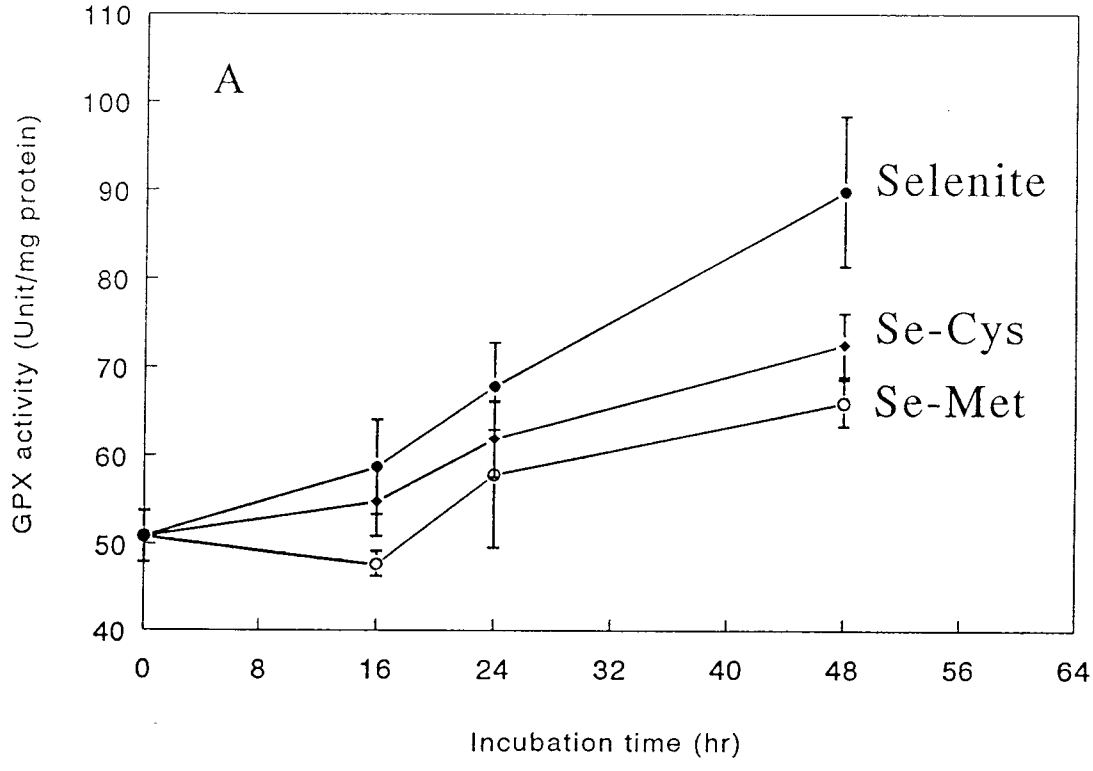

Figure 4-4A. Effects of different forms of Se on GPX activities (nmol NADPH oxidized/min/mg protein) in 4-day differentiated L8 myotubes cultured for various times (16, 24 or 48 hr). Culture media is differentiation media (basal media + 2% CS) with 10^{-6}M Se as selenite, L-selenomethionine or L-selenocystine.

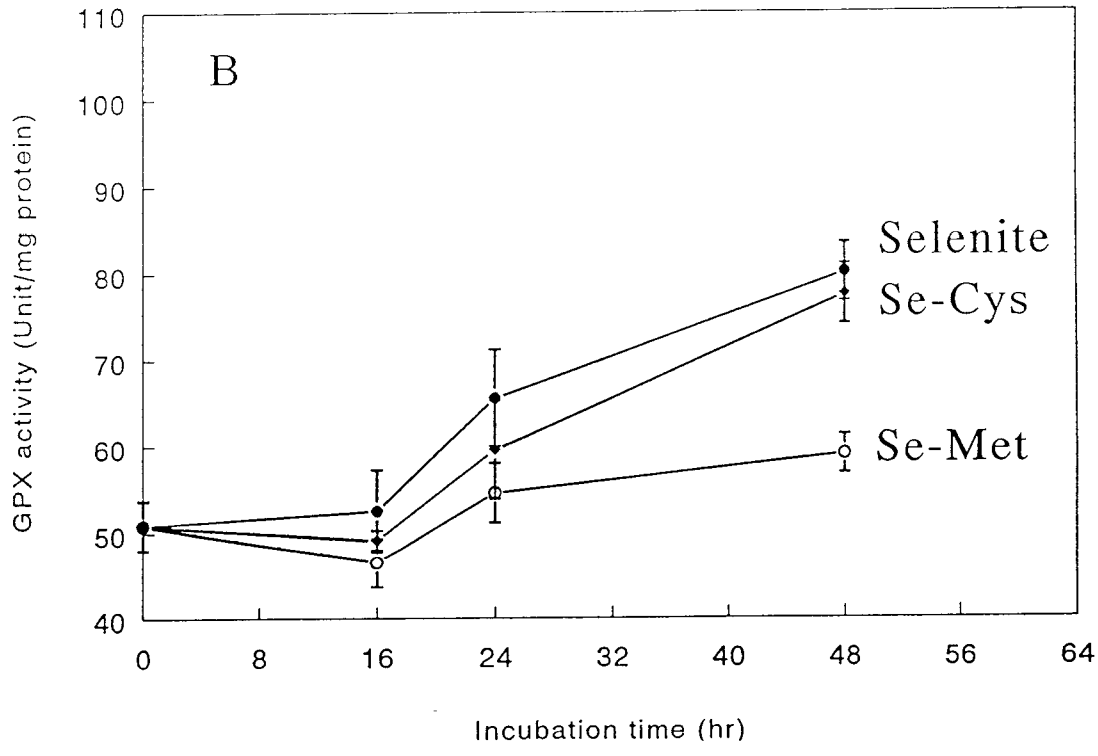

Figure 4-4B. Effects of different forms of Se on GPX activities (nmol NADPH oxidized/min/mg protein) in 4-day differentiated L8 myotubes cultured for various times (16, 24 or 48 hr). Culture media is differentiation media with 10^{-7}M Se as selenite, L-selenomethionine or L-selenocystine.

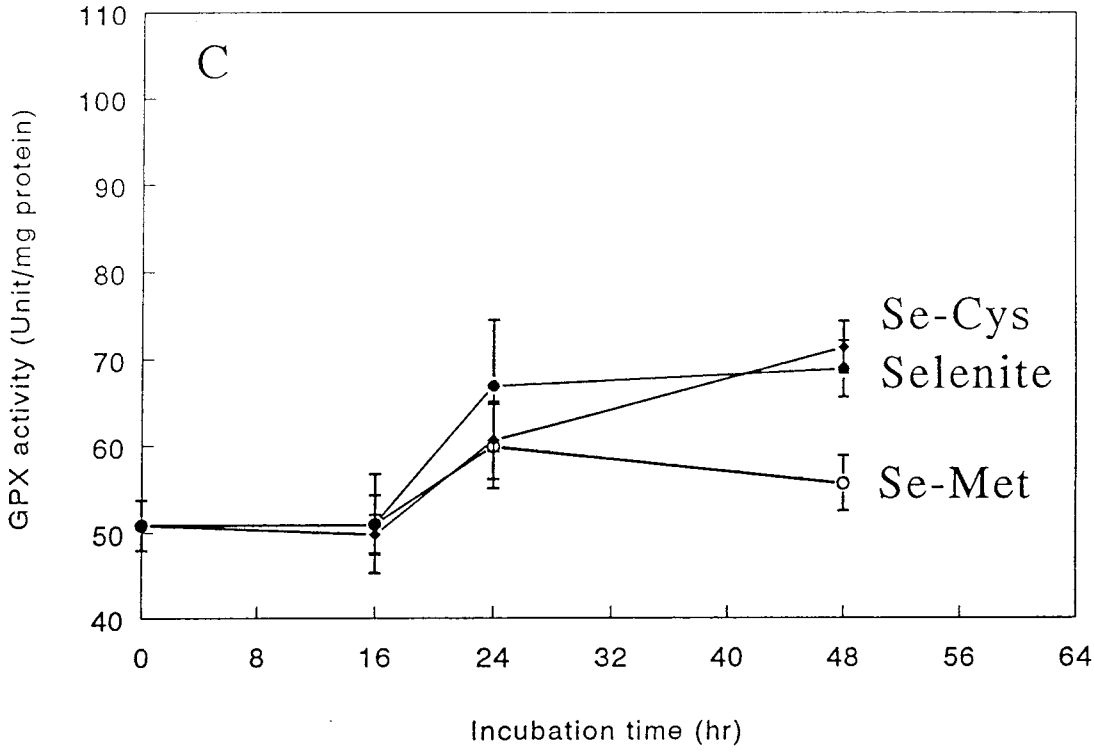

Figure 4-4C. Effects of different forms of Se on GPX activities (nmol NADPH oxidized/min/mg protein) in 4-day differentiated L8 myotubes cultured for various times (16, 24 or 48 hr). Culture media is differentiation media with 10^{-8}M Se as selenite, L-selenomethionine or L-selenocystine.

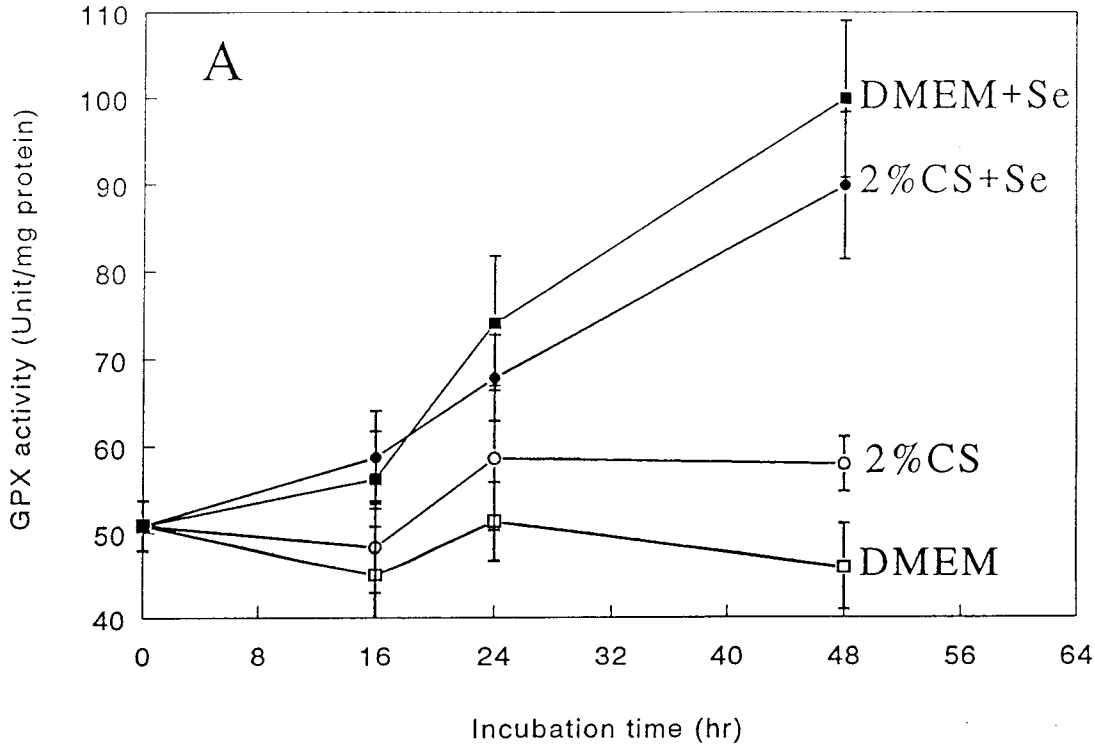

Figure 4-5A. Effects of serum and selenium concentrations on GPX activities (nmol NADPH oxidized/min/mg protein) in 4-day differentiated L8 myotubes cultured for various times (16, 24 or 48 hr). Culture media is basal media (without serum), basal media with 10^{-6} M selenite, differentiation media (basal media + 2%CS), or differentiation media with 10^{-6}M selenite.

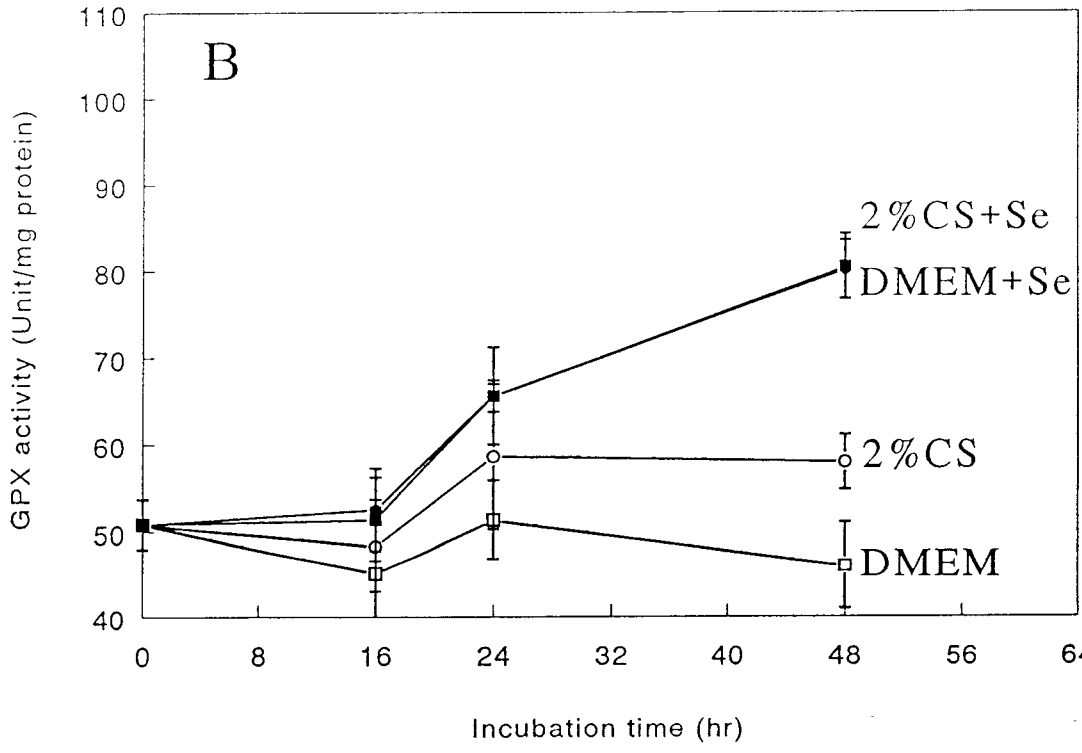

Figure 4-5B. Effects of serum and selenium concentrations on GPX activities (nmol NADPH oxidized/min/mg protein) in 4-day differentiated L8 myotubes cultured for various times (16, 24 or 48 hr). Culture media is basal media (without serum), basal media with 10^{-6} M selenite, differentiation media (basal media + 2%CS), or differentiation media with 10^{-7}M selenite.

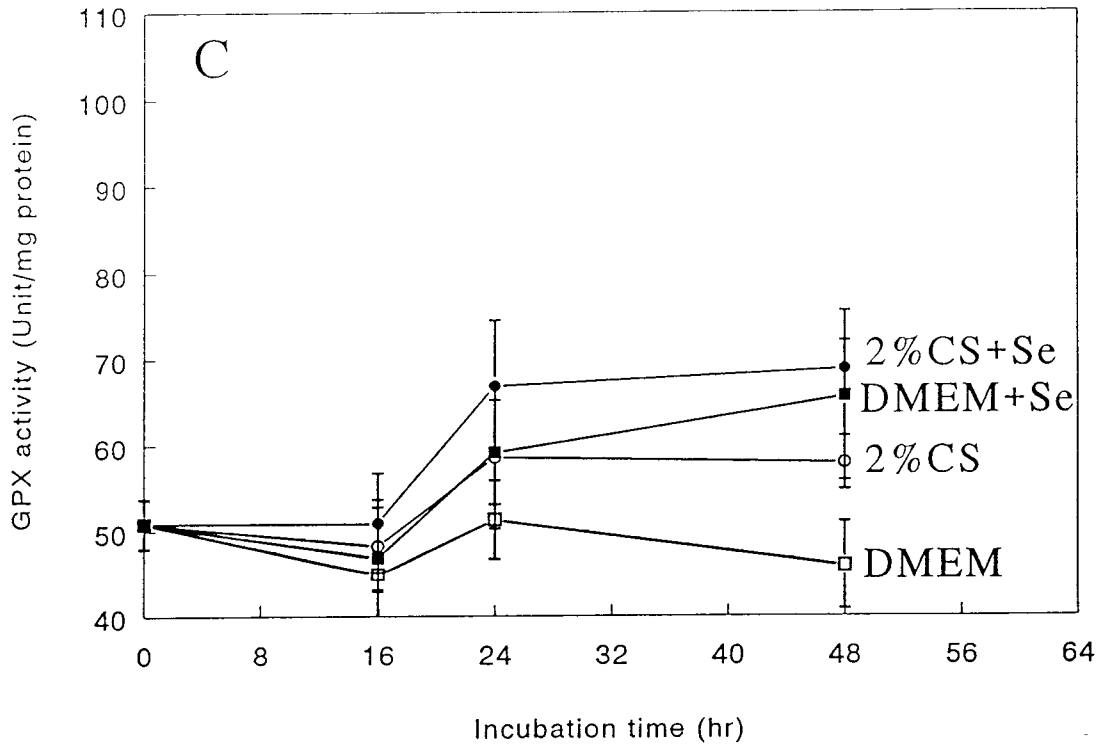

Figure 4-5C. Effects of serum and selenium concentrations on GPX activities (nmol NADPH oxidized/min/mg protein) in 4-day differentiated L8 myotubes cultured for various times (16, 24 or 48 hr). Culture media is basal media (without serum), basal media with 10^{-6} M selenite, differentiation media (basal media + 2%CS), or differentiation media with 10^{-8}M selenite.

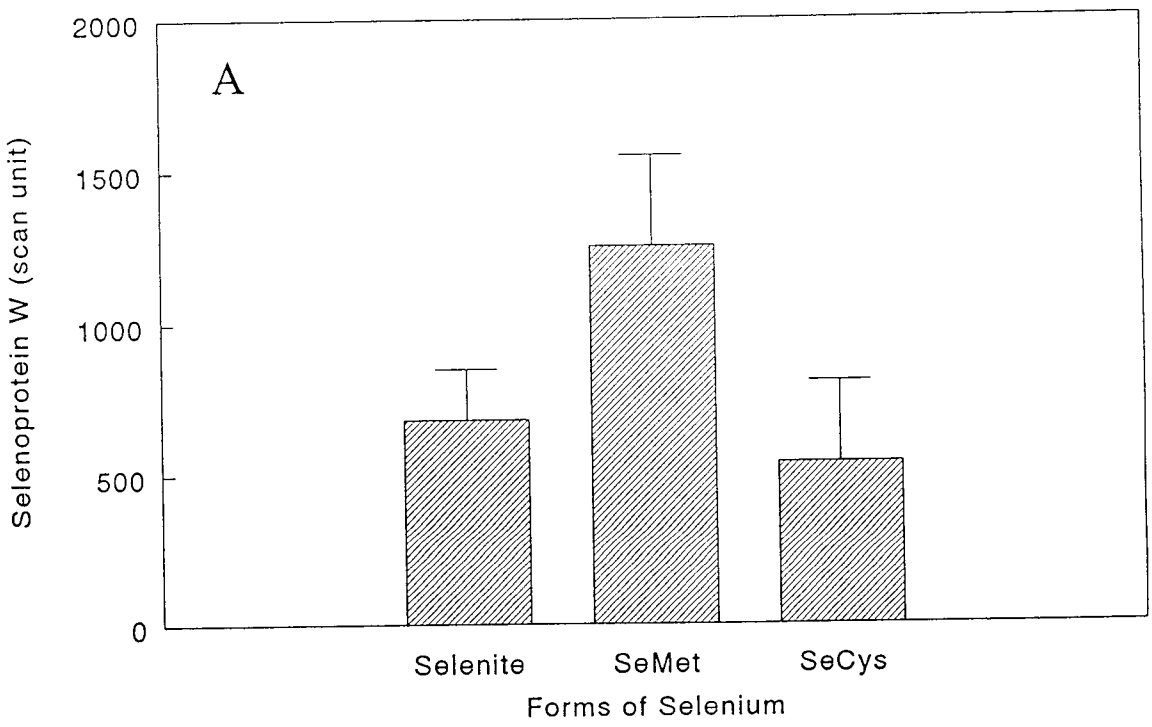

Figure 4-6A. Effects of different forms of selenium (10^{-6}M of selenite, L-selenomethionine or L-selenocystine) on selenoprotein W levels of 4-day differentiated L8 myotubes incubated for 16 hr). Different electrophoresis was performed for the various time period, thus the difference can only be compared within the same incubation time.

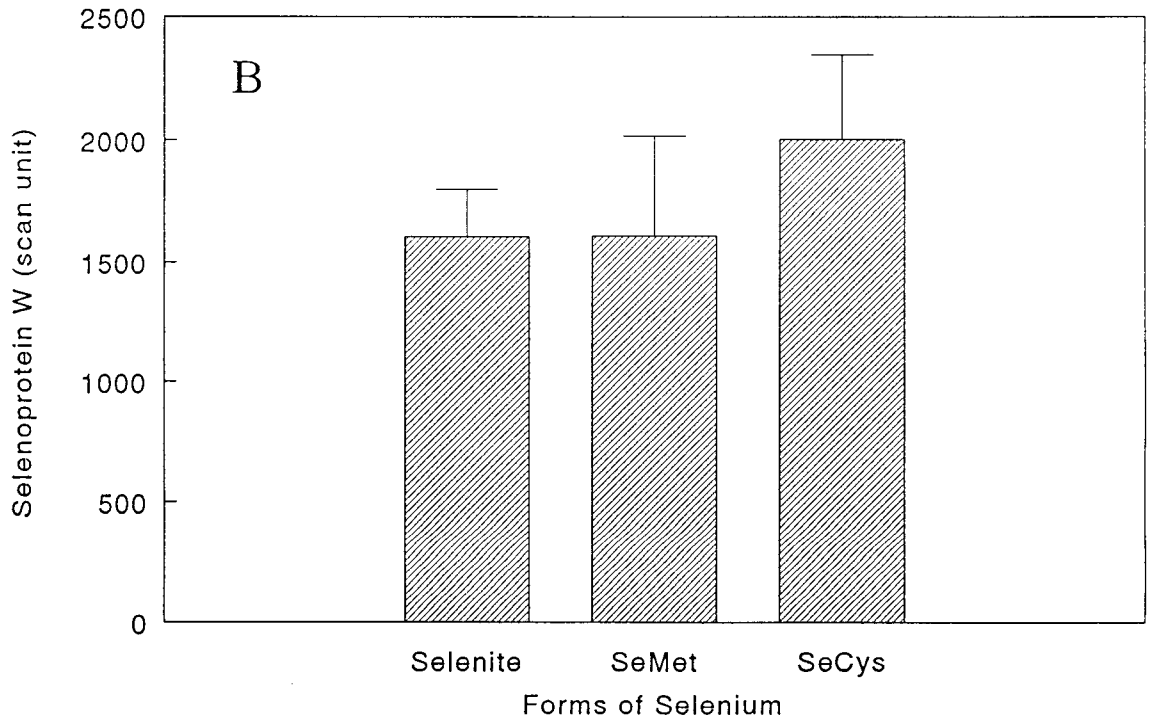

Figure 4-6B. Effects of different forms of selenium (10^{-6}M of selenite, L-selenomethionine or L-selenocystine) on selenoprotein W levels of 4-day differentiated L8 myotubes incubated for 24 hr. Different electrophoresis was performed for the various time period, thus the difference can only be compared within the same incubation time.

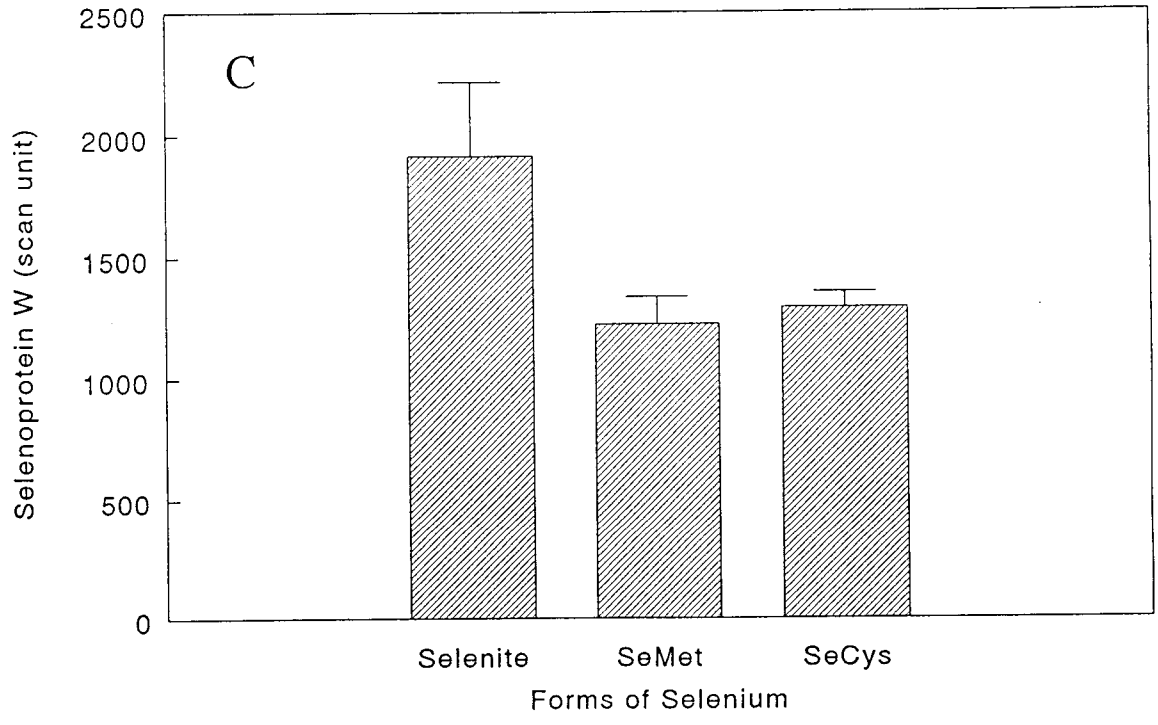

Figure 4-6C. Effects of different forms of selenium (10^{-6}M of selenite, L-selenomethionine or L-selenocystine) on selenoprotein W levels of 4-day differentiated L8 myotubes incubated for 48 hr. Different electrophoresis was performed for the various time period, thus the difference can only be compared within the same incubation time.

differences among these 3 selenium forms at 16 and 24 hr incubation ($p > .05$). After 48 hr incubation, selenite appeared to be the most available form of selenium for muscle cell usage ($p < .05$). There were no differences between selenium forms as selenocystine and selenomethionine ($p > .05$). The availability of different selenium forms for selenoprotein W synthesis is sodium selenite > selenocystine = selenomethionine ($p < .05$).

Effects of different selenium concentrations and type of media on selenoprotein W level at various times are shown in Fig. 4-7. Addition of selenium in incubation media caused an increase ($p < .05$) in selenoprotein W level (Fig. 4-7), but there was no difference among myotubes incubated with various selenium concentration at 16 hr (Fig. 4-7A). Selenium concentration as 10^{-6} M caused the highest selenoprotein W level ($p < .05$) at 24 hr incubation (Fig. 4-7B) whereas selenium concentration of 10^{-6} M and 10^{-7} M gave similar results ($p > .05$) at 48 hr incubation (Fig. 4-7C).

Table 4-4 shows the effects of selenium concentration at 10^{-6} M and serum on selenoprotein W levels at 48 hr of incubation. Selenoprotein W level in myotubes incubated with basal media plus selenium was significantly higher than other incubation media ($p < .05$). There were no significant differences among myotubes incubated in other media ($p > .05$). Similar to GPX activity, the selenoprotein W levels between myotubes incubated in basal media with selenium and differentiation media with selenium did not differ significantly ($p > .05$).

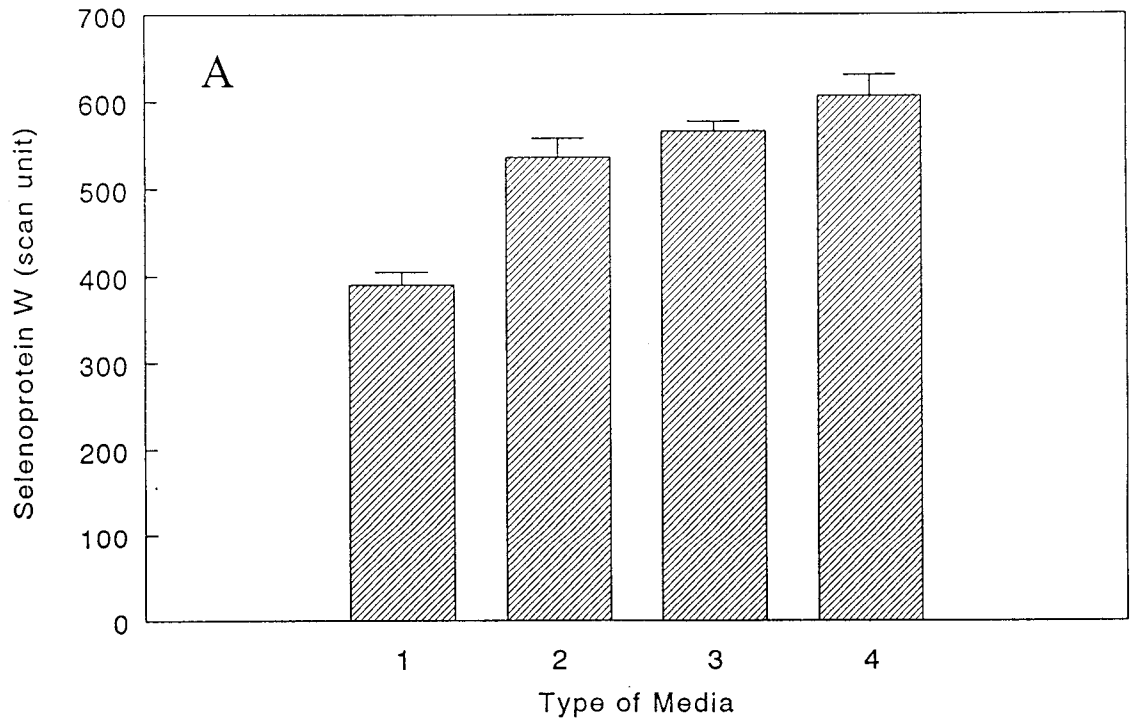

Figure 4-7A. Effects of different selenium concentrations (10^{-8} M, 10^{-7} M or 10^{-6} M Se as selenite) on selenoprotein W levels of 4-day differentiated L8 myotubes incubated for 16 hr. Bar 1: differentiation media (basal media + 2%CS), Bar 2: differentiation media + 10^{-8} M Se, Bar 3: differentiation media + 10^{-7} M Se, Bar 4: differentiation media + 10^{-6} M Se. Different electrophoresis was performed for the various time period, thus the difference can only be compared within the same incubation time.

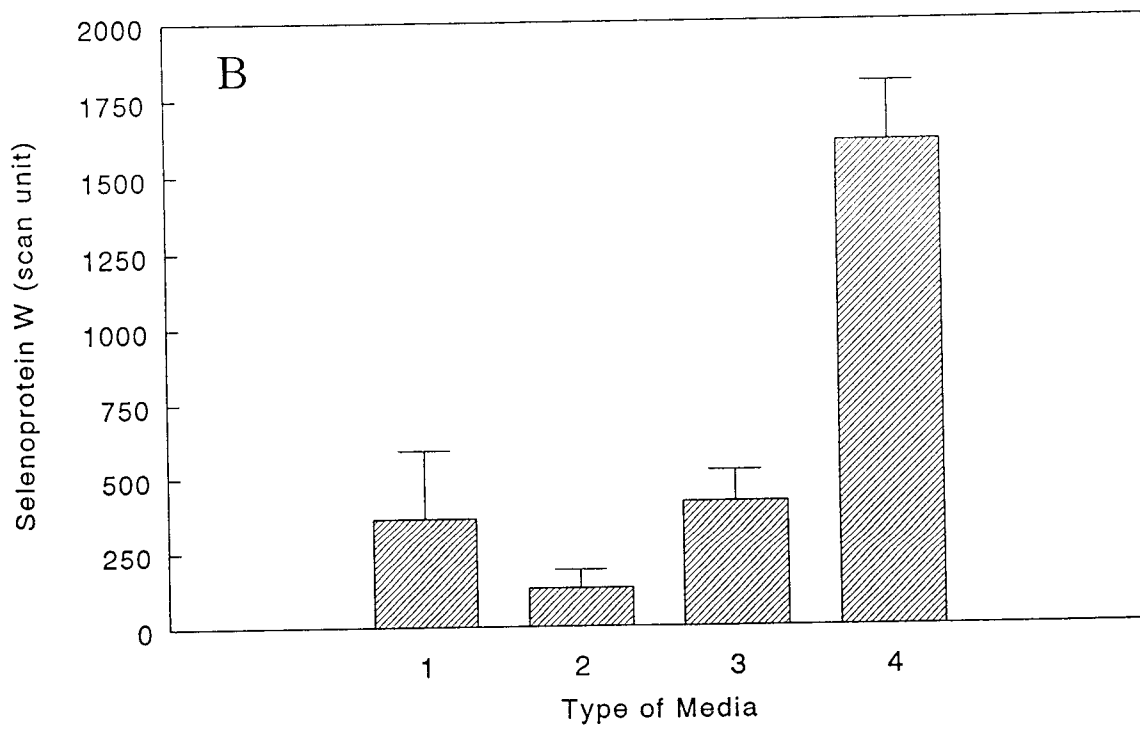

Figure 4-7B. Effects of different selenium concentrations (10^{-8} M, 10^{-7} M or 10^{-6} M Se as selenite) on selenoprotein W levels of 4-day differentiated L8 myotubes incubated for 24 hr. Bar 1: differentiation media (basal media + 2%CS), Bar 2: differentiation media + 10^{-8} M Se, Bar 3: differentiation media + 10^{-7} M Se, Bar 4: differentiation media + 10^{-6} M Se. Different electrophoresis was performed for the various time period, thus the difference can only be compared within the same incubation time.

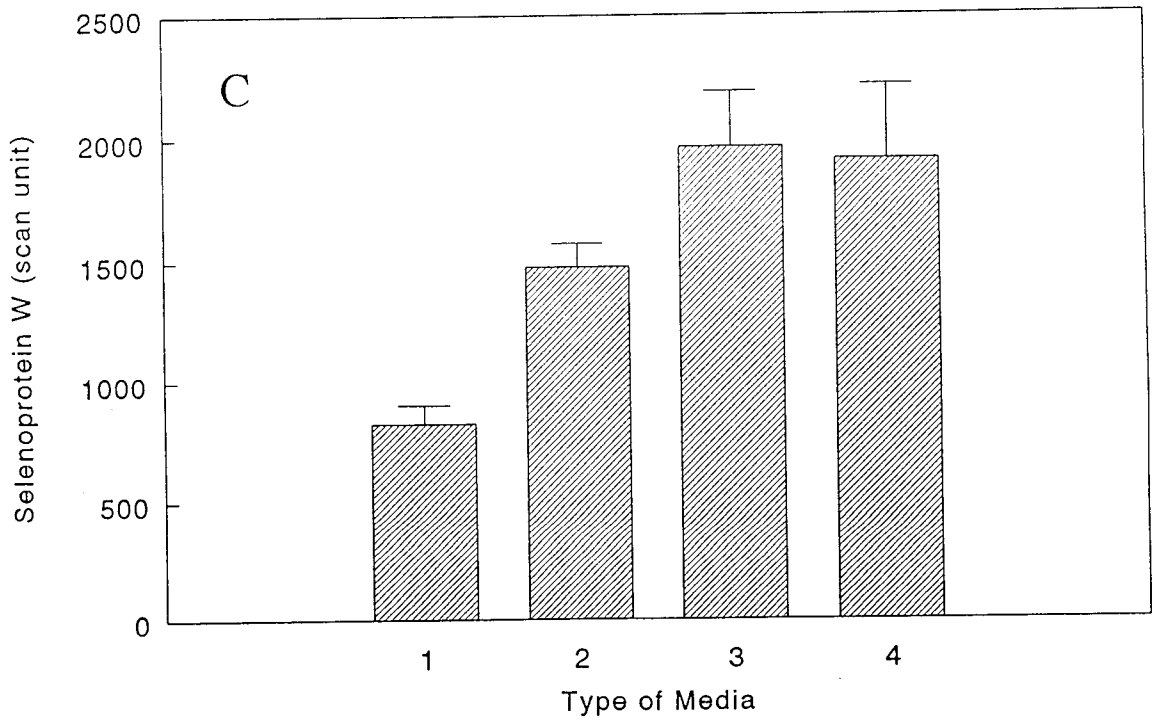

Figure 4-7C. Effects of different selenium concentrations (10^{-8} M, 10^{-7} M or 10^{-6} M Se as selenite) on selenoprotein W levels of 4-day differentiated L8 myotubes incubated for 48 hr. Bar 1: differentiation media (basal media + 2%CS), Bar 2: differentiation media + 10^{-8} M Se, Bar 3: differentiation media + 10^{-7} M Se, Bar 4: differentiation media + 10^{-6} M Se. Different electrophoresis was performed for the various time period, thus the difference can only be compared within the same incubation time.

Table 4-4. Effects of serum and selenium concentrations on selenoprotein W level and GPX activity in 6-day differentiated L8 myotubes.

Media	Se-W level	GPX activity
Basal media + 10%CS	392±49[a]	99.2±2.4[a]
Differentiation media (DM)	141±65[b]	83.3±7.8[b]
DM + 10^{-8} M Selenite	442±116[a]	109.9±5.3[a]

* Values are expressed as mean ± S.E. Different superscripts denote significant difference ($p < .05$). Selenoprotein W level is expressed as scan unit whereas GPX activity is expressed as nmol NADPH oxidized/min/mg protein.

** L8 muscle cells were cultured in basal media with 10% CS to reach confluence, then incubated in various media to differentiate for 6 days.

*** Differentiation media = basal media + 2% CS.

Another study was conducted to establish that selenium was the major factor controlling selenoprotein W content rather than serum concentration. Table 4-5 showed the selenoprotein W level and GPX activity for myotubes in various differentiation media for 6 days. The selenoprotein W levels and GPX activities between myotubes incubated in basal media with high serum concentration (10% CS) and low serum concentration (2%CS) differed significantly ($p < .05$). However, addition of selenium to low serum media resulted a significant increase ($p < .05$) of selenoprotein W level and GPX activity, but these increases in selenoprotein W level and GPX activity did not differ significantly from those of high serum media ($p > .05$). These results suggested that the lower selenoprotein W level and GPX activity found in myotubes incubated with lower serum media was due to the decreased selenium concentration which was provided by serum in media.

Northern blot analysis indicated that selenium influenced the mRNA level for selenoprotein W (Fig. 4-8). The differences in intensity of the bands indicate that selenium affected the mRNA levels.

The calculated results are shown in Table 4-6. There is no difference in 18 S rRNA levels among different media ($p > .05$). This was used as an internal control probe. The results indicated that selenoprotein W mRNA of L8 myotubes incubated in various selenium concentrations reached a plateau at concentration of 10^{-8} M in response to selenium.

Table 4-5. Effect of serum and selenium concentrations on selenoprotein W levels in 4-day differentiated L8 myotubes incubated for 48 hours.

Media	Se-W level (scan unit)
Basal media	359±31[a]
Basal media $+10^{-6}$M Se	1446±576[b]
Differentiation media	210±26[a]
Differentiation media $+10^{-6}$M Se	318±113[a]

* Values are expressed as mean ± S.E. Different superscripts denote significant difference ($p < .05$). Selenoprotein W level is expressed as scan unit whereas GPX activity is expressed as nmol NADPH oxidized/min/mg protein.

** Basal media = without serum. Differentiation media = basal media + 2%CS.

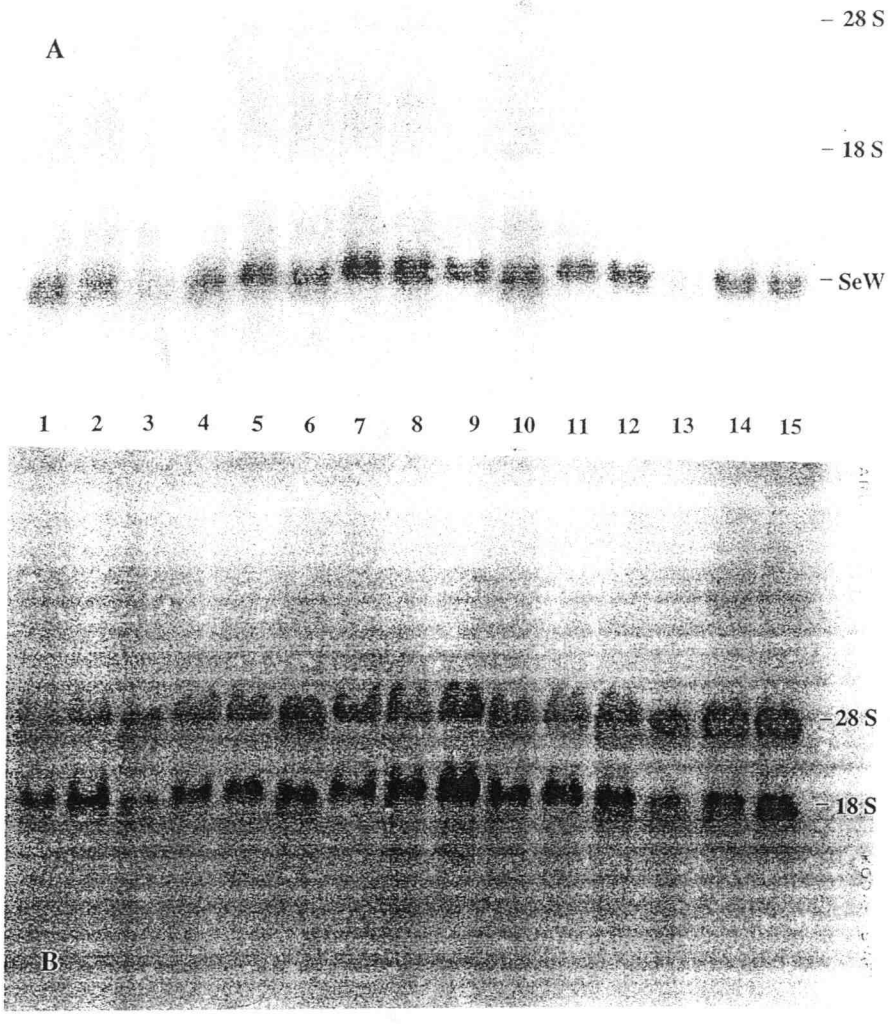

Figure 4-8. Northern blot analysis of total RNA from rat L8 myotubes. 4-day differentiated myotubes were incubated in differentiation media with additions of various concentrations of selenium as selenite for 48 hr. A: hybridization with selenoprotein W cDNA probe. B: hybridization with internal control probe (16 S rRNA oligonucleotides). Lanes 1-3: initial control, lanes 4-6: differentiation media (basal media + 2%CS), lanes 7-9: differentiation media + 10^{-6} M Se, lanes 10-12: differentiation media + 10^{-7} M Se, lanes 13-15: differentiation media + 10^{-8} M Se.

Table 4-6. Effect of various selenium concentrations on selenoprotein W mRNA level in 4-day differentiated L8 myotubes incubated for 48 hours, as shown in figure 4-8.

Media	Se-W mRNA	18 S rRNA	Ratio
Differentiation media (DM)	1684±121^{ac}	3525±158	0.48±0.03^{a}
DM + 10^{-8}M Se	1367±133^{a}	1803±537	0.81±0.10^{b}
DM + 10^{-7}M Se	2008±50^{bc}	2169±103	0.93±0.02^{b}
DM + 10^{-6}M Se	2499±221^{b}	3159±650	0.93±0.02^{b}

* Values are expressed as mean ± S.E. Different superscripts denote significant difference ($p < .05$). Selenoprotein W mRNA and 18 S rRNA are expressed as scan units. Ratio = Se-W mRNA/ 18 S rRNA.

DISCUSSION

In general, L8 muscle cells were cultured in basal media with 10% calf serum to reach confluence and then serum concentration of media was reduced to 2% to induce the differentiation of myotubes (Fig. 4-1; Table 4-1). The decrease in selenoprotein W level during muscle cell differentiation was due to the lower selenium concentration in media. However, selenoprotein W level did not change during muscle cell differentiation (Table 4-2). In addition, selenoprotein W level and GPX activity in myotubes cultured in high serum media (10%CS) were higher than those cultured in low serum media (2%CS), whereas selenoprotein W level and GPX activity were similar in myotubes cultured in low serum media with selenium addition (2%CS + 10^{-8} M Se) and high serum media (Table 4-4). These data indicated that the decrease in selenoprotein W during differentiation was due to the lower selenium concentration which was provided by serum in the media. Selenium is obviously involved in regulation of selenoprotein W in L8 muscle cells. Thus, L8 muscle cells can be used as a model to study the influence of selenium on selenoprotein W levels.

Concentration of selenium in media regulated the level of selenoprotein W in L8 muscle cells (Fig. 4-3; Table 4-3; Fig. 4-7). When selenium was reduced in the media, the selenoprotein W content decreased. When selenium was higher in the culture media, the levels of this protein likewise increased. This is similar to the results obtained in vivo. Supplementation of diets with selenium for rats (17) and sheep (18) result in increased levels of selenoprotein W in tissues. Muscle is more responsive than other tissues and thus the selenoprotein W level increased to a greater extent in muscle upon

selenium addition. Likewise, when selenium is limited in the diet, the levels of this selenoprotein were low. These results indicates that selenium regulates selenoprotein W both in vivo and in vitro, and indicates that in vitro results mimics the in vivo results.

In general, the pattern for GPX activity was similar to that for selenoprotein W levels in the present work. When this was studied in various tissues of sheep, there was no correlation between these two selenoproteins in various tissues examined, whereas the patterns for these two selenoproteins were similar in ovine muscle (18). This suggests differential regulations of selenoprotein W and GPX among the various tissues. However, the regulation of these two selenoproteins appears to be similar in muscle both in vivo and in vitro.

Among the three different forms of selenium, selenite is the most available one for both selenoprotein W and GPX synthesis in L8 muscle cells, whereas selenomethionine is the least effective form (Fig. 4-4; Fig. 4-6). The reduced ability of L8 cells to utilize selenomethionine for selenoprotein W and for GPX activity is in agreement with prior work on GPX induction (12, 13, 26). In the work with human lymphocytes, there was no correlation between uptake of selenium and induction of GPX activity. Selenocysteine was incorporated at three times the rate of selenite, and selenomethionine at 15 times the rate of selenite. Thus, the lower activity of GPX with addition of selenomethionine is not due to less uptake by the cells. This is likely due to the non-specific incorporation of selenomethionine in place of methionine during selenoprotein synthesis (12). However, selenite was found to be more effective than selenomethionine for GPX induction in lymphocytes (26).

To verify the specific incorporation of selenocystine and selenomethionine, L8 myotubes were incubated in media with addition of cysteine or methionine for 48 hr. However, selenoprotein W levels and GPX activities did not differ significantly ($p > .05$) between cells incubated with these amino acids (data not shown). These results indicate that the incorporation of selenocystine and selenomethionine into selenoproteins is selenium-dependent.

It had been reported that only one-third of whole body selenium was used for GPX synthesis and two-third of whole body selenuim was used for synthesis of other selenoproteins in rat tissues (31). Our data indicated that the response of selenoprotein W to selenium in muscle cells was more sensitive and rapid than was GPX. Selenoprotein W showed responses to selenium addition as low as 10^{-8} M for 16 hr (Fig. 4-7A), whereas GPX activity did not show differences until additions at 10^{-7} M selenium for 48 hr were used (Fig. 4-5B). This implies that the priority usage for selenium in media is for selenoprotein W synthesis, followed by GPX synthesis in L8 muscle cells.

Among various selenium concentration, 10^{-7} M selenium is the optimum concentration for maximum synthesis of selenoprotein W and GPX in L8 muscle cells (Fig. 4-4; Fig. 4-5; Fig 4-7). This is consistent with work on GPX induction. Based on GPX activity, the selenium requirements of four cell types was 10^{-7} M when selenite was used as the selenium source (13, 27). This is also consistent with growth patterns. Selenium requirements appeared to be maximum for growth at 10^{-7} M for human fibroblast, mouse myoblast and ovine fibroblast, but the Chang liver cells grew just as well on a basal media, which contained 10^{-8} M selenium, as on the selenium

supplemented ones. This suggests that the selenium requirements vary between different cell types (27).

The results in our study indicated that selenium regulates selenoprotein W synthesis both in transcription and translation. Northern blot analysis showed that selenoprotein W mRNA increased to maximum at selenium concentration of 10^{-8} M in cultured L8 myotubes for 48 hr (Fig. 4-8; Table 4-6), whereas selenoprotein W level reached a plateau at selenium concentration of 10^{-7} M (Fig. 4-7). During selenoprotein synthesis, selenium is co-translationally inserted into selenoprotein sequence. However, in our study the results indicated that in addition to translational regulation, selenium also regulates selenoprotein W synthesis at the transcriptional level. This is consistent with the study in kidney cells which showed that mRNAs of GPX and 5'-DI were regulated by selenium concentration in cultured media (32). Whether selenium regulates gene expression of selenoprotein W directly or indirectly still needs to be further investigated.

Selenoprotein W level in L8 myotubes incubated in basal media (without serum) with addition of selenium for 48 hr was significantly higher ($p < .05$) than that in myotubes incubated in low serum media (2% CS) with addition of selenium (Table 4-4). This result implies that serum may have a negative effect. To investigate the possibility that serum protein may bind selenium and reduce the availability of selenium for cell usage, differentiation media with and without serum were used. Each media were supplemented with 3 different concentrations (10^{-6}M, 10^{-7}M and 10^{-8}M) of selenium as selenite, then incubated at 37°C for 48 hr. TCA were added to precipitate proteins, and the mixtures were centrifuged at 4°C for 10 mins. Supernatants and precipitates were separated and the selenium content measured. If serum proteins do not interfer with

selenium availability, the selenium content among precipitates of differentiation media with various selenite concentrations should remain unchanged. However, the preliminary result indicated that selenium content in precipitate of differentiation media increased as selenium concentration increased (data not shown). This implies that proteins in serum may be involved in binding the selenium and reduces its availability to the muscle cells. However, further experiments are needed to investigate the serum effect on selenium.

Like selenoprotein P and MCS protein, the metabolic function of selenoprotein W is unknown. Selenium deficiency in lambs and calves results in white muscle disease, a disorder characterized by degeneration of both skeletal and cardiac muscles (28). In humans, severe dietary selenium deprivation in discrete regions of China is associated with an endemic juvenile cardiomyopathy called Keshan disease (29). Muscle weakness in patients on long-term parenteral nutrition can be alleviated and prevented by selenium supplementation (30). Therefore, there is sufficient evidence to indicate that selenium is important for normal muscle metabolism. Selenoprotein W is present in muscle at higher concentration than in other tissues, and in selenium deficient animals selenoprotein W level decreased dramatically in muscle (18). It is unknown whether selenoprotein W has any significant role in muscle metabolism, but its sensitive and rapid responses to selenium status indicates that it may play a role in muscle metabolism. Further studies to identify the possible function of selenoprotein W are necessary.

REFERENCES

1. Rotruch, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. and Hoekstra, W. G. (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179, 588-590.

2. Takahashi, K., Avissar, N., Whitin, J., and Cohen, H. (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch. Biochem. Biophys. 256, 677-686.

3. Ursini, F., Maiorino, M., and Gregolin, C. (1985) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim. Biophys. Acta 839, 62-70.

4. Chu, F-F, Doroshow, J. H., and Esworthy, R. S. (1993) Expression, characterization and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. J. Biol. Chem. 268, 2571-2576.

5. Arthur, J. R., Nicol, F. and Beckett, G. J. (1990) Hepatic iodothyronine 5'-deiodinase. Biochem. J. 272,537-540.

6. Croteau, W., Whittemore, S. L. , Schneider, M. J. and Germains, D. L. (1995) Cloning and expression of a cDNA for a mammalian type III iodothyronine deiodinase. J. Biol. Chem. 270, 16569-16575.

7. Hill, K. E., Lloyd, R. S., Yang, J-G., Read, R., and Burk, R. F. (1991) The cDNA for rat selenoprotein P contains 10 TGA codons in the open reading frame. J. Biol. Chem. 266, 10050-10053.

8. Karimpour, I., Culter, M., Shih, D., Smith, J. and Keene, K. C. (1992) Sequence of the gene encoding the mitochondrial capsule selenoprotein of mouse sperm: identification of three in-phase TGA selenocysteine codons. DNA Cell Biol. 11, 692-699.

9. Vendeland, S. C., Beilstein, M. A., Chen, C. L., Jensen, O. N., Barofsky, E. and Whanger, P. D. (1993) Purification and properties of selenoprotein W from rat muscle. J. Biol. Chem. 268, 17103-17107.

10. Vendeland, S. C., Beilstein, M. A., Yeh, J-Y., Ream, W. and Whanger, P. D. (1995) Rat skeletal muscle selenoprotein W: cDNA clone and mRNA modulation by dietary selenium. Proc. Natl. Acad. Sci., USA, 92, 8749-8753.

11. Behne, D., Hilmert, H., Scheid, S., Gessner, H. and Elger, W. (1988) Evidence for specific selenium target tissues and new biologically important selenoproteins. Biochim. Biophys. Acta 966, 12-21.

12. White, C. L. and Hoekstra, W. G. (1979) The metabolism of selenite and selenomethionine by mouse fibroblasts grown in tissue culture. Biol. Trace Elem. Res. 1, 253-257.

13. Beilstein, M. A. and Whanger, P. D. (1987) Metabolism of selenomethionine and effects of interacting compounds by mammalian cells in culture. J. Inorgan. Biochem. 29, 137-152

14. Beilstein, M. A. and Whanger, P. D. (1992) Selenium metabolism and glutathione peroxidase activity in cultured human lymphoblasts. Biol. Trace Elem. Res. 35, 105-118.

15. Berry, M. J., Banu, L., Chen, Y., Mandel, S. J., Kieffer, J. D., Harney, J. W. and Larsen, P. R. (1991) Recognition of UGA as a selenocysteine codon in type I deiodinase in the 3' untranslated region. Nature 353, 273-276.

16. Yaffe, D. (1968) Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Natl. Acad. Sci. USA 61: 477-483.

17. Yeh, J-Y, Beilstein, M. A., Andrews, J. S. and Whanger, P. D. (1995) Tissue distribution and influence of selenium status on levels of selenoprotein W. The FASEB J. 9, 392-396.

18. Yeh, J-Y, Gu, Q., Beilstein, M. A., Forsberg, N. E. and Whanger, P. D. (1995) Selenium supplementation results in increased levels of selenoprotein W in sheep tissues. J. Nutr. (to be submitted) and Chapter 5.

19. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72, 248-254.

20. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

21. Towbin, H., T. Staehelin and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamine gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci., U. S. A. 76, 4350-4354.

22. Paglia, D. E. and Valentine, W. N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158-169.

23. Brown, M. W. and Watkinson, J. H. (1977) An automated fluorimetric method for the determination of nanogram quantities of selenium. Anal. Chim. Acta. 89, 29-35.

24. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

25. Steel, R. G. D. and L. H. Torrie. 1980. Principles and Procedures of Statistics. Mcgraw-Hill Book Co., Inc., New York.

26. Karle, J. A., Kull, F. J. and Shrift, A. (1983) Uptake of selenium-75 by PHA-stimulated lymphocytes: Effect on glutathione peroxidase. Biol. Trace Elem. Res. 5, 17-24.

27. Beilstein, M. A., Whanger, P. D. and Wong, L. (1987) Selenium requirement and metabolism by mammalian cells in vitro. In: Selenium and biology and medicine (G. F. Combs, J. E. Spallholz, O. A. Levander and J. E. Oldfield, eds), An Avi Book, Van Nostrand Reinhold Comp., New York, pp 197-212.

28. Schubert, J. R., Muth, O. H., Oldfield, J. E. and Remmert. L. F. (1961) Experimental results with selenium in white muscle disease of lambs and calves. Fed. Proc. 10, 689-692.

29. Chen, X. S., Yang, G. Q., Chen, J. S., Chen, X. C., Wen, Z. M. and Ge, K. Y. (1980) Studies on the relations of selenium and Keshen disease. Biol. Trace Elem. Res. 2, 91.

30. Brown, M. R., Cohen, J. J., Lyons, J. M., Curtis, T. W., Thunberg, B., Cochran, W. J. and Klish, W. J. (1986) Proximal muscle weakness and selenium deficiency associated with long term parenteral nutrition. Amer. J. Nutr. 43, 549-554.

31. Hawkes, W. C., Wilhelmsen, E. C., Tappel, A. L. (1985) Abundance and tissue distribution of selenocysteine-containing proteins in the rat. J. Inorg. Biochem. 23:77-92.

32. Gross, M., Oertel, M. and Kohrle, J. (1995) Differential selenium-dependent expression of type I 5'-deiodinase and glutathione peroxidase in the porcine epithetial kidney cell lne LLC-PK_1. Biochem. J. 306-851-856.

33. Giovannoni, S. J. (1991) The polymerase chain reaction. In: Sequencing and hybrid techniques in bacterial systematics (Stackebrandt, E. and Goodfellow, M. eds.). John Wiley and Sons, New York. 176-201.

CHAPTER 5

SELENIUM SUPPLEMENTATION RESULTS IN INCREASED LEVELS OF SELENOPROTEIN W IN SHEEP TISSUES[1,2,3]

Jan-Ying Yeh, Qui-ping Gu, Michael A. Beilstein, Neil E. Forsberg and Philip D. Whanger

Departments of Agricultural Chemistry and Animal Sciences,
Oregon State University, Corvallis, OR 97330

Shorten title: Selenoprotein W in ovine tissues

Contact person: P. D. Whanger at the above address

Phone: 503-737-1803

Fax: 503-737-0497

email: whangerp@bcc.orst.edu

ABSTRACT

Since selenium increases the levels of other selenoproteins, the influence of this element on selenoprotein W content was examined. The effect of dietary selenium on tissue selenoprotein W was examined in wether sheep fed either a low selenium diet or the same diet supplemented with 3 mg selenium as selenite per kg diet. Muscle biopsies were taken initially and at 3.5, 7.0 and 10.5 weeks afterwards. The sheep were killed after the last muscle biopsy and samples from nine tissues were taken. Selenoprotein W was determined in tissues by western blots hybridized with a polyclonal antibody against a synthetic peptide based on the protein sequence of the homologous rat selenoprotein W. In supplemented animals, muscle selenoprotein W significantly increased over initial levels ($p < .05$) whereas in animals consuming the low selenium diet muscle selenoprotein W levels declined significantly ($p < .05$). This selenoprotein exists in various amounts among tissues examined. Highest levels were found in skeletal muscles and heart. Liver had the lowest level of selenoprotein W among the tissues examined. In supplemented sheep, the selenoprotein W tissue levels and tissue selenium concentrations were significantly higher ($p < .05$) than those in unsupplemented sheep, except for brain. The selenoprotein W levels in brains of both groups were not significantly different ($p > .05$), whereas selenium concentrations in brains of supplemented animals were significantly higher than those of unsupplemented animals ($P < .05$).

INTRODUCTION

Evidence for a low molecular weight selenium-containing protein in muscle, now called selenoprotein W, was first reported in 1969 (Pedersen et al., 1969). The presence of this protein was noted in subsequent work (Black et al., 1978; Pedersen et al., 1972), but it was only purified recently (Vendeland et al., 1993). Interest in this protein arose from possible involvement in the etiology of the sheep nutritional myopathy, white muscle disease. Failure to incorporate selenium into this protein in selenium deficient lambs is associated with the myopathy (Pedersen et al., 1972). Demonstration that the selenium in a partially pure preparation of the protein was in the form of selenocysteine (Beilstein et al., 1981) provided supporting evidence that the protein was a true selenoprotein as opposed to selenium bound to protein sulfhydryls (Ganther and Corcoran, 1969), or random incorporation of selenomethionine for methionine in proteins (McConnell et al., 1974).

Synthesis of several other selenocysteine containing proteins is regulated by dietary selenium (Arthur et al., 1990; Behne et al., 1990; Burk and Hill, 1993 and Sunde, 1990). Previous studies of the relationship of selenoprotein W to dietary selenium in sheep (Black et al., 1978) and rats (Beilstein and Whanger, 1985) were based on the observation of the incorporation of radiolabeled ^{75}Se into a low molecular weight fraction of muscle cytosol. The availability of antibodies which recognized the selenoprotein in a number of species (Yeh et al., 1995) made it feasible to precisely determine levels of the protein during dietary manipulation.

In the current study selenoprotein W was measured in muscle biopsies collected from sheep as they were either supplemented or depleted of selenium. After 10.5 weeks of feeding these diets, the sheep were killed and the selenoprotein was examined in other tissues.

MATERIALS AND METHODS

Six wether sheep of mixed breed about six months of age were divided into two groups. One group was fed the basal diet of pelleted alfalfa (Vernell Pellet Company, Corvallis, OR) which contained 15% protein, 1% fat, 35% fiber and 0.020 mg selenium per kg. The other group was fed the same pellets with selenite added at 3 mg selenium per kg. Feed was provided ad libitum, but there was no difference in feed consumption between the two groups during the study ($p > .05$).

Biopsies of the biceps femoris muscle were taken from each sheep initially and at 3.5, 7.0 and 10.5 weeks after beginning the experimental diets. Blood was taken from the jugular vein at the same time of biopsy. After a sample was taken for selenium analysis, the blood was centrifuged to separate the plasma from erythrocytes. The plasma was frozen at -70^0 C until analysis. Selenium concentration and glutathione peroxidase (GPX, EC 1.11.1.9) activity were determined on the plasma samples. For surgery, sheep were first anaesthetized with Biotal (Boehringer Ingelheim Animal Health, Inc., St. Louis, MO) and maintained under anaesthesia with Halothane (Halocarbon Laboratory, River Edge, NJ). A lateral incision was made to access the biceps femoris. Fascia and connective tissue were cleaned to remove a 1 g portion of the caudal biceps femoris. This muscle was then immediately frozen at -70° C until analysis. The remaining biceps femoris was sutured and the wound closed. Animals were sprayed with Furox topical antibiotic (Solvay Animal Health, Inc., Mendota Heights, MN) and injected intramuscularly with Liquimycin LA-200 antibiotic (Pfizer Animal Health, New York, NY) to prevent infection. Animals were monitored for two weeks following surgery to

ensure complete recovery. At the end of the experiment, animals were sacrified by exsanguination after being stunned with a stun-gun and samples of two muscles (biceps femoris and semitendinosus), heart, tongue, brain, lung, spleen, kidney and liver were quickly removed and frozen on dry ice. Tissue samples were subsequently stored at -70° C until analysis.

Tissues were homogenized in 6 volumes of buffer (20 mmol/L Tris [pH 7.5], 0.25 mol/L sucrose, 1 mmol/L EGTA, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L 2-mercaptoethanol and 0.025 g/L leupeptin) and the homogenates were centrifuged at 17,000 x g for 10 minutes at 4°C to obtain cytosolic extracts. GPX enzyme activity was determined in extracts by the method of Paglia and Valentine (1967) and protein concentrations were measured by the dye-binding assay (Bradford, 1976) using bovine serum albumin as standards. After digestion with nitric and perchloric acids, selenium content of whole blood, tissue samples and extracts were determined by a semi-automated fluorometric assay (Brown and Watkinson, 1977) with an Alpchem II system (Alpchem Corp., Milwaukie, OR).

Tissue extracts 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 um, BA-S83; Schleicher & Schuell, Keene, NH) according to the method of Towbin et al. (1979). Pure rat selenoprotein W ranged from 5 to 20 ng were included in each gel to use as standards. Selenoprotein W contents in tissue extracts were determined in Western Blot analysis as described by Yeh et al. (1995), using a rabbit polyclonal antibody against the peptide sequence corresponding to amino acid residues 13 to 31 of rat selenoprotein W. After hybridization with horseradish peroxidase-

conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA), blots were incubated with ECL chemiluminescent reagent (Amersham Life Sciences Inc., Arlington Heights, IL) and exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester NY). Developed films were scanned with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuaNT program (Molecular Dynamics, Sunnyvale, CA).

Statistical analyses were performed by the Student-Newman-Keuls t-test and analysis of variance (ANOVA) with Fisher's least-significant difference (LSD) method for comparing groups (Steel and Torrie, 1980). A significant level of 5 % was adopted for all comparisons. This study was approved by the animal care committee of Oregon State University.

RESULTS

There were no significant differences in muscle GPX activity, muscle selenium contents, muscle selenium selenoprotein W content, whole blood selenium concentration, plasma selenium concentration or plasma GPX activity between the sheep at the initiation of this experiment, indicating that their selenium status was similar. The selenium concentration in the whole blood is shown in figure 5-1. The selenium concentration in the deficient sheep gradually dropped from 3.4×10^{-6} mol/L to 1.9×10^{-6} mole/L at the end of the experiment ($p < .05$). As expected, the selenium concentration in the whole blood of the supplemented sheep increased significantly ($p < .05$) throughout the study, reaching a mean level of 1.1×10^{-5} mol/L at the end of the study.

Figure 5-2 shows the Western blot for muscle biopsy samples at various times from the two groups of sheep. Based on the intensity of the bands, the levels of selenoprotein W increased over time in supplemented sheep whereas this decreased in deficient sheep.

The selenoprotein W in the muscle (figure 5-3) followed similar patterns as the blood. The concentration of this selenoprotein gradually dropped from about 24 to 15 ng per mg protein at the end of the study ($p < .05$). In contrast, there was a significant increase ($p < .05$) of this selenoprotein in the muscle of supplemented sheep. The selenoprotein W concentrations in muscles of supplemented sheep were significantly higher ($p < .05$) at 3.5 weeks and 7.0 weeks in comparison to the initial values and the 10.5 weeks values were significantly higher ($p < .05$) as compared to the earlier ones. This is similar to the blood selenium patterns.

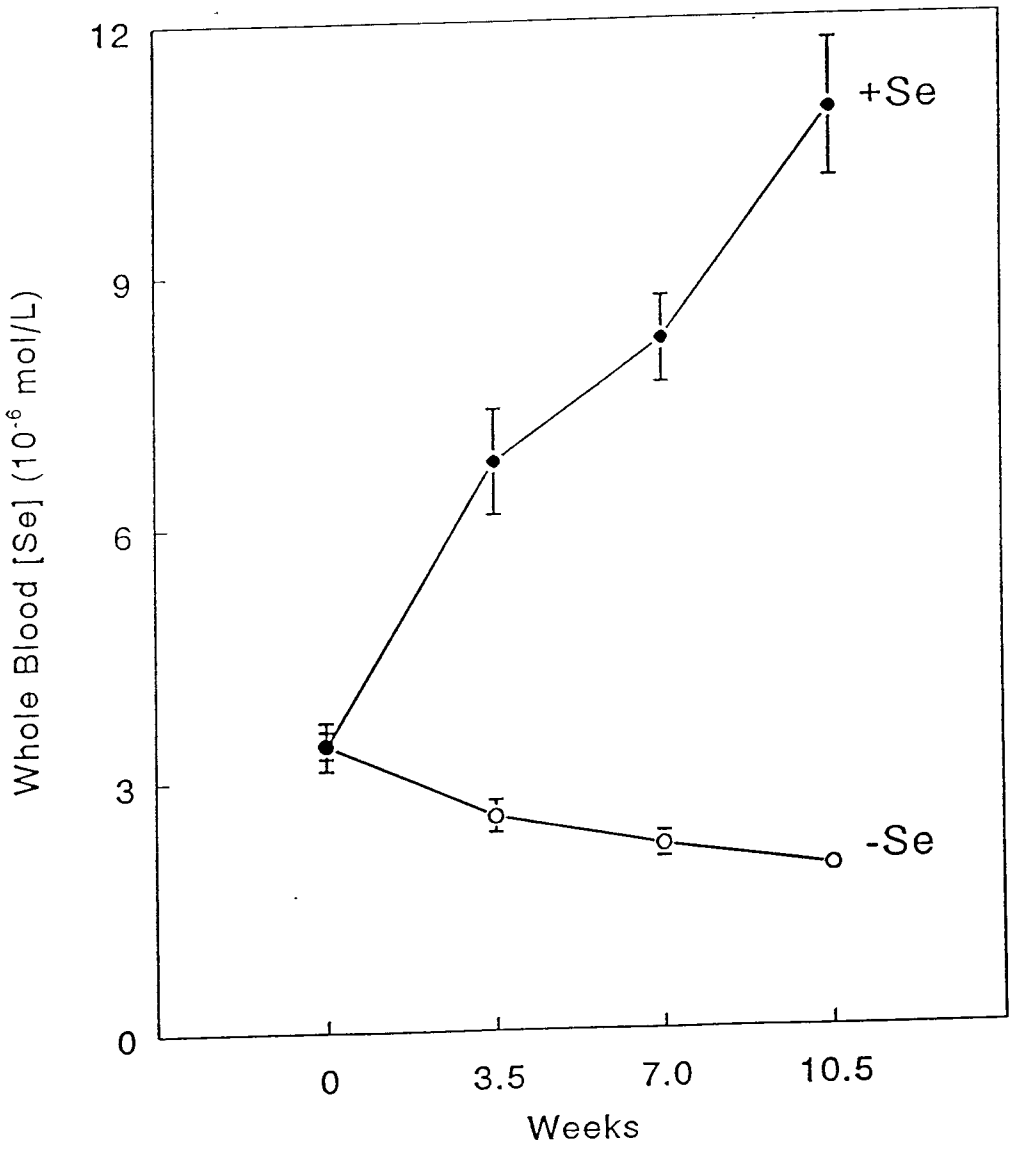

Figure 5-1. Blood selenium levels in sheep fed for various times the basal diet or those fed this diet plus 3 mg selenium per kg. The values are presented as means ± SEM (as indicated by error bars).

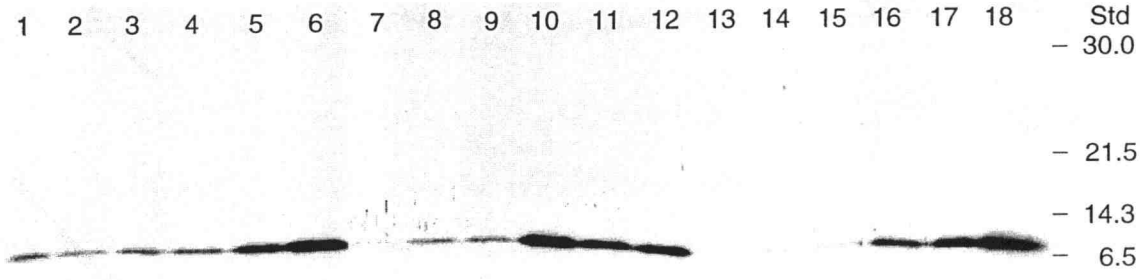

Figure 5-2. Western blot of muscle samples taken from sheep fed the deficient or supplemented diets for various times. Lanes 1-3: sheep fed Se-deficient diet for 3.5 wks. Lanes 4-6: sheep fed Se-supplemented diet for 3.5 wks. Lanes 7-9: sheep fed Se-deficient diet for 7.0 wks. Lanes 10-12: sheep fed Se-supplemented diet for 7.0 wks. Lanes 13-15: sheep fed Se-deficient diet for 10.5 wks. Lanes 16-18: sheep fed Se-supplemented diet for 10.5 wks.

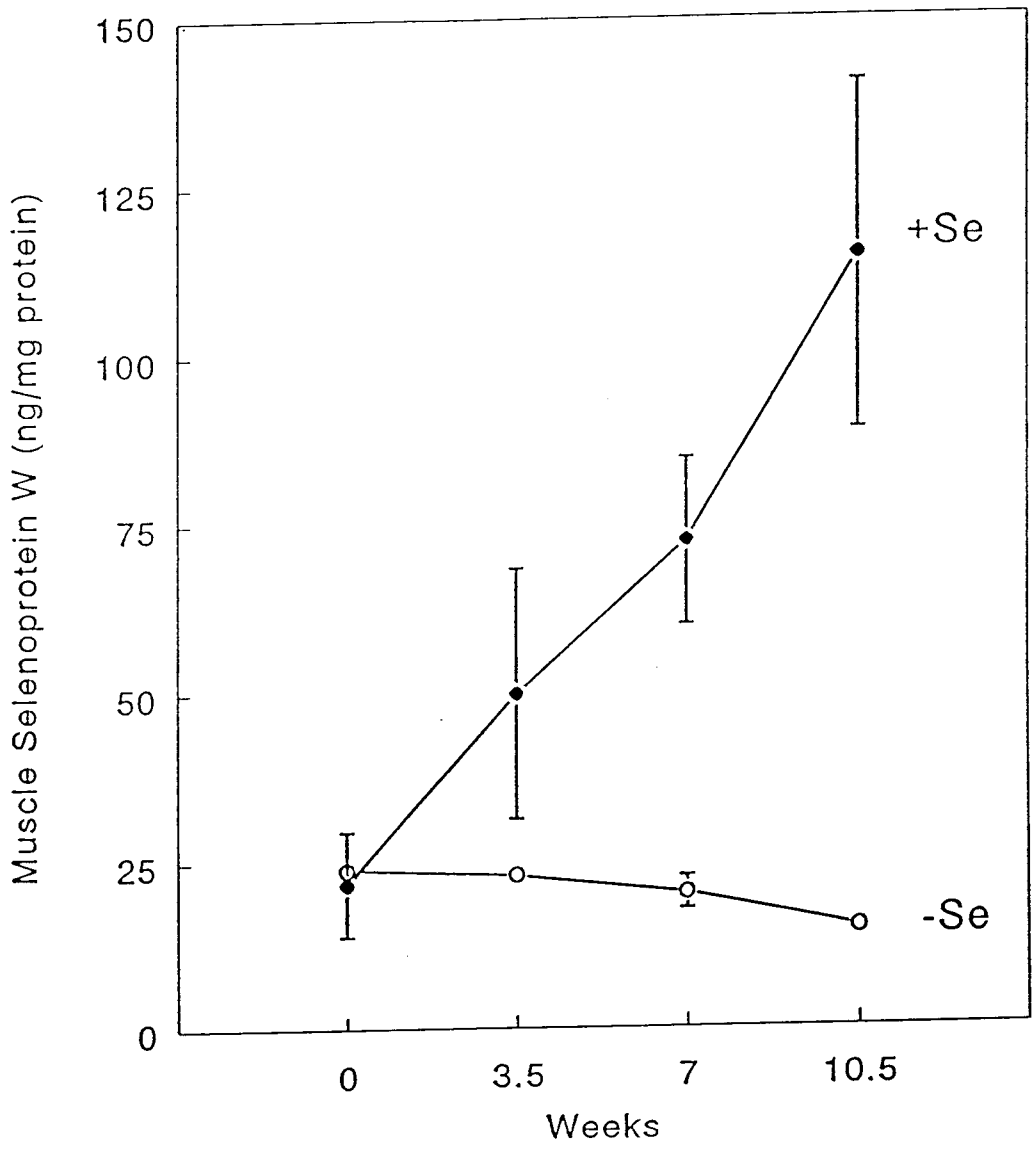

Figure 5-3. Selenoprotein W content in muscle from sheep fed for various times the basal diet or those fed this diet with 3 mg selenium per kg. The values are presented as means ± SEM (as indicated by the error bars).

The selenium content in muscles of deficient sheep decreased gradually ($p < .05$) whereas those in muscles of supplemented sheep increased dramatically ($p < .05$) and reach a plateau after 3.5 weeks of supplementation (figure 5-4). The selenium content in the muscle cytosols followed the same patterns as the selenoprotein W content (data not shown).

The GPX activity of the muscle followed similar trends but did not duplicate the results with selenoprotein W (figure 5-5). As expected, GPX activity dropped ($p < .05$) to the lower point after 3.5 weeks in the deficient sheep whereas GPX activity of supplemented sheep increased dramatically ($p < .05$) and reached a plateau after 3.5 weeks of supplementation. The patterns of muscle GPX activitiy and muscle selenium content in supplemented sheep were similar.

The selenoprotein W content of the various tissues is shown in figure 5-6. The selenoprotein W content was significantly lower ($p < 0.05$ to 0.01) in all tissues of the control, except for the brain, than the supplemented sheep. The selenoprotein W content was highest in muscle and heart, following by brain, tongue, spleen, kidney, lungs and liver in decreasing order. Since the selenoprotein W content was the same for the biceps femoris and semitendinosus muscles, the average values for each of these are presented in this figure.

The selenium content in the whole tissues followed a different pattern than selenoprotein W (figure 5-7). The selenium content was highest in the liver, followed by kidney, lung, spleen, heart, tongue, brain, and the muscles (combined biceps femoris and semitendinosus) in decreasing order. The selenium content was significantly higher

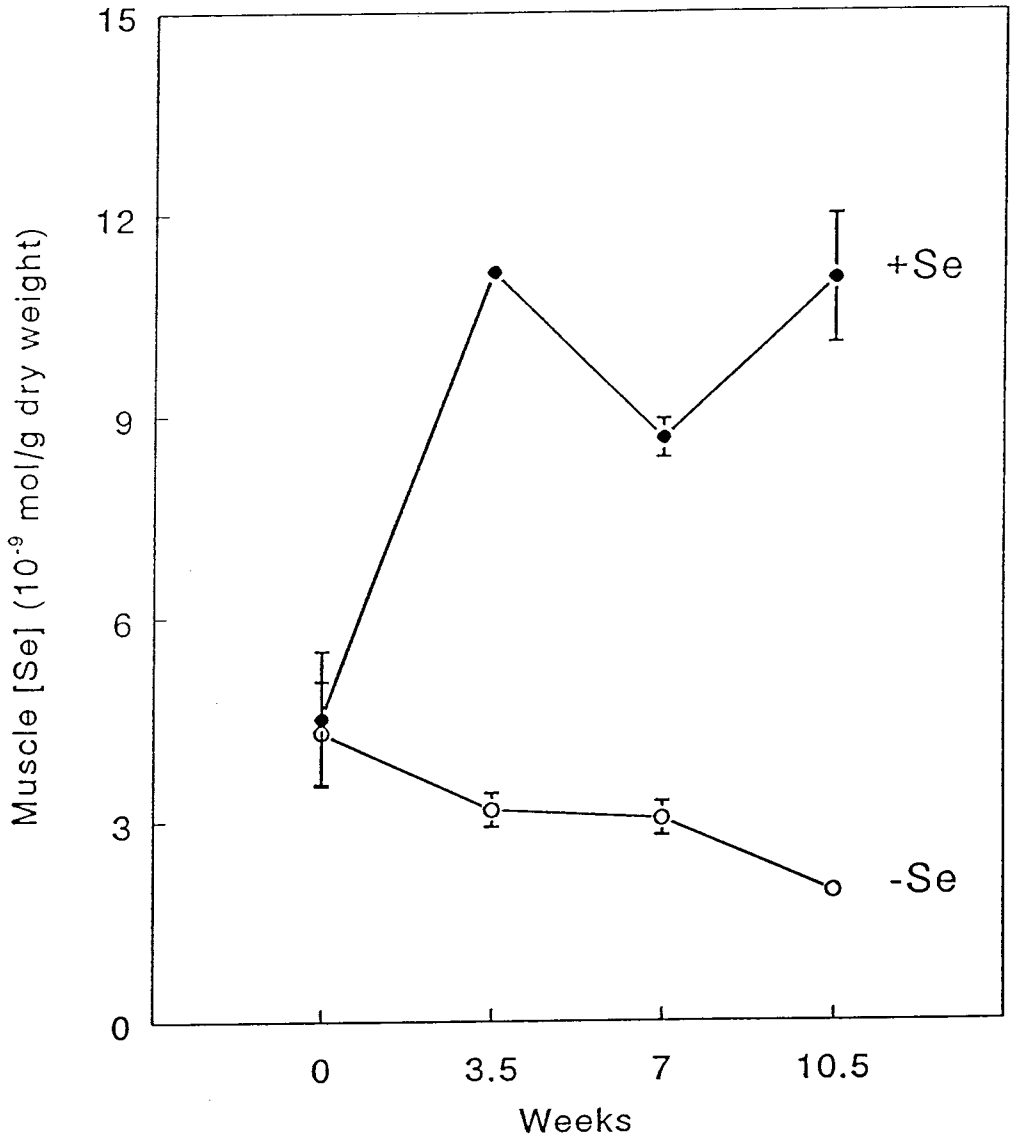

Figure 5-4. Selenium content in muscle from sheep fed for various times the basal diet or those fed this diet with 3 mg selenium per kg. The values are presented as mean ± SEM (as indicated by the error bars).

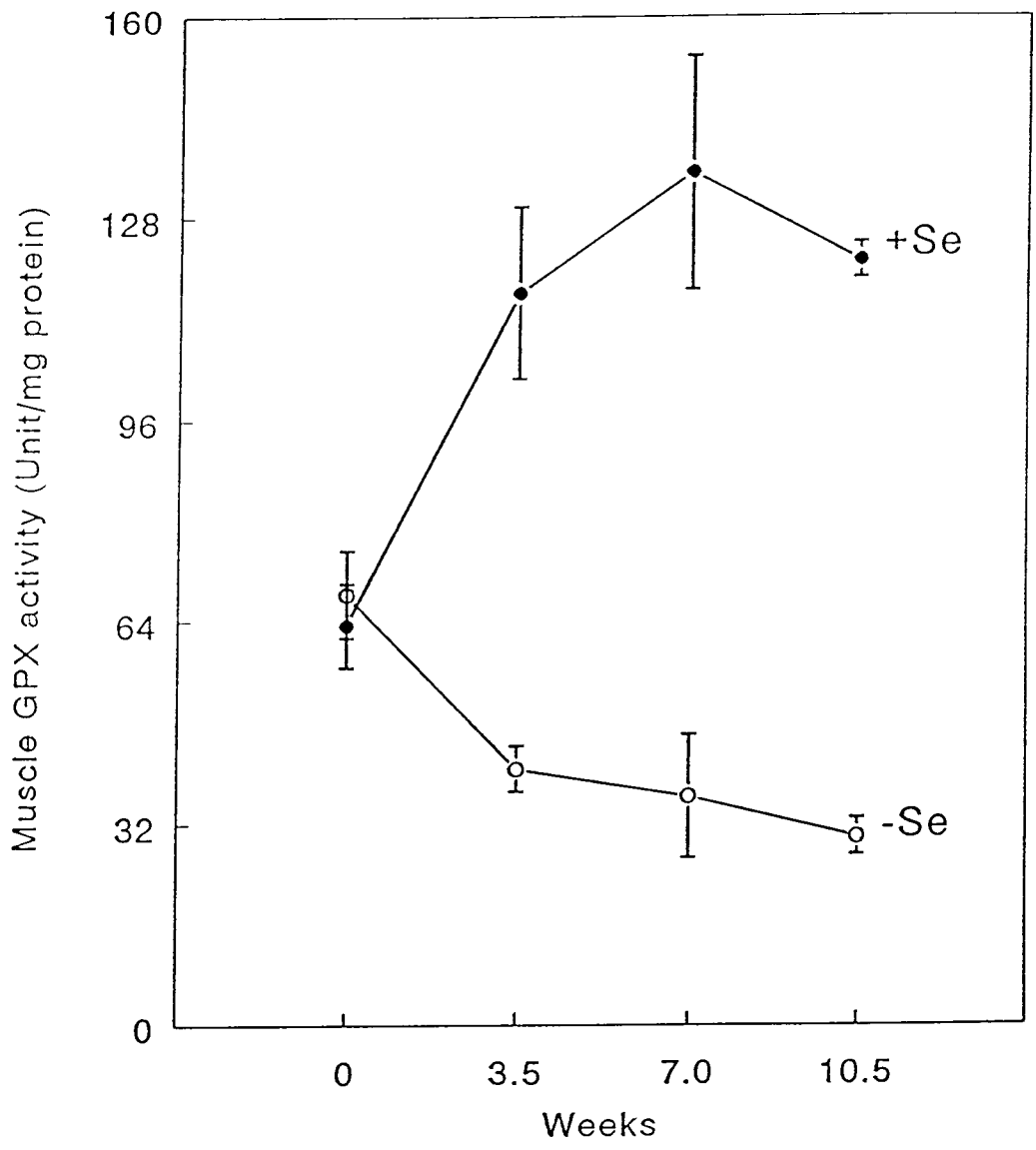

Figure 5-5. Glutathione peroxidase activity in muscle from sheep fed for various times the basal diet and those fed this diet with 3 mg selenium per kg. The GPX activity is expressed as nmol NADPH oxidized/min/mg protein. The values are presented as means ± SEM (as indicated by the error bars).

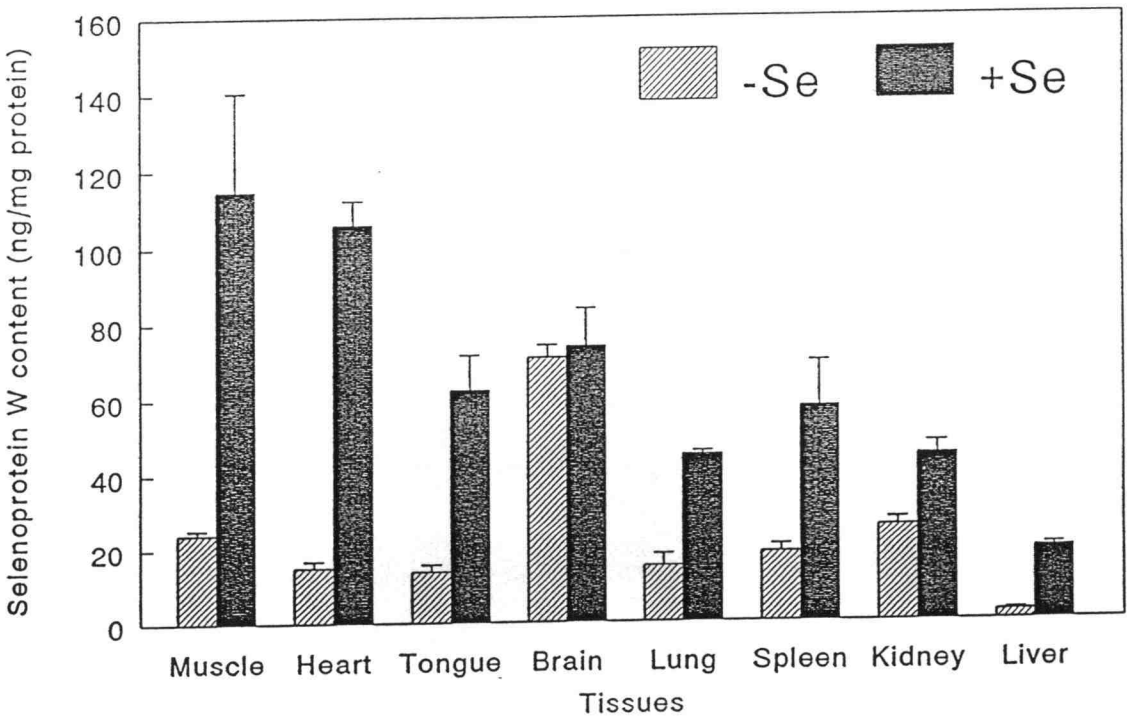

Figure 5-6. Selenoprotein W content in different tissues from sheep fed the basal diet or those fed the basal diet with 3 mg selenium per kg. The values are presented as means ± SEM (as indicated by error bars).

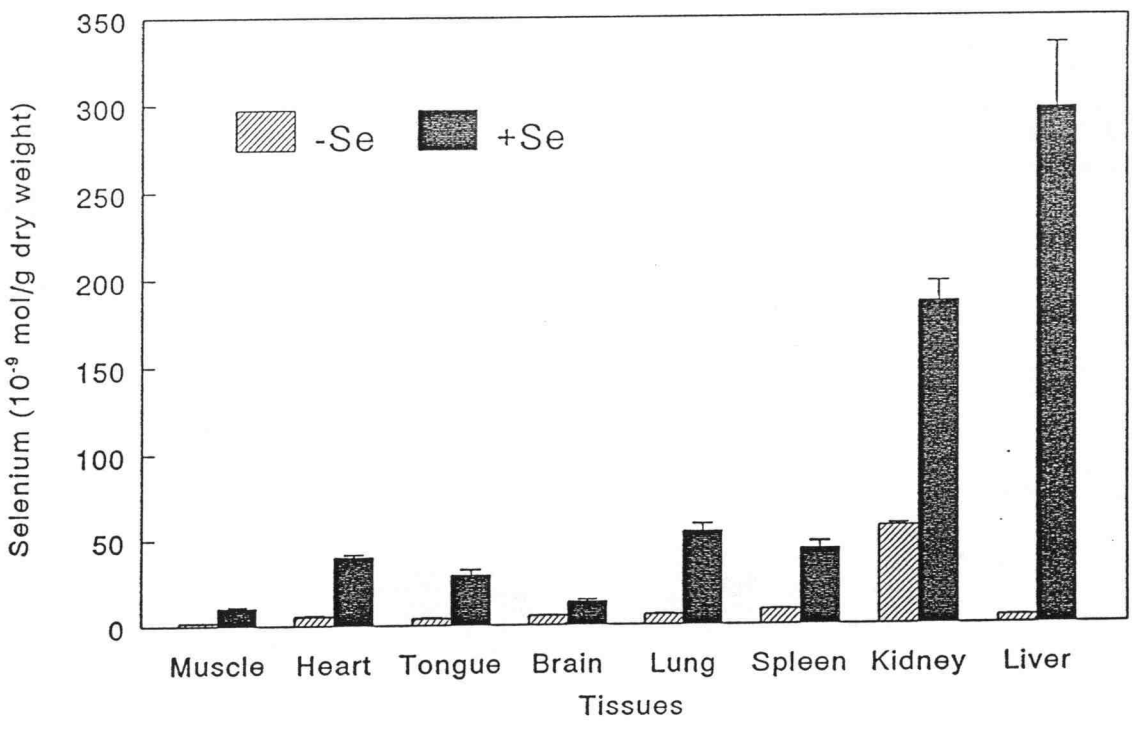

Figure 5- 7. Selenium content of various whole tissues from sheep fed the basal diet and those fed this diet plus 3 mg selenium per kg. The values are presented as means ± SEM (as indicated by error bars).

($p < 0.05$ to 0.01) in all tissues from the supplemented sheep than in those from sheep fed the deficient diet. The selenium in the cytosols from these tissues followed the same patterns (data not shown).

The GPX activity in the various sheep tissues did not follow the pattern of either the selenium content or the selenoprotein W levels (figure 5-8). This activity was lower in all tissues, except brain, from the deficient sheep than those from the supplemented ones ($p < .05$ to 0.01). GPX activity was highest in the spleen, followed by lung, heart, tongue, kidney, brain, muscles (combined biceps and semitendinosus) and liver in decreasing order. Thus, it is evident that the distribution of selenium, selenoprotein W and GPX between tissues do not follow similar patterns. There are no correlations among the relative tissue distributions of selenium, selenoprotein W content and GPX activity.

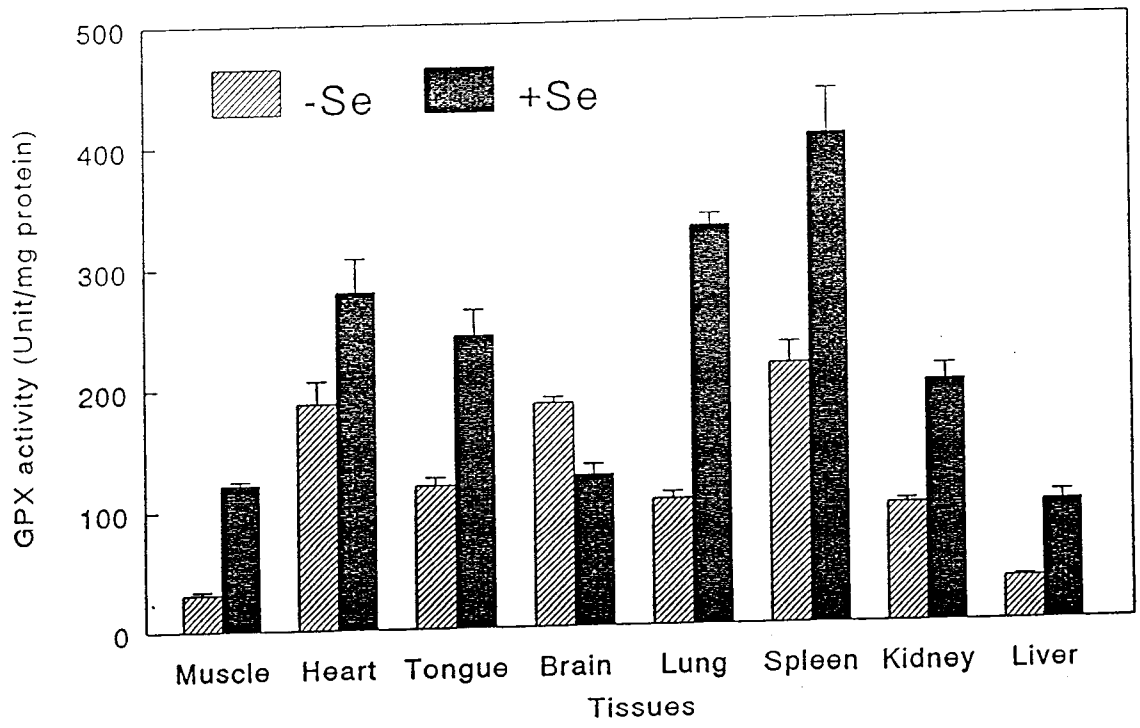

Figure 5-8. Glutathione peroxidase activity in different tissues from sheep fed the basal diet or those fed the diet with 3 mg selenium per kg. The GPX activity is expressed as nmol NADPH oxidized/min/mg protein. The values are presented as means ± SEM (as indicated by error bars).

DISCUSSION

The cDNA for selenoprotein W from sheep muscle has now been sequenced, and this selenoprotein was found to be conserved between the rat and sheep (Gu et al., 1996). The antibody raised against the peptide sequence of rat selenoprotein W was shown to cross-react with ovine tissues (Yeh et al., 1995). Thus, this antibody was used in Western blot analysis in the present study.

These results indicate that there are no correlations among the relative tissue distribution of selenium, GPX and selenoprotein W. Selenoprotein W was highest in muscle and heart (figure 5-6), selenium was highest in liver (figure 5-7), whereas GPX was highest in the spleen (figure 5-8) for selenium-supplemented sheep. There was a significant correlation of muscle selenoprotein W content with the selenium levels ($P < 0.002$), with muscle cytosolic selenium ($P < 0.005$), and with muscle GPX activity ($P < 0.003$). As expected, when all tissues were considered there was no correlation of tissue selenoprotein W content with tissue selenium, tissue cystosol selenium or with tissue GPX activity. White muscle disease is a selenium deficient myopathy where lesions occur in the muscle and heart (Schubert et al., 1961). Because selenoprotein W is highest in these organs in sheep given selenium, it is tempting to speculate its involvement in the prevention of this disorder in these organs.

Deficient and 3 mg selenium per kg diet were compared to determine whether there was a difference in selenoprotein W levels between extremely wide selenium status. Now that it has been established that selenoprotein W levels increase with supernutritional levels, nutritional levels will now be tested. However, it should be noted that in rats

selenoprotein W levels in muscle reaches a plateau only after 1 mg selenium per kg diet is fed (Yeh et al., unpublished data). It will be of interest to determine whether similar patterns are obtained with sheep.

There were no difference ($p > .05$) in the selenoprotein W content in the brain between the sheep fed the deficient diet and those given selenium (figure 5-6), but there was a 53% difference in the total selenium content (figure 5-7). The GPX activity was higher ($p < .05$) in this organ of the deficient sheep than that of supplemented sheep (figure 5-8). However, the regulations of selenium and selenoprotein synthesis in brain appear to be different from those in other tissues. Davidson and Kennedy (1993) indicated that there is greater synthesis of the selenoproteins in deficient sheep. Brain may regulate its selenoprotein levels to balance the selenium content during selenium deficiency or selenium toxicity. In our study, it shows that selenoprotein W content in brain did not change due to selenium deficiency or supplementation. However, GPX activity was higher and the selenium content was 53% lower in brain of deficient sheep. Therefore, the regulations of these two selenoproteins are different in brain. Further studies are needed to investigate the significance of these observations.

Neither the tissue distribution or its characteristics offer any clues on the metabolic functions of selenoprotein W. It contains one gram atom of selenium per mole protein (Vendeland et al, 1993) and reduced glutathione is bound to one species of this protein (Beilstein et al, 1995). Since all the selenoenzymes which have been identified thus far are involved in redox reactions (Burk and Hill, 1993; Sunde, 1990), it appears advantageous to postulate a role for selenoprotein W as an antioxidant, especially since glutathione is one of its binding moieties.

Selenoprotein W is one of three mammalian selenoproteins which have been well characterized without identified functions. Of these, selenoprotein P has been studied to the greatest extent. The majority of the selenium in plasma of rats (Read et al, 1990) and humans (Deagen et al, 1993) has been shown to be associated with this protein. It is the highest selenium containing protein known, with ten selenocysteine residues per mole protein (Hill et al, 1991). Similar to selenoprotein W in tissues, the level of selenoprotein P in plasma is affected by the selenium status of the animal (Yang et al, 1969). The only other selenoprotein known to possess more than one selenocysteine residues per subunit is mitochondrial capsule selenoprotein (MCS) from mouse sperm, which interestingly contains all three selenocysteines in the first 35 amino acids of a total of 197 residues (Karimpour et al, 1992). The cDNA for selenoprotein W has been recently cloned and it is similar to MCS in that the selenocysteine residue is in the early part (number 13) of the molecule (Vendeland et al, 1995). This is in contrast to selenoprotein P where most of the selenocysteine residues are in the last part of the protein (Hill et al, 1991).

A family of GPXs and type 1 iodothyronine 5'-deiodinase are the only mammalian selenoenzymes presently known. The cellular GPX was recognized about 20 years ago (Rotruck et al, 1973) and represented the only biochemical function of selenium for many years. A plasma GPX was shown to be immunochemically distinct from the cellular enzyme (Takahashi et al, 1987), and is clearly a separate gene product from the cellular one (Takahashi et al, 1990). A third GPX was identified which reduces fatty acid hydroperoxides esterified to phospholipids (PHGPX), and assumed to play a significant role in reducing peroxides in membranes (Schuckelt et al, 1991). A fourth GPX has been found predominantly in the gastrointestinal mucosa (Chu et al, 1993). Except for

PHGPX, which is a monomer, all GPXs are tetramers and have one selenocysteine residue per subunit. The deiodinase contains one selenocysteine per mole protein (Behne et al, 1990; Berry et al, 1991) and converts thyroxine to triiodothyronine, providing a metabolic link between selenium and iodine. The relationship of selenoprotein W to these selenoenzymes is not known, but the present results indicate a different tissue distribution from them.

Up to 13 selenium containing proteins have been reported in rat tissues (Behne et al, 1988) and up to 9 have been reported in ovine tissues (Davidson and Kennedy, 1993). Three to four selenium containing proteins were found in ovine heart and muscle. Thus far only GPX and selenoprotein W have been identified in these tissues, which suggests that there are at least two more to be identified in these tissues. More selenium containing proteins were present in other tissues such as the liver and pituitary, providing sufficient reasons to believe that the identification of more selenoproteins is forthcoming.

REFERENCES

Arthur, J. R., Nicol, F. and Beckett, G. J. (1990) Hepatic iodothyronine 5'-deiodinase. Biochem. J. 272: 537-540.

Behne, D., Hilmert, H, Scheid, S. Gessner, H. and Elger, W. (1988) Evidence for specific selenium target tissues and new biologically important selenoproteins. Biochim. Biophys. Acta 966: 12-21.

Behne, D., Kyriakopoulos, A., Meinhold, H. and Kohrie, J. (1990). Identification of type I iodothyronine 5'-deiodinase as a selenoenzyme. Biochem. Biophys. Res. Commun. 173: 1143-1149.

Beilstein, M. A., Tripp, M. J. and Whanger, P. D. (1981) Evidence for selenocysteine in ovine tissue organelles. J. Inorgan. Biochem. 15: 339-347.

Beilstein, M.A. and Whanger P. D. (1985) Selenium accumulation in tissues, tissue fractions and cytosolic proteins in rats. Biochem. Arch. 1: 153-162.

Beilstein, M. A., Vendeland, S. C., Barofsky, E., Jensen, O. N. and Whanger, P. D. (1995) Selenoprotein W of rat muscle binds glutathione and an unknown small molecular weight moiety. J. Inorgan. Biochem. 59: 256-262.

Berry, M. L., Banu, L. and Larsen, P. R. (1991) Type I iodothyronne deiodinase is a selenocysteine-containing enzyme. Nature 349: 438-440.

Black, R. S., Tripp, M. J., Whanger, P. D. and Weswig, P. H. (1978). Selenium proteins in ovine tissues: III. Distribution of selenium and glutathione peroxidases in tissue cytosols. Bioinorgan. Chem. 8: 161-170.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72: 248-254.

Brown, M. W. and Watkinson, J. H. (1977). An automated fluorimetric method for the determination of nanogram quantities of selenium. Anal. Chim. Acta. 89: 29-35.

Burk, R. F. and Hill, K. E. (1993). Regulation of selenoproteins. Annu. Rev. Nutr. 13: 65-81.

Chu, F. F., Doroshow, J. H. and Esworthy, R. S. (1993) Expression, characterization and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. J. Biol. Chem. 268: 2571-2576.

Davidson, W. B. and Kennedy, G. (1993) Synthesis of [^{75}Se] selenoproteins is greater in selenium-deficient sheep. J. Nutr. 123: 689-694.

Deagen, J. T., Butler, J. A., Zachara, B. A. and Whanger, P. D. (1993) Determination of the distribution of selenium between glutathione peroxidase, selenoprotein P and albumin in plasma. Anal. Biochem. 208: 176-184.

Forstrom, J. W., Sakowski, J. J. and Tappel, A. L. (1978). Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. Biochemistry 17: 2639-2644.

Ganther, H.E. and C. Corcoran (1969) Selenotrisulfides. II. Cross-linking of reduced pancreatic ribonuclease with selenium. Biochemistry 8: 2557-2563.

Gu, Q., Beilstein, M. A., Vendeland, S. C., Ream, L. M., and Whanger, P. D. (1996) Nucleotide sequences of selenoprotein W cDNAs from skeletal muscle of rat, mouse, sheep, monkey and human. Nucleic Acid Res. (Submitted)

Hill, K. E., Lloyd, R. S., Yang, J-G, Read, R. and Burk, R. F. (1991) The cDNA for rat selenoprotein P contains 10 TGA condons in the open reading frame. J. Biol. Chem. 266: 10050-10053.

Karimpour, I, Cutler, M., Shih, D., Smith, J. and Keene, K. C. (1992) Sequence of the gene encoding the mitochondrial capsule selenoprotein of mouse sperm: identification of three in-phase TGA selenocysteine codons. DNA Cell Biol. 11: 692-699.

Kraus, R. J., Foster, S. J. and Ganther, H. E. (1983). Identification of selenocysteine in glutathione peroxidase by mass spectroscopy. Biochemistry 22: 5853-5858.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

McConnell, K. P., Hsu, J. M., Herrman, J. L. and Anthony W. L. (1974) Parallelism between sulfur and selenium amino acids in protein synthesis in the skin of zinc-deficient rats. Proc. Soc. Exptl. Biol. Med. 145: 970-974.

Paglia, D. E. and Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70:158-169.

Pedersen, N. D., Whanger, P. D. and Weswig, P. H. (1969) Distribution of ^{75}Se-bound proteins in tissues of normal and myopathic lambs. Pacific Slope Biochem. Conf. p. 49.

Pedersen, N. D., Whanger, P. D., Weswig, P. H. and Muth, O. H. (1972). Selenium binding proteins in tissues of normal and selenium responsive myopathic lambs. Bioinorgan. Chem. 2: 33-45.

Read, R., Bellew, T., Yang, Y-G., Hill, K. E., Palmer, I. S. and Burk, R. F. (1990) Selenium and amino acid composition of selenoprotein P, the major selenoprotein in rat serum: J. Biol. Chem. 265: 17899-17905.

Rotruck, J. T., Pope, A. L. Ganther, H. E., Swanson, A. B., Hafeman, D. and Hoekstra, W. G. (1973). Selenium: biochemical role as a component of glutathione peroxidase. Science 179: 588-590.

Schubert, J. R., Muth, O. H., Oldfield, J. E. and Remmert, L. F. (1961) Experimental results with selenium in white muscle disease of lambs and calves. Fed Proc. 20: 689-692.

Schuckelt, R., Brigelius-Flohe, R., Maiorino, M., Roveri, A. and Reumkens, J. (1991) Phospholipid hydroperoxide glutathione is a selenoenzyme distinct from the classical glutathione peroxidase as evident from cDNA and amino acid sequencing. Free Radical Res. Commun. 14: 343-346.

Stadtman, T. C. (1990) Selenium biochemistry. Annu. Rev. Biochem. 59:11-127.

Steel, R. G. D. and Torrie, J. H. (1980). Principles and Procedures of Statistics, 2nd edition, p. 186-190, McGraw-Hill, New York, NY, USA.

Sunde, R. A. (1990). Molecular biology of selenoproteins. Annu. Rev. Nutr. 10: 451-474.

Takahashi, K., Avissar, N., Whitin, J. and Cohen, H. (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch. Biochem. Biophys. 256: 677-686.

Takahashi, K., Akasaka, M., Yamamoto, Y., Koyayahi, C., Mizoguchi, J. and Koyama, J. (1990) Primary structure of human plasma glutathione peroxidase deduced from cDNA sequences. J. Biochem. 108: 145-148.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamine gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354.

Vendeland, S. C., Beilstein, M. A., Chen, C. L., Jensen, O. N., Barofsky, E. and Whanger, P. D. (1993). Purification and properties of selenoprotein W from rat muscle. J. Biol. Chem. 268: 17103-17107.

Vendeland, S. C., Beilstein, M. A., Yeh, J-Y, Ream, L. W. and Whanger, P. D. (1995) Rat skeletal muscle selenoprotein W: cDNA clone and mRNA modulation by dietary selenium. Proc. Natl. Acad. Sci., USA 92: 8749-8753.

Yang, J-G, Hill, K. E. and Burk, R. F. (1989) Dietary selenium intake controls rat plasma selenoprotein P concentration. J. Nutr. 119: 1010-12.

Yeh, J-Y, Beilstein, M. A., Andrews, J. S. and Whanger, P. D. (1995). Tissue distribution and influence of selenium status on levels of selenoprotein W. The FASEB J. 9: 392-396.

CHAPTER 6

CONCLUSION

Although evidence for selenoprotein W was obtained over 20 years ago, it was only recently purified and characterized. This selenoprotein was identified in the present study by Western blots. It was not feasible to purify enough of this protein to raise antibodies against the native protein. Thus, an alternate procedure was used. Antibodies were raised against a peptide of amino acids 13 to 31. The antibody to this peptide was used in the Western blots to detect the presence of this selenoprotein and to obtain quantitative estimates of its concentration. However, a more desirable method needs to be developed to quantitate this selenoprotein. As discussed in the appendix, attempts were made to develop such a method by an ELISA procedure. Efforts were not successful in development of a method sensitive enough for this purpose and this should be one of the primary goals for future work.

The antibody raised against the peptide appears to be sufficient for detecting selenoprotein W in tissues of rats, mice, sheep, cattle and guinea pigs. However, it may not be sufficient for human and higher primate tissues. This antibody reacted with a protein about three times the molecular weight of selenoprotein W in primate tissues, but no reaction occurred at the molecular weight of selenoprotein W in the Western blots. The deduced amino acid sequence has now been determined from the nucleotide sequence of the cDNA. Since there are three major differences in the amino acid composition of this peptide region between rats and primates, this may account for these results.

Antibodies have now been raised against the peptide from primates and these gave bands at the place were selenoprotein W should be in Western blots. Therefore, the antibody situation needs to be investigated more thoroughly. One approach would be to have E. coli synthesize a mutant protein and use this as the antigen to raise antibodies. This would appear to be a worthy goal to pursue in the future.

There are four species of selenoprotein W. One of these does not have any adducts bound to it. The second one has reduced glutathione as the adduct. The third species has a 45 dalton moiety bound to it and very recent evidence suggest that it is calcium. The fourth species has both adducts bound to it. It is not known how calcium, reduced glutathione, selenium or other factors affect the relative amounts of this selenoprotein. A convenient method needs to be developed to quantitate these different species so the effects of nutritional factors on their relative concentrations can be assessed.

The present work indicates that selenoprotein W is present in muscle, brain, testis and spleen of rats as determined by Western blot analysis. In contrast, its level is highest in muscle and heart of selenium-supplemented sheep. Selenium deficiency results in White Muscle Disease (WMD) which is a degenerative process predominatly in heart and skeletal muscle of lambs and calves. However, selenium deficiency does not cause the degeneration in heart of rats, but causes cardiac and skeletal muscle degeneration in lambs. The different tissue distribution of selenoprotein W in rats and sheep may account for this difference in affected tissues between these animals.

The results reported in chapter 3 indicate that selenoprotein W and cGPX are regulated differently by selenium. Although cGPX activity in tissues reached a plateau

with 0.1 mg selenium per kg diet or less, higher levels of selenium were required for selenoprotein W to reach a plateau. There may be some differences in the regulation of this selenoprotein between tissues because 1.0 mg selenium per kg was required for this plateau to be reached in the muscle whereas this plateau was reached with lower levels in other tissues. Therefore, studies on the regulation of selenoprotein W by selenium would appear to be a fruitful area to pursue.

The function of this selenoprotein is still unknown. Selenoprotein W is absent in muscle and heart of the WMD lambs, whereas it is present in muscle and heart of normal animal. This may imply that selenoprotein W is involved in the prevention of WMD. Contractions in cardiac and skeletal muscles are controlled by the calcium transport system of sarcoplasmic reticulum. Calcium metabolism is altered in WMD lambs due to the markedly decrease in calcium transport by sarcoplasmic reticulum. Injection of selenium into WMD lambs results in the rapid recovery of this disease and the greater increase of calcium uptake by sarcoplasmic reticulum.

The present work indicate that the response of selenoprotein W to selenium status was rapid and selenium-dependent both in animals and in cell culture. In rat studies, selenoprotein W mRNA increased significantly in muscle from those fed a diet with 0.1 ppm selenium, and this selenoprotein level in muscle increased dramatically and reached a plateau with supplementation of 1 ppm selenium. In L8 muscle cells, selenoprotein W mRNA increased markedly and reached a plateau with addition of 10^{-8} M selenium in media, whereas the selenoprotein W level reached a plateau with addition of 10^{-7} M selenium in media. The relationship of mRNA content to the protein levels needs to be investigated in more detail. Experiments need to be conducted to determine whether

selenium affects transcription or mRNA stability. The available data, however, indicate that selenium can regulate selenoprotein W synthesis at both transcriptional and translational levels, but this needs to be confirmed.

Because of its small molecular weight and low concentration in tissues, selenoprotein W probably acts as a regulatory protein. Recently, a part of selenoprotein W sequence (amino acids 60-71) was discovered to be highly homologous to the sequence of calcium-binding domains in several calcium binding proteins. Therefore, the gene for selenoprotein W may also be regulated by calcium binding to the calcium-binding domain. The increase of calcium uptake in sarcoplasmic reticulum by selenium supplementation to WMD animals may in turn increase selenoprotein W gene expression in muscle and heart of selenium-deficient animals. It would therefore be prudent to consider calcium in addition to selenium in the regulation of selenoprotein W. The response of selenoprotein W to selenium status in L8 muscle cells mimics the patterns found in animals. Thus, L8 muscle cells can be used as a model to investigate the possible role of selenoprotein W in muscular degeneration.

BIBLIOGRAPHY

American Institute of Nutrition. (1977) Report of the AIN Ad Hoc Committee on standards for nutritional studies J. Nutr. 107: 1340-1348.

Arthur, J. R., Nicol, F. and Beckett, G. J. (1990a) Hepatic iodothyronine 5'-deiodinase. Biochem. J. 272: 537-540.

Arthur, J. R., Nicol, F., Hutchinson, A. R. and Beckett, G. J. (1990b) The effects of selenium depletion and repletion on the metabolism of thyroid hormones in the rat. J. Inorg. Chem. 39: 101-108.

Axley, M. J. and Stadtman, T. C. (1989) Selenium metabolism and selenium-dependent enzymes in microorganisms. Annu. Rev. Nutr. 9: 127-137.

Bansal, M. P., Cook, R. C., Danielsom, K. G. and Medina, D. (1989) A 14-kilodalton selenium-binding protein in mouse liver is fatty acid-binding protein. J. Bio. Chem. 264: 13780-13784.

Baron, C. and Böck, A. (1991) The length of the aminoacyl-acceptor stem of the selenocysteine-specific $tRNA^{Sec}$ of Escherichia coli is the determinant for binding to elogation factors SELB ot Tu. J. Biol. Chem. 266: 20375-20379.

Beckett, G. T., Beddows, S. E., Morrice, P. C., Nicol, F. and Arthur, J. R. (1987) Inhibition of hepatic deiodination of thyroxine is caused by selenium deficiency in rats. Biochem. J. 248: 443-447.

Behne, D., Hilmert, H., Scheid, S., Gessner, H. and Elger, W. (1988) Evidence for specific selenium target tissues and new biologically important selenoproteins. Biochim Biophys. Acta 966: 12-21.

Behne, D., Kyriakopoulos, A., Meinhold, H. and Köhrle, J. (1990) Identification of type 1 iodothyronine 5'-deiodinase as a selenoenzyme. Biochem. Biophys. Res. Commun. 173: 1143-1149.

Behne, D., Weiss-Nowak, C., Kalcklosch, M., Westphal, C., Gessner, H. and Kyriakopoulos, A. (1995) Studies on the distribution and characteristics of new mammalian selenium-containing proteins. Analyst 120: 823-825.

Beilstein, M. A., Tripp, M. J. and Whanger, P. D. (1981) Evidence for selenocysteine in ovine tissue organelles. J. Inorgan. Biochem. 15: 339-347.

Beilstein, M. A. and Whanger, P. D. (1984) Effects of cyanide on selenium metabolism in rats. J. Nutr. 114: 929-937.

Beilstein, M. A. and Whanger, P. D. (1985) Selenium accumulation in tissues, tissue fractions and cytosolic proteins in rats. Biochem. Arch. 1: 153-162.

Beilstein, M. A. and Whanger, P. D. (1987) Metabolism of selenomethionine and effects of interacting compounds by mammalian cells in culture. J. Inorgan. Biochem. 29: 137-152.

Beilstein, M. A. and Whanger, P. D. (1992) Selenium metabolism and glutathione peroxidase activity in cultured human lymphoblasts. Biol. Trace Elem. Res. 35: 105-118.

Beilstein, M. A., Whanger, P. D. and Wong, L. (1987) Selenium requirement and metabolism by mammalian cells in vitro. In: Selenium and biology and medicine (G. F. Combs, J. E. Spallholz, O. A. Levander and J. E. Oldfield, eds), An Avi Book, Van Nostrand Reinhold Comp., New York, pp 197-212.

Beilstein, M. A., Vendeland, S. C., Andrews, J. and Whanger, P. D. (1993) Immunization with a synthetic peptide to produce antibodies to a rat muscle selenoprotein. The FASEB J. 7: A289.

Beilstein, M. A., Vendeland, S. C., Barofsky, E., Jensen, O. N. and Whanger, P. D. (1995) Selenoprotein W of rat muscle binds glutathione and an unknown small molecular weight moiety. J. Inorgan. Biochem. 59: 256-262.

Berry, M. J., Banu, L., Chen, Y., Mandel, S. J., Kieffer, J. D., Harney, J. W. and Larsen, P. R. (1991b) Recognition of UGA as a selenocysteine codon in Type 1 deiodinase requires sequences in the 3' untranslated region. Nature 353: 273-276.

Berry, M. J., Banu, L., Harney, J. W. and Larsen, P. R. (1993) Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. EMBO J. 12: 3315-3322.

Berry, M. J., Banu, L. and Larsen, P. R. (1991a) Type 1 iodothyronine deiodinase is a selenocysteine-containing enzyme. Nature 349: 438-440.

Black, R. S., Tripp, M. J., Whanger, P. D. and Weswig, P. H. (1978). Selenium proteins in ovine tissues: III. Distribution of selenium and glutathione peroxidases in tissue cytosols. Bioinorgan. Chem. 8: 161-170.

Blot W. J. et al. (1993) Nutrition intervention trials in Linxian, China: Supplementation with specific vitamin/ mineral combinations, cancer incidence, and disease-specific mortality in the general population. J. Natl. Cancer Inst. 85: 1483-1498.

Böck, A., Forchhammer, K., Heider, L. and Baron, C. (1991b) Selenoprotein synthesis: an expansion of the genetic code. Trends Biochem. Sci. 16: 463-467.

Böck, A., Forchhammer, K., Heider, L., Leinfelder, W., Sawers, G., Veprek, B. and Zinoni, F. (1991a) Selenocysteine: the 21st amino acid. Mol. Microbiol. 5: 515-520.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72: 248-254.

Brodt, P. (1983) Tumor immunology--three decades in review. Ann. Rev. Microbiol. 37: 447-476.

Brown, M. W. and Watkinson, J. H. (1977). An automated fluorimetric method for the determination of nanogram quantities os selenium. Anal. Chim. Acta. 89: 29-35.

Brown, M. R., Cohen, H. J., Lyons, J. M., Curtis, T. W., Thunberg, B., Cochran, W. J. and Klish, W. J. (1986) Proximal muscle weakness and selenium deficiency associated with long term parenteral nutrition. Amer. J. Clin. Nutr. 43: 549-554.

Burk, R. F. and Hill, K. E. (1993) Regulation of selenoproteins. Annu. Rev. Nutr. 13: 65-81.

Calvin, H. I., Cooper, G. W. and Wallace, E. (1981) Evidence that selenium in rat sperm is associated with a cysteine-rich structural protein of the motichondrial capsules. Gamete Res. 4: 139-149.

Chen, X. S., Yang, G. Q., Chen, J. S., Chen, X. C., Wen, S. M. and Ge, K. Y. (1980) Studies on the reactions of selenium and Keshan disease. Biol. Trace Elem. Res. 2:91.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

Christensen, J. J. and Burgener, K. W. (1992) Dietary selenium stabilizes glutathione peroxidase mRNA in rat liver. J. Nutr. 122: 1620-1626.

Chu, F-F., Dorooshow, J. H. and Esworthy, R. S. (1993) Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. J. Biol. Chem. 268: 2571-2576.

Combs, G. F. and Combs, S. B. (1986) The role of selenium in nutrition. Academic Press, New York. pp. 413-461.

Cone, J. E., del Rio, M. R. Davis, J. N. and Stadtman, T. C. (1976) Chemical characterization of the selenoprotein component of clostridial glycine reductase: identification of selenocysteine as the organoselenium moiety. Proc. Natl. Acad. Sci. USA 73: 2659-2663.

Croteau, W., Whittemore, S. L., Schneider, M. J. and St. Bermain, D. L. (1995) Cloning and expression of a cDNA for a mammalian type III iodothyronine deiodinase. J. Biol. Chem. 270: 16569-16575.

Davidson, W. B. and Kennedy, G. (1993) Synthesis of [^{75}Se] selenoproteins is greater in selenium-deficient sheep. J. Nutr. 123: 689-694.

Deagen, J. T., Butler, J. A., Zachara, B. A. and Whanger, P. D. (1993) Determination of the distribution of selenium between glutathione peroxidase, selenoprotein P and albumin in plasma. Anal. Biochem. 208: 176-184.

Farese, R. V., Standaert, M. L., Francois, A. J., Ways, K., Arnold, T. P., Hernandez, H. and Cooper, D. R. (1992) Effects of insulin and phorbol esters on subcellular distribution of protein kinase C isoforms in rat adipocytes. Biochem. J. 288, 319-323.

Fields, C. G., Lloyd, D. H., MacDonald, R. L., Otteron, K. M. and Noble, R. L. (1991) HBTV activation for automated Fmoc solid-phase peptide synthesis. Peptide Research 4: 95-101.

Flohe, L. Gunzler, W. A. and Schock, H. H. (1973) Glutathione peroxidase: a selenoenzyme. FEBS Lett. 32: 132-134.

Forchhammer, K. and Böck, A. (1991) Biology and biochemistry of selenium. Naturwissenschaften 78: 497-504.

Forstrom, J. W., Sakowski, J. J. and Tappel, A. L. (1978). Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. Biochemistry 17: 2639-2644.

Ganther, H.E. and C. Corcoran (1969) Selenotrisulfides. II. Cross-linking of reduced pancreatic ribonuclease with selenium. Biochemistry 8: 2557-2563.

Garcia, G. E. and Stadtman, T. C. (1992) *Clostridium sticklandii* glycine reductase selenoprotein A gene: cloning, sequencing and expression in *Escherichia coli*. J. Bacteriol. 174: 7080-7089.

Giovannoni, S. J. (1991) The polymerase chain reaction. In: Sequencing and hybrid techniques in bacterial systematics (Stackebrandt, E. and Goodfellow, M. eds.). John Wiley and Sons, New York. pp. 176-201.

Gomez, B. Jr. and Tappel, A. L. (1989) Selenoprotein P receptor from rat. Biochim. Biophys. Acta 979: 20-26.

Gross, M., Oertel, M. and Kohrle, J. (1995) Differential selenium-dependent expression of type I 5'-deiodinase and glutathione peroxidase in the porcine epithetial kidney cell lne LLC-PK$_1$. Biochem. J. 306:851-856.

Gu, Q., Beilstein, M. A., Vendeland, S. C., Ream, L. M., and Whanger, P. D. (1996) Nucleotide sequences of selenoprotein W cDNAs from skeletal muscle of rat, mouse, sheep, monkey and human. Nucleic Acid Res. (Submitted)

Gunzler, W. A., Steffens, G. J., Grossmann, A., Kim, S. M. A., Otting, F., Wendel, A. and Flohe, L. (1984) The amino acid sequence of bovine glutathione peroxidase. Hoppe-Zeyler's Z. Physiol. CHem. 365: 195-212.

Hafeman, D. G., Sunde, R. A. and Hoekstra, W. G. (1974) Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. J. Nutr. 104: 580-587.

Hawkes, W. C., Lyons, D. E. and Tappel, A. L. (1982) Identification of a selenocystein-specific aminoacyl transfer RNA from rat liver. Biochim. Biophys. Acta 699: 183-191.

Hawkes, W. C., Wilhelmsen, E. C. and Tappel, A. L. (1985) Abundance and tissue distribution of selenocysteine-containing proteins in the rat. J. Inorg. Biochem. 23: 77-92.

Heider, J. and Bock, A. (1992) Targeted insertion of selenocysteine into the α-subunit of formate dehydrogenase from *Methanobacterium formicicum*. J. Bacteriol. 174: 659-663.

Hill, K. E., Lloyd, R. S. and Burk, R. F. (1993) Conserved nucleotide sequences in the open reading frame and 3' untranslated region of selenoprotein P mRNA. Proc. Natl. Acad. Sci. USA 90: 537-541.

Hill, K. E., Lloyd, R. S., Yang, J. G., Read, A. and Burk, R. F. (1991) The cDNA for rat selenoprotein P contains 10 TGA codons in the open reading frame. J. Biol. Chem. 266: 10050-10053.

Ho, Y-S., Howard, A. J. and Crapo, J. D. (1988) Nucleotide sequence of a rat glutathione peroxidase cDNA. Nucleic Acids Res. 16: 5207-5210.

Hoekstra, W. G. (1975) Biochemical function of selenium and its relation to vitamin E. Fed. Proc. 34: 2083.

Jones, J. B. and Stadtman, T. C. (1981) Selenium-dependent and selenium-independent formate dehydrogenases of *Methanococcus vannielii*. Separation of the two forms and characterization of the purified selenium-independent form. J. Biol. Chem. 256: 656-663.

Karimpour, I., Cutler, M., Shih, D., Smith, J. and Keene, K. C. (1992) Sequence of the gene encoding the mitochonidrial capsule selenoprotein of mouse sperm: identification of three in-phase TGA selenocysteine codons. DNA Cell Biol. 11: 692-699.

Karle, J. A., Kull, F. J. and Shrift, A. (1983) Uptake of selenium-75 by PHA-stimulated lymphocytes: Effect on glutathione peroxidase. Biol. Trace Elem. Res. 5: 17-24.

Knight, S. A. B. and Sunde, R. A. (1987) The effect of progressive selenium deficiency on anti-glutathione peroxidase antibody reactive protein in rat liver. J. Nutr. 117: 732-738.

Kraus, R. J., Foster, S. J. and Ganther, H. E. (1983). Identification of selenocysteine in glutathione peroxidase by mass spectroscopy. Biochemistry 22: 5853-5858.

Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105-132.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lee, B. J., Worland, P. J., Davis, J. N., Stadtman, T. C. and Hatfield, D. L. (1989) Identification of a selenocysteyl-tRNA^{ser} in mammalian cells that recognizes the nonsense codon, UGA. J. Biol. Chem. 264: 9724-9727.

Lei, X. G., Evenson, J. K., Thompson, K. M. and Sunde, R. A. (1995) Glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase are differentially regulated in rats by dietary selenium. J. Nutr. 125: 1438-1446.

Leinfelder, W., Forchhammer, K., Veprek, B., Zehelein, E. and Böck, A. (1990) In vitro synthesis of selenocysteinyl-tRNA$_{UCA}$ from seryl-tRNA$_{UCA}$: Involvement and characterization of the selD gene product. Proc. Natl. Acad. Sci. USA 87: 543-547.

Leinfelder, W., Forchhammer, K., Zinoni, F., Sawers, G., Mandrand-Berthelot, M-A. and Böck, A. (1988) Escherichia coli genes whose products are involved in selenium metabolism. J. Bacteriol. 170: 540-546.

McConnell, K. P., Hsu, J. M., Herrman, J. L. and Anthony, W. L. (1974) Parallelism between sulfur and selenium amino acids in protein synthesis in the skin of zinc-deficient rats. Proc. Soc. Exptl. Biol. Med. 145: 970-974.

Mills, G. C. (1957) Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J. Biol. Chem. 229: 189.

Mizutani, T., Kurata, H., Yamada, K. and Totsuka, T. (1992) Some properties of murine selenocysteine synthase. Biochem. J. 284: 827-834.

Oh, S-H, Ganther, H. E. and Hoekstra, W. G. (1974) Selenium as a component of glutathione peroxidase isolated from ovine erythrocytes. Biochemistry 13: 1825-1829.

Paglia, D. E. and Valentine, W. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70: 158-169.

Pedersen, N. D., Whanger, P. D. and Weswig, P. H. (1969) Distribution of ^{75}Se-bound proteins in tissues of normal and myopathic lambs. Pacific Slope Biochem. Conf. p. 49.

Pedersen, N. D., Whanger, P. D., Weswig, P. H. and Muth, O. H. (1972). Selenium binding proteins in tissues of normal and selenium responsive myopathic lambs. Bioinorgan. Chem. 2: 33-43.

Pond, F. R., Tripp, M. J., Wu, A. S. H., Whanger, P. D. and Schmitz, J. A. (1983) Incorporation of selenium-75 into semen and reproductive tissues of bulls and rams. J. Reprod. Fert. 69: 411-418.

Read, R., Bellew, T., Yang, J-G., Hill, K. E. Palmer, I. S. and Burk, R. F. (1990) Selenium and amino acid composition of selenoprotein P, the major selenoprotein in rat serum. J. Biol. Chem. 265: 17899-17905.

Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G. and Hoekstra, W. G. (1973) Selenium: Biochemical role as a component of glutathione peroxidase. Science 179: 588-590.

Schuber, A. P., Orr, E. C., Recny, M. A., Schendel, P. F., May, H. D., Schauer, N. L. and Ferry, J. G. (1986) Cloning, expression, and nucleotide sequence of the formate dehydrogenase genes from Methanobacterium formicicum. J. Biol. Chem. 261:1 2942-12947.

Schubert, J. R., Muth, O. H., Oldfield, J. E. and Remmert, L. F. (1961) Experimental results with selenium in white muscle disease of lambs and calves. Federation Proc. 20: 689-692.

Schuckelt, R., Brigelius-Flohè, R., Maiorino, M., Roveri, A., Reumkens, J., StraßBurger, W., Ursini, F., Wolf, B. and Flohè, L. (1991) Phospholipid hydroperoxide glutathione peroxidase is a selenoenzyme distinct from the classical glutathione peroxidase as evident from cDNA and amino acid sequencing. Free Rad. Res. Comms. 14: 343-361.

Schwarz, K. and Foltz, C. (1957) Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J. Amer. Chem. Soc. 79: 3292.

Shen, Q., Chu, F-F. and Newburger, P. E. (1993) Sequences in the 3'-untranslated region of the human cellular glutathione peroxidase gene are necessary and sufficient for selenocysteine incorporation at the UGA codon. J. Biol. Chem. 268: 11463-11469.

St. Germain, D. L. (1994) In: Thyroid hormone metabolism: molecular biology and alternative pathway (Wu, S-Y. and Visser, T. J. eds.). CRC Press, Ann Arbor, MI. pp. 45-66.

Stadtman, T. C. (1987) Specific occurence of selenium in enzymes and amino acid tRNAs. FASEB J. 1: 375-379.

Stadtman, T. C. (1990) Selenium biochemistry. Annu. Rev. Biochem. 59: 11-127.

Steel, R. G. D. and Torrie, L. H.(1980) Principles and Procedures of Statistics, 2nd edition, Mcgraw-Hill Book Co., Inc., New York. pp. 186-190.

Sunde, R. A. (1990). Molecular biology of selenoproteins. Annu. Rev. Nutr. 10: 451-474.

Sunde, R. A. and Evenson, J. K. (1987) Serine incorporation into the selenocysteine moiety of glutathione peroxidase. J. Biol. Chem. 262: 933-937.

Sunde, R. A. and Hoekstra, W. G. (1980) Incorportaion of selenium into liver glutathione peroxidase in the Se-adequate and Se-deficient rat. Proc. Soc. Exp. Biol. Med. 165: 291-297.

Takahashi, K., Akasaka, M., Yamamoto, Y., Koyayashi, C., Mizoguchi, J. and Koyama, J. (1990) Primary structure of human plasma glutathione peroxidase deduced from cDNA sequences. J. Biochem. 108: 145-48.

Takahashi, K., Avissar, N., Whitin, J. and Cohen, H. (1987) Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch. Biochem. Biophys. 256: 677-686.

Takahashi, K. and Cohen, H. J. (1986) Selenium-dependent glutathione peroxidase protein and activity: Immunological investigations on cellular and plasma enzymes. Blood 68: 640-645.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamine gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci., U. S. A. 76: 4350-4354.

Toyoda, H., Himeno, S. and Imura, N. (1990) Regulation of glutathione peroxidase mRNA level by dietary selenium manipulation. Biochim. Biophys. Acta. 1049: 213-215.

Turner, D. C. and Stadtman, T. C. (1973) Purification of protein components of the clostridial glycine reductase system and characterization of protein A as a selenoprotein. Arch. Biochem. Biophys. 154: 366-381.

Ullrey, D. E., Combs, J. F., Conrad, H. R., Hoekstra, W. G., Jenkins, K. J. W., Levander, O. A. and Whanger, P. D. (1983) Selenium in nutrition. National Academy Press, Washington, D. C.

Ursini, F., Maiorino, M. and Gregolin, C. (1985) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim. Biophys. Acta 839: 62-70.

van Rij, A. M., McKenzie, J. M. Thomson, C.D. and Robinson, M. F. (1981) Selenium supplementation in total parenteral nutrition. JPEN 5: 120-124.

Vendeland, S. C., Beilstein, M. A., Chen, C. L., Jensen, O. N., Barofsky, E. and Whanger, P. D. (1993) Purification and properties of selenoprotein W from rat muscle. J. Biol. Chem. 268: 17103-17107.

Vendeland, S. C., Beilstein, M. A., Yeh, J-Y., Ream, W. and Whanger, P. D. (1995) Rat skeletal muscle selenoprotein W: cDNA clone and mRNA modulation by dietary selenium. Proc. Natl. Acad. Sci. USA 92: 8749-8753.

Whanger, P. D. (1985) Metabolic interactions of selenium with cadmium, mercury and silver. In: Advances in nutritional research (Draper, H. H. ed.). Plenum Publishing corp. 7: 221-250.

Whanger, P. D. (1987) Evidence for non-glutathione peroxidase selenium-containing proteins in mammalian tissues. In Selenium in Biology and Medicine, Third International Symp., Beijing, PRC (Combs, G. F., et al., eds.), Avi Book, van Nostrand Reinhold Comp., New York. pp. 197-212.

Whanger, P. D. (1992) Selenium in the treatment of heavy metal poisoning and chemical carcinogenesis. J. Trace Elem. Electrolytes Health Dis. 6: 209-221.

Whanger, P. D. (1993) Some biochemical properties of selenoprotein W. TEMA-8 (Anke, M. et al., eds.) Verlag Media Touristik, Gersdorf, Germany, pp.119-126.

White, C. L. and Hoekstra, W. G. (1979) The metabolism of selenite and selenomethionine by mouse fibroblasts grown in tissue culture. Biol. Trace Elem. Res. 1: 253-257.

Wilhelmsen, E. C., Hawkes, W. C., Tappel, A. L. (1985) Substitution of selenocysteine for cysteine in a reticulocyte lysate protein synthesis system. Biol. Trace Elem. Res. 7:141-151.

Yaffe, D. (1968) Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc. Natl. Acad. Sci. USA 61: 477-483.

Yamazaki, S. (1982) A selenium-containing hydrogenase from *Methanococcus vannielii*: identification of the selnium moiety as a selenocysteine residue. J. Biol. Chem. 257: 7926-7929.

Yang, G. and Zhou, R. (1994) Further observations on the human maximum safe dietary selenium intake in a seleniferous area of China. J. Trace Elem. Electrolytes Health Dis. 8: 159-165.

Yang, J-G., Hill, K. E. and Burk, R. F. (1989) Dietary selenium intake controls rat plasma selenoprotein P concentration. J. Nutr. 119: 1010-1012.

Yang, J-G., Morrison-Plummer, J. and Burk, R. F. (1987) Purification of quantitation of a rat plasma selenoprotein distinct from glutathione peroxidase using monoclonal antibodies. J. Biol. Chem. 262: 13372-13375.

Yang, J-G., Morrison-Plummer, J. and Burk, R. F. (1987) Purification of quantitation of a rat plasma selenoprotein distinct from glutathione peroxidase using monoclonal antibodies. J. Biol. Chem. 262: 13372-13375.

Yeh, J-Y., Beilstein, M. A., Andrews, J. S. and Whanger, P. D. (1995) Tissue distribution and influence of selenium status on levels of selenoprotein W. The FASEB J. 9: 392-396.

Yeh, J-Y, Gu, Q-P., Beilstein, M. A., Forsberg, N. E. and Whanger, P. D. (1996) Selenium supplementation results in increased levels of selenoprotein W in sheep tissues. J. Nutr. (to be submitted) and Chapter 5.

Yoshimura, S., Wantanabe, K., Shemizu, H., Onozawa, T., Mizoguchi, J., Tosuda, K. Hatta, H. and Morinchi, T. (1991) Tissue specific expression of the plasma glutathione peroxidase gene in rat kidney. J. Biochem. 109: 918-923.

Zinoni, F., Birkmann, A., Leinfelder, W. and Böck, A. (1987) Cotranslational insertion of selenocysteine into formate dehydrogenase from Escherichia coli directed by a UGA codon. Proc. Natl. Acad. Sci. USA 84: 3156-3160.

Zinoni, F., Birkmann, A., Stadtman, T. C. and Böck, A. (1986) Nucleotide sequence and expression of the selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from Escherichia coli. Proc. Natl. Acad. Sci. USA 83: 4650-4654.

Zinoni, F., Heider, L. and Böck, A. (1990) Features of the formate dehydrogenase mRNA necessary for decoding of the UGA codon as selenocysteine. Proc. Natl. Acad. Sci. USA 87: 4660-4664.

APPENDICES

APPENDIX 1.

GENERATION AND PURIFICATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST RAT SELENOPROTEIN W

The hydrophilicity was calculated from the amino acid sequence of selenoprotein W and two peptides were synthesized corresponding to the first two hydrophilic regions (amino acid residues 13-31 for peptide 1 and amino acid residues 51-69 for peptide 2) by Central Service Laboratory of Center for Gene Research and Biotechnologyatn Oregon State University. These peptides were synthesized with cysteine added to the amino-terminal ends for conjugation to keyhole limpet hemocyanin (KLH). These KLH-conjugated synthetic peptides were used for immunization of mice and rabbits respectively to generate monoclonal and polyclonal antibodies. The monoclonal antibodies were purified by protein A columns and polyclonal antibodies by peptide-coupled Sulfolink affinity columns. After purification, antibody solutions were concentrated by centrifugation with centrifuge tubes and 50% glycerol was subsequently added to the concentrated antibody solution. These antibody solutions were divided into aliquots and frozen at -20°C for later analysis.

Generation and purification of monoclonal antibody

Mice were immunized at 3-week intervals with KLH-conjugated peptides and bled 7-10 days following each injection. The antisera were titered against the peptides in a particle concentration fluorescence immunoassay (PCFIA). After 4 rounds of

immunizations, the mice were given a final injection of the antigen and the apleens were removed 3 days later for fusions with myeloma cells. Hybridoma were selected by cultuing in HAT media and then cultured in RPMI media. The supernatants from single clonal hybridoma were assayed after 10 days growth for reactivities in the PCFIA. These procedures were repeated until positive clones were found. To produce ascites fluid, nude mice were inoculated intraperitoneally each with 2 ml of monoclonal antibody-producing hybridoma cells (2.5 X 10^6 cells/ml in Phosphate Buffered Saline [PBS]) and the ascites formed 1-2 weeks after injection of the hybridoma cells. The ascites were harvested by inserting a needle into the abdominal cavity and then centrifuged at 1500 xg for 10 min at room temperature. The supernatants were harvested and stored at 4°C until all collection was completed within one week. The ascites fluid were pooled and heat-inactivated in a water bath at 56°C for 45 min, and the titer was determined.

The ascites were first determined by the subclass kit (Pierce, Rockford, IL). The subclass of the monoclonal antibody appeared to be IgG1. The ascites were subsequently purified by using high salt conditions on protein A columns. The NaCl concentration of the ascites were adjusted to 3.3 M and 1/10 volume of 1.0 M sodium borate (pH 8.9) added to the ascites solution. This antibody solution was passed through a protein A bead column (Pierce, Rockford, IL). The protein A column was washed with 10 column volumes of buffer A (3.0 M NaCl, 50 mM sodium borate [pH 8.9]) following by 10 column volumes of buffer B (3.0 M NaCl, 10 mM sodium borate [pH 8.9]). 0.1 M glycine (pH 3.0) was added stepwise to elute the antibodies which were collected in tubes containing 1/10 volume of 1.0 M Tris (pH 7.5). This resulted in a neutral pH. The fractions containing antibody were identified by absorbance at 280 nm (1 OD = 0.8

mg/ml). The excellulose desalting columns were used to desalt the antibody fractions and exchange its buffer to PBS.

Generation and purification of polyclonal antibody

Rabbits were immunized at 3-week intervals with KLH-conjugated synthetic peptides in Freund's adjuvant for 9 weeks. Blood was taken one week after the last injection and the serum was collected. The polyclonal antibodies were purified from rabbit sera by Sulfolink affinity columns (Pierce, Rockford, IL) coupled with synthetic peptides.

The synthetic peptides were dissolved in buffer A (50 mM Tris, 5 mM EDTA, pH 8.5). These peptide solutions were mixed with Sulfolink coupling gels for 15 min at room temperature and incubated for 30 min without further mixing. The Sulfolink affinity columns were washed with 3 column volumes of buffer A. After applying one column volume of freshly prepared 50 mM cysteine solution in buffer A to the column, the peptide-coupled gels were mixed for 15 min at room temperature and incubated for 30 min without mixing at room temperature in order to block non-specific binding sites on the gels. These peptide-coupled columns were washed with 16 column volumes of 1 M NaCl and equilibrated with 3 column volumes of PBS. Rabbit sera were diluted with 2 volumes of PBS and applied to the peptide-coupled columns. The columns were washed with 6 column volumes of PBS and the antibodies eluted with 4 column volumes of 0.1 M glycine (pH 2.8). Forty 1-ml fractions were collected and neutralized by adding 1/20 volume of 1 M Tris (pH 9.5). The antibody-containing fractions were identified by

absorbance at 280 nm (1 OD = 0.8 mg/ml). The excellulose desalting columns were used to desalt and exchange buffer of antibody fractions to PBS.

Assays of monoclonal and polyclonal antibodies

The antibody solutions were assayed by Western blotting technique using purified selenoprotein W and tissue cytosols as antigens. The results from Western blot analysis indicated that the monoclonal antibodies recognized purified selenoprotein W but with very low affinity. Because of this low affinity, these monoclonal antibodies failed to recognize selenoprotein W in tissue supernatants. The polyclonal antibodies against both synthetic peptides recognized selenoprotein W either as pure selenoprotein W or in tissue supernatants. However, polyclonal antibody against peptide 1 produced less non-specific background than polyclonal antibody against peptide 2 in the Western blot assay.

APPENDIX 2.

DEVELOPMENT OF SANDWICH ELISA ASSAY USING MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST SELENOPROTEIN W

To develop a quick method for quantitating amount of selenoprotein W in animal tissues, two-antibodies sandwich ELISA assay was tested. Monoclonal and polyclonal antibodies were generated against both peptide 1 and peptide 2 of selenoprotein W and each of them used in the sandwich ELISA assay.

ELISA plates were coated with purified monoclonal antibody in PBS (20 mM sodium phosphate, 100 mM NaCl, pH 7.4) and kept at 4°C overnight. These plates were blocked with SupperBlock solution (Pierce, Rockford, IL) at RT for 1 hr. Either synthetic peptides, pure selenoprotein W or different tissue supernatants were added to the plates and incubated at 37°C for 1 hr. A polyclonal antibody was added to the plates and incubated at 37°C for an additional 1 hr. Then goat-anti rabbit IgG-HRP (Bio-Rad, Richmond, CA) was added to the plates and these plates were incubated at 37°C for another 1 hr. After adding the ABTS substrate, the plates were incubated at RT for 10 min. Absorbance was measured by an ELISA plate reader at 405 nm. The concentration of selenoprotein W in tissue supernatants could be calculated from the standard curve of pure selenoprotein W.

Different combinations of monoclonal and polyclonal antibodies (monoclonal and polyclonal antibodies against peptide 1, monoclonal antibody against peptide 2 and polyclonal antibody against peptide 1, monoclonal antibody against peptide 1 and polyclonal antibody against peptide 2, monoclonal and polyclonal antibodies against

peptide 2) were used to develop the sandwich ELISA assay. The sandwich ELISA assay worked using synthetic peptides as antigens, but did not work using either pure selenoprotein W or tissue supernatants as antigens.

Both polyclonal antibodies functioned in Western blot assay with pure selenoprotein W and tissue supernatants, but monoclonal antibodies did not give bands in Western blot assay with tissue supernatants. Therefore, it is not certain whether the monoclonal antibodies can or can not work in the sandwich ELISA assay. Due to the different states of proteins in the two assays (native state for ELISA assay and denatured state for Western blot analysis), monoclonal and polyclonal antibodies may only recognize the denatured state of selenoprotein W. Thus, the denatured selenoprotein W and tissue supernatants were also used in the sandwich ELISA assay. Unfortunately, this approach was still unsuccessful. Presumably this is due to the low affinity of monoclonal antibodies. To develop the sandwich ELISA assay successfully, regeneration of monoclonal antibody with greater affinity will be necessary.