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The use of anti-glutathione peroxidase antibodies in the study of selenium-dependent glutathione peroxidase

Knight, Simon Alexander Bowles, M.S.

The University of Arizona, 1988
THE USE OF ANTI-GLUTATHIONE PEROXIDASE ANTIBODIES
IN THE STUDY OF
SELENIUM-DEPENDENT GLUTATHIONE PEROXIDASE

by

SIMON ALEXANDER BOWLES KNIGHT

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APPROVAL BY THESIS DIRECTOR

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Liver glutathione peroxidase activity is affected by changes in selenium (Se) status. To investigate the effect of Se status on GSH-Px protein we prepared antibodies against rat liver GSH-Px and used them in an ELISA. The immunoreactivity of the anti-GSH-Px antibodies against GSH-Px was both tissue and species specific. When rats were depleted of Se, liver GSH-Px activity decreased exponentially to zero with a half-life of 2.8 d. Liver GSH-Px protein also decreased exponentially, but not to zero, with a longer half-life of 5.2 d. Dietary repletion of Se-deficient rats with 0.5 mg Se/kg diet increased GSH-Px protein and activity after 1 d. After 14 d of repletion the levels of GSH-Px protein and activity had plateaued at the levels present in Se-adequate rats. When Se-deficient rats were injected with 15 or 60 ug Se, only rats injected with 60 ug Se and killed 24 h later showed an increase in GSH-Px protein and activity. These results suggest that when Se is limiting, GSH-Px protein and GSH-Px activity are coordinately regulated by the available Se, but in Se-adequacy homeostatic processes control the level of GSH-Px.
INTRODUCTION

The micro trace element selenium (Se) is essential for the enzymatic activity of the enzyme glutathione peroxidase (GSH-Px; glutathione:H₂O₂ oxidoreductase, EC 1.11.1.9)(1). A number of studies have demonstrated that during Se-deficiency in animals GSH-Px activity decreases, in some tissues to undetectable levels (2,3). When Se-deficient animals are repleted with Se GSH-Px activity returns to the level present in Se-sufficient rats (2,3). Se is present at the active site of GSH-Px, but unlike other trace metals, which associate with apoproteins in an ionic manner, Se in GSH-Px is covalently bound to carbon in a selenocysteine ([Se]Cys) residue (4). The mechanism for Se incorporation into GSH-Px is not known, however it has recently been discovered that the [Se]Cys is specified by a UGA codon (5).

Although the effect of Se status on GSH-Px activity has been studied extensively, there have been few studies examining the effect of Se status on the protein portion of GSH-Px. The first evidence that the level of GSH-Px protein was less in the Se-deficient rat liver than in the Se-adequate rat liver was provided by Yosida and coworkers (6), using immunoprecipitation. More recently Takahashi, Newburger and Cohen (7) reported that Se repletion of a Se-deficient individual restored both GSH-Px activity and GSH-Px protein levels in the patient's erythrocytes. This suggested that Se status can control the level of GSH-Px protein as well as GSH-Px activity.
To investigate the time course of changes in GSH-Px protein levels with changes in Se status we prepared polyclonal antibodies against rat liver GSH-Px and used them in an enzyme-linked immunosorbant assay (ELISA) for GSH-Px protein. The anti-GSH-Px antibodies were also used to determine if GSH-Px protein present in the tissues of rat and other species had similar epitypes to that of GSH-Px in rat liver.

This thesis is organized into five chapters: chapter I is a literature review; chapters II and III were prepared as papers and have been published by the Journal of Nutrition; chapter IV contains work examining the cross-reactivity of the anti-GSH-Px antibodies with GSH-Px present in other tissues and species; chapter V is a general discussion.
CHAPTER I

LITERATURE REVIEW
The essentiality of selenium

Selenium (Se) was identified in 1818 by the Swedish chemist Berzelius. It is in the same group as sulfur in the periodic table (group VIB) and exhibits similar physical and chemical properties to sulfur. Se has metallic properties as well as non-metallic properties and is thus considered a metalloid (8).

The first biological effect of Se to be observed was that of toxicity, identified in the 1930's. "Alkali disease" in Nebraska and South Dakota was believed by settlers to be caused by water with a high salt content. In fact the disease was caused by a high Se content in the plants of this area (9,10). This evidence was substantiated when Franke and Potter (11) fed either large amounts of sodium selenide or sodium selenite to rats and produced the characteristic toxic symptoms, including atrophied and necrotic livers.

This negative image of Se lasted until the early 1950's when Klaus Schwarz (12) reported on a third factor (Factor 3) present in American brewers yeast that could protect rats from dietary necrotic liver degeneration. The other two previously identified factors that prevented this condition were cystine and vitamin E. Factor 3 was found to be of low molecular weight, acid stable and water soluble (13). In addition, concentrated preparations of Factor 3 developed a garlic-like odor upon addition of alkali (14). It was this odor, also present on the breath of cattle that had consumed seleniferous herbage, that led Schwarz and Foltz (14) to analyze Factor 3 preparations for Se, which
they detected. Furthermore Se supplementation as sodium selenite gave complete protection against necrotic liver degeneration in rats. Thus a definitive nutritional role for Se was established (14).

Following this discovery it was shown that Se could alleviate other diseases. In vitamin E deficient chicks, Se supplementation prevented exudative diathesis (15,16). Se supplementation also protected lambs from white muscle disease (muscular dystrophy) (17) and caused increase growth in lambs suffering from "ill thrift" on the South Island of New Zealand (18).

Confirmation that Se was indeed an essential element, whose role could not be replaced by vitamin E, was provided some years later. Thompson and Scott (19) demonstrated that chicks fed a highly purified diet deficient in Se, exhibited stunted growth and a decreased survival rate. Furthermore, pancreatic atrophy occurred along with decreased levels of pancreatic lipase and trypsin activity (20). Although nutritional pancreatic atrophy is considered a pathological condition of uncomplicated Se deficiency, it is noteworthy that high levels of vitamin E or synthetic oxidants such as butylated hydroxytoluene can also prevent this condition (21).

The essentiality of Se for rats was demonstrated by McCoy and Weswig (22) using second generation Se-deficient rats, which failed to reproduce, developed poor hair coats and showed defective eye development. Resupplementation of these rats with 0.1 mg Se/kg diet as sodium selenite restored hair coat and reproductive capabilities (22).
The importance of Se in humans was concluded from studies in China. An endemic disease of juvenile cardiomyopathy (Keshan disease) occurred in a rural region that runs from the northeast to the central south. Se was suspected as a causative agent because of the low levels of Se in the soil and the prevalence of Se-deficiency diseases in livestock. In a study of over 12,000 children from the region, weekly supplementation of 0.5 to 1.0 mg Se as sodium selenite significantly decreased the incidence of Keshan disease and improved survival compared to a placebo treatment (23). As yet the mechanism by which Se prevents this disease as not been determined.

Glutathione peroxidase

By coincidence, in the same year that Schwarz and Foltz (14) discovered a nutritional role for Se, Mills (24) published a paper describing the enzyme GSH-Px. At the time however, these were both very unrelated events. Mills (24) demonstrated the presence of an enzyme in erythrocyte hemolysates that catalyzed the oxidation of reduced glutathione by hydrogen peroxide. This reaction reduces hydrogen peroxide to water and protects hemoglobin from oxidation to methemoglobin. GSH-Px has been shown to catalyse the final step in a series of reactions that provide a major pathway in erythrocytes for the reduction of hydrogen peroxide (25); NADPH generated by the pentose phosphate pathway is oxidized, in a reaction catalysed by glutathione reductase, to maintain levels of reduced glutathione. The
reduced glutathione can then be utilized by GSH-Px to reduce hydrogen peroxide to water (25).

A role for Se in preventing erythrocyte hemolysis was investigated by Rotruck and colleagues (26) who determined that Se was protective, provided that glucose was present in the incubation medium. Moreover, glucose protected erythrocytes against hemolysis, when rats received adequate Se, but did not protect the erythrocytes of Se-deficient rats (26). The action of Se was believed to be involved in the utilization of reduced glutathione, for erythrocytes from Se-deficient rats had a greater concentration of glutathione than erythrocytes from rats fed a diet adequate in Se (26). This observation led Rotruck and colleagues to focus their attention on GSH-Px. Dialysis of hemolysates from Se-adequate rats did not decrease enzyme activity, and addition of Se to Se-deficient hemolysates did not increase activity, indicating that the Se was tightly associated with the GSH-Px. Partial purification of GSH-Px from the erythrocytes of rats injected with $^{75}$Se as sodium selenite led to the cochromatography of the $^{75}$Se label with GSH-Px (1). Thus Se was shown to be an integral part of GSH-Px.

GSH-Px has a molecular weight of approximately 88,000 Da, dependent on species and consists of 4 equally sized subunits each of a molecular weight of 22,000 Da. The enzyme contains stoichiometric amounts of Se, 4 g-atoms Se per mole of enzyme with each subunit possessing one Se atom (27-29). In comparison to other trace metals that associate with apoproteins, Se is different in that it is covalently bound to carbon,
forming a [Se]Cys residue present at the active site of GSH-Px (4).

**Incorporation of Se into GSH-Px**

The discovery that Se in GSH-Px was present as a [Se]Cys moiety (4) led to two schools of thought regarding the mechanism of Se incorporation. These were, translational incorporation (ie direct incorporation of [Se]Cys during translation) versus post-translational incorporation (ie modification of an amino acid after translation).

The evidence supporting a translational mechanism came from Tappel's group. Hawkes, Lyons and Tappel (30) isolated a [75Se]Cys-specific aminoacyl tRNA from liver slices incubated with either [75Se]Cys or [75Se]selenite. Addition of this [75Se]Cys-tRNA to an in vitro translation system gave better 75Se incorporation into GSH-Px than 75Se from 75[Se]selenite, suggesting translational incorporation (31).

In opposition to the findings of the above experiments, studies investigating the incorporation of Se from selenite and selenocystine [Se]Cys2 into GSH-Px suggested a post-translational mechanism (32). These studies indicated that Se as selenite or selenide was metabolically closer than Se as [Se]Cys2 to the form of Se used for incorporation into GSH-Px. This indicated that exogenous [Se]Cys was not directly incorporated into GSH-Px during translation, but rather that a post-translational modification of an existing amino acid occurred (32).
This theory was supported when Sunde and Evenson (33) perfused rat livers with $^{75}\text{Se}$ selenite, $[\text{U-}^{14}\text{C}]$ serine and $[3-^{3}\text{H}]$ serine. Amino acid analysis of the GSH-Px, purified from the perfused livers showed coelution of $^{75}\text{Se}$, $^{14}\text{C}$ and $^{3}\text{H}$ at the position of the $[\text{Se}]\text{Cys}$ derivative carboxymethylselenocysteine (33). This demonstrated that serine provides the carbon skeleton for $[\text{Se}]\text{Cys}$ (33).

The debate of a post-translational versus a translational mechanism of Se incorporation into GSH-Px was answered in a rather fortuitous manner. Chambers and coworkers (5) when examining expression of proteins during reticulocyte maturation, cloned a mouse genomic DNA recombinant encoding a 19 kDa polypeptide whose mRNA was highly expressed. When it was sequenced and the implied amino acid sequence determined, it was revealed that the sequence was homologous to bovine GSH-Px (5). The sequence contained a surprising piece of information; the $[\text{Se}]\text{Cys}$ at the active site of GSH-Px was encoded by the termination codon UGA. This finding was confirmed in the bacterial selenoenzyme formate dehydrogenase, when it was shown that the $[\text{Se}]\text{Cys}$ was also encoded by a UGA codon (34). The discovery of the UGA codon indicated that the incorporation of $[\text{Se}]\text{Cys}$ into GSH-Px was by a translational mechanism, however, it did not explain Sunde and Evenson's (33) result that serine provides the carbon skeleton for $[\text{Se}]\text{Cys}$. It was known that a certain seryl-tRNA, present in bovine liver could read UGA codons and suppress the termination function (35). Furthermore the serine could be phosphorylated to phosphoserine whilst esterified to the tRNA by a
specific phosphotransferase (36,37). Sunde and Evenson (33) thus proposed a co-translational mechanism in which the phosphate group of the phosphoseryl-tRNA is replaced by a \(-\text{SeH}\) to form the \([\text{Se}]\text{Cys}-\text{tRNA}\), which then inserts the newly formed \([\text{Se}]\text{Cys}\) into GSH-Px during translation. Indeed this may be the mechanism, since a novel tRNA species that is charged with serine and then modified to \([\text{Se}]\text{Cys}\) while esterified to the tRNA has been recently discovered in bacteria (38). It is probable that eukaryotes also possess such a novel tRNA, though this remains to be proven.

**Bacterial selenoproteins**

Stadtman (39) recently reviewed the specific occurrence of selenium in bacterial proteins and listed five. These were formate dehydrogenase, glycine reductase and hydrogenase all of which have Se present as \([\text{Se}]\text{Cys}\). Nicotinic acid hydroxylase and xanthine oxidase do not contain detectable \([\text{Se}]\text{Cys}\), in these two enzymes the Se is present as part of a labile cofactor (39), which has yet to be characterized. Conspicuous by its absence from this recent listing of bacterial selenoproteins is thiolase. The Se is present in thiolase as selenomethionine ([Se]Met) and it would appear that rather than the [Se]Met being located at a specific site, it is inserted in place of methionine in a competitive manner during translation (40). Thus, enzymes and proteins that incorporate [Se]Met as a methionine analogue in a non-specific manner, such as thiolase and potentially mammalian
muscle proteins (41), should not be considered true selenoproteins, unless Se is essential to their function.

Mammalian selenoproteins

Although GSH-Px may be the most studied mammalian selenoprotein, it is by no means the only one. As early as 1972 Pederson and coworkers (42) reported the existence of a 10 kDa selenoprotein in lamb muscle. Of note was that this selenoprotein was not detected in lambs suffering from muscular dystrophy, a symptom associated with severe Se deficiency in lambs. Purification and spectral analysis of this selenoprotein suggested that it contained a heme chromophore similar to that bound to cytochrome C (43). However, later evidence indicates that a heme group is not present (44). The presence of a 17 kDa $^{75}\text{Se}$ containing protein in rat sperm was discovered by Calvin (45). The absence of this selenoprotein may account for the immotile and structurally defective sperm produced by Se-deficient rats.

Injection of $[^{75}\text{Se}]$ selenite into Se-adequate rats revealed a serum selenoprotein having a molecular weight of greater than 67 kDa (the highest molecular weight standard) (46). The molecular weight of this protein, now termed $^{75}\text{Se}-\text{P}$, was determined by sephadex G-150 chromatography to be 79 kDa (47). The use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicates that the Se is present in a 55 kDa subunit (48). Although in Se adequacy the pool size of liver GSH-Px is larger than that of $^{75}\text{Se}-\text{P}$, under conditions of Se-
deficiency the $^{75}$Se-P in both plasma and liver has as an apparent priority for the available Se (47). The function of $^{75}$Se-P is not known. Motsenbocker and Tappel (49) have suggested that it may be a Se transport protein from the liver to extrahepatic tissue. There is some evidence that the Se in $^{75}$Se-P is present as [Se]Cys (50), the existence of a TGA codon in the open reading frame of the gene for $^{75}$Se-P would confirm this.

Using the technique of SDS-PAGE and counting of gel slices to detect proteins that incorporate $^{75}$Se, Evenson and Sunde (48) detected a 65 kDa $^{75}$Se-selenoprotein in the liver, heart and to a lesser extent the testis. The characteristics of this selenoprotein are unknown. Whether it is related to a 75 kDa selenoprotein observed in the kidney and liver (51) remains to be established.

In the last three years there have been reports of selenoproteins with peroxidase activity, but which differ from the classical liver GSH-Px. In pig heart a phospholipid hydroperoxide GSH-Px has been identified (52). The enzyme is a monomer with a molecular weight of approximately 23 kDa, though Ursini, Maiorino and Gregolin (52) define the Se content as 1 g-atom Se/22,000 g protein, rather than 23,000 g protein. Ursini, Maiorino and Gregolin (52) suggested that the phospholipid hydroperoxide GSH-Px acts on lipophylic interfacial
substrates, since its activity is stimulated by the presence of Triton-X.

Another distinct GSH-Px has been reported by Takahashi and coworkers (53). This GSH-Px, purified from human plasma, has a molecular weight and subunit structure similar to classical liver GSH-Px. It differs from liver GSH-Px in that it is a glycoprotein and that it is not immunoreactive with antibodies produced against red blood cell GSH-Px (53). Follow-up work has shown that it is synthesized and secreted by a human liver cell line (54), suggesting the liver also synthesizes this protein in vivo.

The effect of Se status on GSH-Px activity

Once the intimate role of Se in GSH-Px had been established a number of reports followed that linked changes in Se status with changes in GSH-Px activity. Feeding a Se-deficient torula yeast based diet to rats caused both erythrocyte and liver GSH-Px activities to decrease (2). When Se-deficient rats were repleted with Se as sodium selenite erythrocyte GSH-Px activity increased (2). Although repletion with 5.0 and 0.5 mg Se/kg diet caused a more rapid initial increase in erythrocyte GSH-Px activity than 0.1 mg Se/kg diet, the maximal activity for all three levels was reached between 60 to 80 d, at which point GSH-Px activity plateaued (2). Because GSH-Px activity in these experiments showed dependence on the Se status of the rats, Hafeman, Sunde and Hoekstra (2) suggested that GSH-Px activity could provide a convenient
assay for Se nutritional status.

Similar studies of Se deficiency followed by Se resupplementation were carried out with [Se]Met (3). Rats were fed a Se-deficient torula yeast based diet for 17 d and then repleted with 2 mg Se/kg diet as [Se]Met. During the 17 d depletion of Se, GSH-PX activity in the liver, kidney, heart, lung and plasma decreased. In contrast, there was no change in GSH-Px activity in erythrocytes and in testis there was an apparent increase in activity. When the rats were repleted with Se-Met, GSH-Px increased in all tissues except testis. The lack of change of GSH-Px activity seen in erythrocytes during Se depletion, may have been due to the long life (60 d) of the red blood cell. The increase in testis GSH-Px activity during Se deficiency may have been a consequence of the testis priority for available Se, over other tissues (55).

During maturation of rats, testis selenium content increased 500 % in 35 d, when an Se-adequate (0.25 mg Se/kg diet) was fed (56). This priority for Se by the testis may in part explain Chow and Tappel's (3) observations with male 4 week old growing rats.

In other species as well, changes in GSH-Px activity occur with alterations in Se status. When chicks were fed a Se-deficient diet for 6 d, the plasma GSH-Px activity decreased to undetectable levels (57). Liver GSH-Px activity also decreased but not as dramatically as plasma GSH-Px. When chicks were repleted with 0.02, 0.04 and 0.06 mg Se/kg diet as sodium selenite or [Se]Met plasma GSH-Px activity increased in a linear dose-dependent manner (57). Omaye and Tappel (58) resupplemented
Se-deficient chicks with levels of Se ranging from 0.025 to 14 mg Se/kg diet as [Se]Met. This caused GSH-Px activity in liver, pancreas, heart, muscle and especially plasma to increase linearly as a function of the logarithm of dietary Se, even at the toxic levels of Se.

Guinea pigs have a substantially lower level of GSH-Px activity than rats (59). Nonetheless feeding guinea pigs an Se-deficient torula yeast based diet decreases GSH-PX activity in the liver and the blood (59).

In humans too, Se status influences GSH-Px activity. A study by Rea and associates (60) showed that blood GSH-Px activity varied directly with red blood cell Se content. However, above 0.14 ug Se/ml erythrocytes, GSH-Px activity plateaued and increasing Se content was not associated with higher level of GSH-Px activities. This would indicate that the Se requirement for GSH-Px in the red blood cells had been met (60).

The combined findings from these different species demonstrate conclusively that Se status has an effect on GSH-Px activity, until a saturation of enzyme activity is reached.

**Regulation of metalloproteins by metals**

There are a number of metalloproteins that are regulated by their associated metal. Metallothionein is induced by copper, cadmium and zinc (reviewed in 61). Rat liver copper-zinc superoxide dismutase activity is increased up to 2-fold by copper administration (62). Iron
deficiency in chicks increases serum transferrin levels by 2 to 3-fold (63) whereas iron administration increases the ferritin content in the liver (64). The mechanisms for regulation of these metalloproteins are not understood. Cadmium administration induces metallothionein I gene transcription and metallothionein I mRNA accumulation in rat liver and kidney (65). To explain how metallothionein synthesis is induced at the transcriptional level Hamer's group (66) have been using the yeast Saccharomyces cerevisiae. The genome of S. cerevisiae can be readily altered through DNA transformation and it possesses the CUP1 gene which encodes a small metallothionein-like protein that is inducible by copper and that binds copper (66). This makes S. cerevisiae a useful model for understanding the regulation of mammalian metallothionein. In their most recent model Hammer and coworkers (67) suggest that metallothionein controls the level of free intracellular copper available to interact with positive transcription factors that can bind to the CUP1 promoter. Where as metals induce the synthesis of metallothionein at the transcriptional level, ferritin is regulated by iron at the translational level (64). The model proposed by Zahringer, Baliga and Munro (68) was that in the absence of iron ferritin subunits adhere to the ferritin mRNA preventing translation. Addition of iron causes aggregation of these subunits into ferritin, thus removing the inhibition and allowing translation.

The mechanism of regulation for GSH-Px by Se is not known. However recently Saedi and coworkers (69) have demonstrated that during Se
deficiency the level of GSH-Px mRNA decreases at the same rate as GSH-Px activity. It remains to be established how Se alters the level of GSH-Px mRNA.

The use of antibodies as biochemical tools

The use of antibodies for the quantitation of specific proteins was developed by Mancini, Carbonara and Heremans (70) and Laurell (71) using techniques based on Ouchterlony's observations of the reaction of antigens and antibodies in gels (72). Mancini's method was based on the radial diffusion of an antigen from a well into a uniformly thin layer of antibody-containing agar. The amount of antigen present was proportional to the area of the antigen-antibody precipitate formed (70). Laurell's (71) technique was a modification of Mancini, Carbonara and Heremans' (70) method. By electrophoresing the antigen into the gels rather than relying on diffusion, ascending antigen-antibody precipitate "rockets" are formed. The height of each rocket is proportional to the amount of antigen present (71).

In this thesis a newer antibody technique was used to quantitate the antigen, namely enzyme-linked immunosorbant assay (ELISA). This technique developed by Engvall & Perlmann (73) utilizes an antibody to which an enzyme marker has been conjugated, typically horseradish peroxidase or alkaline phosphatase, which catalyse colorometric reactions. There are a number of variations of the ELISA (reviewed in 74). The one used in this thesis is termed a non-competitive ELISA, in
which the antigen is bound to a solid support (the wall of a plastic well). The specific antibody (antibody\textsubscript{1}) then binds to the immobilized antigen and a second antibody, produced against the first antibody, to which an enzyme marker has been conjugated (antibody\textsubscript{2}-enzyme) binds to the antigen-antibody\textsubscript{1} complex. Thus forming an antigen-antibody\textsubscript{1}-antibody\textsubscript{2}-enzyme complex, which allows detection of the antigen on addition of the marker-enzyme's substrate.

A second immunological procedure used in this thesis is that of immunoblotting or western blotting. This is a qualitative technique and allows examination of the specificity of the antibody for its antigen and examination of other tissues for the presence of a specific antigen. Immunoblotting was developed by Towbin, Staehelin & Gordon (75), proteins which have been separated by PAGE are electrophoretically transferred from the gel to the surface of nitrocellulose membranes. This exposes the immobilized enzymes and allows them to be detected by specific antibodies by using similar techniques to that described for the ELISA.

The use of anti-GSH-Px antibodies

The first group to produce anti-GSH-Px antibodies demonstrated that the antigenic sites for these antibodies and the catalytic site of GSH-Px were at different locations (76). In Japan, Watanabe and a number of coworkers developed anti-GSH-Px antibodies and used them to localize GSH-Px in rat liver (77), adrenal cortex (78) and kidney (79).
Immunohistochemical staining of the rat liver showed that the GSH-Px was located in the parenchymal cells but not in the endothelial nor Kupffer cells (77). Furthermore the immunohistochemical staining showed a zonal distribution of GSH-Px, with greater amounts at the peripheries of the liver lobules (77).

The first demonstration that there was less GSH-Px protein in Se-deficient rats than in Se-adequate rats was provided by Yoshida, Iwami and Yasumoto (6). They showed that 10 times the amount of liver extract was required from a Se-deficient rat, than from a Se-adequate rat, to "neutralize" a given amount of antiserum. This observation was important, because it was the first indication that Se status may effect the level of GSH-Px protein as well as GSH-Px activity.
CHAPTER II

THE EFFECT OF PROGRESSIVE SELENIUM DEFICIENCY
ON ANTI-GLUTATHIONE PEROXIDASE ANTIBODY
REACTIVE PROTEIN IN RAT LIVER.¹

SIMON A.B. KNIGHT and ROGER A. SUNDE

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A number of experiments have linked changes in Se status of animals to changes in GSH-Px activity (2,3,58). When animals are placed on a Se-deficient diet they exhibit a decline in GSH-Px activity, and when animals are repleted with dietary Se they show a restoration of GSH-Px activity.

In contrast to the intense research on the effect of Se status on GSH-Px activity, few experiments have studied changes in the concentration of the protein portion of GSH-Px. In 1982 Yoshida, Iwami and Yasumoto (6) used anti-GSH-Px antibodies to show that the low amount of GSH-Px activity in Se-deficient rat liver is accompanied by a similar low level of GSH-Px protein. This suggested that Se status could control the level of GSH-Px protein as well as GSH-Px activity.

To investigate the effect of Se status on GSH-Px protein level, we prepared antibodies to rat liver GSH-Px and used them in an ELISA to quantitate the level of GSH-Px protein. This experiment studied the effect of progressive Se deficiency on the level of GSH-Px protein in weanling rats fed a Se-deficient diet for 28 days. The initial 28 days of Se deficiency were examined, because within this period Se-deficient rats develop liver necrosis (80) and liver GSH-Px activity falls to undetectable levels (2). This suggests that large changes in GSH-Px protein might also occur within this time.
MATERIALS AND METHODS

Animals and diets. Male-21-day old weanling rats, (Holtzman Co., Madison, WI) weighing from 65 to 70 g were housed individually in hanging wire mesh cages. Diet and deionized water were provided ad libitum. The basal diet was that of Schwarz (80) as modified by Hafeman and Hoekstra (81) with alterations in some of the specific salts used for the mineral mix as indicated in Table 1. This diet was supplemented with 0.4% D,L-methionine (U.S. Biochem. Corp., Cleveland, OH) and 100 IU/Kg all-rac-α-tocopheryl acetate (Sigma, St. Louis, MO.) to prevent liver necrosis. Fluorometric analysis (82) showed the basal diet to contain 0.008 ppm Se. Se-adequate rats were fed the basal diet supplemented with 0.2 ppm Se as Na₂SeO₃ (Sigma, St. Louis, MO). Long-term Se-deficient rats, fed the Se-deficient diet for 125 days, were used to characterize the anti-GSH-Px antibodies to insure that the full extent of the change in GSH-Px protein with Se-deficiency could be examined.

Experimental design. The weanling rats were fed the Se-adequate diet overnight and then divided randomly into 11 groups, five rats per group. Five groups were fed the Se-deficient diet, five groups were fed the Se-adequate diet, and the remaining group was killed. At 3, 7, 14, 21 and 28 days after the start of dietary treatment, one Se-deficient group and one Se-adequate group were killed.

Rats were anesthetized with ether and blood was drawn by cardiac puncture with a heparinized syringe. The livers were perfused in situ
### Table 1. Composition of Basal Diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percent of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torula yeast</td>
<td>30.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>58.59</td>
</tr>
<tr>
<td>Lard</td>
<td>5.00</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>5.00</td>
</tr>
<tr>
<td>Vitamin premix</td>
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</tr>
<tr>
<td>D,L-Methionine</td>
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</tr>
<tr>
<td>Choline chloride</td>
<td>0.10</td>
</tr>
<tr>
<td>all-rac-α-tocopheryl acetate</td>
<td>0.01</td>
</tr>
</tbody>
</table>

3Lake State, Rhinelander, WI

4Morrell, Northfield, IL

5Mineral mix (g/kg mix): CaCO₃, 526.76; MgCO₃, 25.0; MgSO₄·7H₂O, 32.76; NaCl, 69.0; KCl, 108.0; KH₂PO₄, 212.0; ferric ammonium citrate-green, 20.5;KI, 0.08; MnSO₄·H₂O, 3.33; NaF, 1.00; Al₃(NH₄)₂(SO₄)₂·12H₂O, 0.16; CuSO₄·5H₂O, 0.90; CrCl₃·6H₂O, 0.51.

6Vitamin premix (mg/kg diet): glucose monohydrate, 8797.5; thiamin·HCl, 4.0; riboflavin, 2.5; pyridoxine·HCl, 2.0; calcium-D-pantothenate, 20; niacin, 100; menadione, 1.0; folic acid, 2.0; d-biotin, 1.0; vitamin B₁₂ (0.1% triturate), 10.0; retinyl acetate and ergocalciferol (500,000 IU vitamin A/g and 50,000 IU ergocalciferol/g), 40.0; retinyl palmitate (250,000 IU vitamin A/g), 20 (providing a total of 25,000 IU vitamin A/kg diet and 2000 IU ergocalciferol/kg diet).

7D,L-Methionine, U.S. Biochem Corp., Cleveland, OH

8all-rac-α-tocopheryl acetate, Sigma, to provide 100 IU vit E/kg diet
with ice cold 0.15 M KCl to flush out erythrocytes, rinsed in distilled water, lightly blotted and weighed. Approximately 0.5 g of each large left liver lobe was homogenized in 9 volumes of 0.25 M sucrose containing 0.25 mM EDTA. The homogenate was centrifuged (7800 x g, 20 min, Model J-21C, JA-20 rotor, Beckman Inst., Palo Alto, CA.) and the supernatant was recentrifuged (105,000 x g, 60 min, model L5-50, rotor 50 Ti, Beckman Inst.). GSH-Px activity of the supernatant was assayed by the coupled assay procedure (82) using \( \text{H}_2\text{O}_2 \) so that only Se-dependent GSH-Px was measured. Protein was determined by the method of Lowry et al. (83). GSH-Px protein was assayed using anti-GSH-Px antibodies as described in the next section.

**Antibody procedures.** Anti-GSH-Px antibodies were produced in rabbits that had been challenged with GSH-Px. GSH-Px (0.25 mg) purified from rat liver (31), was emulsified with complete Freund's adjuvant and injected into the hind leg lymph nodes of an anesthetised 8 mo old rabbit. Secondary and tertiary challenges of 0.25 mg GSH-Px in incomplete Freund's adjuvant were given 3 wk and 11 mo later. The rabbit was bled 9 days after the third injection and thereafter weekly. The blood was centrifuged (3000 x g, 20 min, Acuspin, Beckman Inst.) and the antiserum was collected. The fresh antisera was assayed for anti-GSH-Px antibodies using a crude preparation of GSH-Px (83 EU/mg) in the following ELISA modified from the method of Engvall and Perlman (73): GSH-Px was serially diluted in 0.05 M carbonate buffer, pH 9.6, to give a range of GSH-Px concentrations of \( 1.25 \times 10^{-5} \) to \( 1.25 \times 10^{-1} \) EU/150
ul. Aliquots of 150 ul diluted GSH-Px were incubated overnight in ELISA plates (Costar, Cambridge, MA) at 4°C. Control wells contained carbonate buffer alone. After each well was washed 3 times with phosphate buffered saline (PBS) containing 0.05% Tween (PBST), 150 ul of 1% bovine serum albumin (BSA) solution in PBST was added, and the ELISA plates were incubated for 30 min at 23°C. After 3 washings, 150 ul per well of antisera diluted 1:1000 in PBST was added. After 2 h incubation at 23°C, the wells were washed as before and 150 ul of goat-anti-rabbit-IgG (GAR) conjugated to horseradish peroxidase (GAR-HRP, Sigma Chem. Co., St. Louis, MO) diluted 1:1000 in PBST was added. After a 2 h incubation at 23°C and 3 washings, 150 ul of the HRP substrate (1 mM 2-2, azinodi-(3-ethylbenzyl-thiazoline sulfonic acid) (ABTS, Sigma), containing 100 mM H₂O₂) was added. The reaction at 23°C was stopped after 10 min by the addition of 50 ul 10% sodium dodecyl sulfate (SDS) per well. The color intensity was measured at 405 nm with a spectrophotometer.

The ability of the antisera to precipitate GSH-Px was investigated by adding increasing levels of antisera to purified ⁷⁵Se-labelled rat liver GSH-Px (⁷⁵Se-GSH-Px) that had been isolated from rats injected with [⁷⁵Se]selenite (33). One EU of ⁷⁵Se-GSH-Px was incubated with 0, 0.001, 0.013, 0.067, 0.133 or 0.267 ml of antisera in 20 mM phosphate buffer, pH 7.0, containing 0.6 mM EDTA in total of 3 ml at 37°C. Controls containing these levels of antisera alone were treated similarly. After 1 h the samples were centrifuged (3000 x g, 20 min)
and the supernatants were assayed for GSH-Px activity and counted for
\(^{75}\text{Se} \) (1275 Minigamma, LKB Instrument Ltd., Gaithersburg, MD).

The immunoglobulin G (IgG) fraction was purified by precipitation
of the antisera with 35\% saturated ammonium sulfate followed by DEAE ion
exchange chromatography (84). The IgG fraction was shown to be specific
for GSH-Px by the procedure of immunoblotting (75). Purified GSH-Px, Se
adequate rat liver cytosol, and Se deficient rat liver cytosol were
subjected to gradient (7.5 - 20\%) SDS-PAGE (85). The polypeptides were
then electrophoretically transferred (Transphor TE52, Hoefer, San
Francisco, CA) to a nitrocellulose membrane (NC) (Bio-Rad, Richmond, CA)
for 2 h at 10\degree C. The NC membrane was immediately washed in tris-
buffered saline (TBS) for 5 min, and incubated with 3\% gelatin in TBS
for 30 min (these and all subsequent steps were carried out at 23\degree C with
gentle agitation). After washing twice with TBS containing 0.05\% Tween
(TBST), the NC membrane was incubated for 12 h with the IgG fraction
containing the anti-GSH-Px antibodies diluted 1:100 in TBST containing
1\% gelatin. After washing twice with TBST, the membrane was incubated
with GAR-HRP diluted 1:1000 in 1\% gelatin TBST for 2 h. After washing
twice with TBST, then twice with TBS, the membrane was incubated with
HRP Color Development Solution (Bio-Rad) for 35 min. The membrane was
finally rinsed in TBS and photographed.

To assay the GSH-Px protein present in the Se-adequate and Se-
deficient rat liver cytosols, the previously described ELISA was used
with the following modifications: liver cytosol, diluted 1:10 in 0.05 M
carbonate buffer, pH 9.6, was used in place of the crude preparation of
GSH-Px; the IgG fraction, diluted 1:100 in PBST, replaced the antisera;
the intensity of the colorimetric reaction (15 min) was measured with an
automatic ELISA plate reader (Titertek Multiscan, Flow Labs, Irvine,
Scotland) at 405 nm. The ELISA reactivity of each liver cytosol from
the experimental animals was determined in duplicate.

**Statistical Analysis.** Student's unpaired t-test was used to
compare levels of GSH-Px activity and protein in Se-adequate versus Se-
deficient rats. For comparison of the half lives of GSH-Px protein and
GSH-Px activity, linear regression of the exponential transformation of
the data was computed, and the slopes were compared using analysis of
covariance and Student's t-test (86).

**RESULTS**

**Characterization of anti-GSH-Px antibody.** When 1 EU of $^{75}$Se-
labelled GSH-Px and 0.267 ml of anti-GSH-Px antisera were incubated
together, 94.6% of the $^{75}$Se and 97.4% of the GSH-Px activity were
precipitated (Fig. 1). When lesser quantities of antisera were used,
the extent of precipitation of $^{75}$Se and GSH-Px activity was linearly
proportional to the amount of antisera added. Thus precipitating
antibodies to rat liver GSH-Px had been produced in the rabbit
challenged with purified GSH-Px.

Specificity of the anti-GSH-Px antibodies was confirmed by
immunobloting (Fig. 2). SDS-PAGE of purified GSH-Px often results in a
Figure 1. Antisera titration of pure $^{75}$Se-labelled GSH-Px. Increasing levels of antiserum were added to tubes containing 1 EU of purified $^{75}$Se-GSH-Px in 20 mM potassium phosphate buffer (pH 7.0), giving a total volume of 3 ml. The tubes were incubated (1 h, 37°C) and then centrifuged (3000 x g, 20 min). Each point indicates the GSH-Px activity (○—○) or $^{75}$Se counts (△—△) detected in the supernatant.
Figure 2. Immunoblot of Se-adequate and long-term Se-deficient rat liver cytosol. Purified GSH-Px (A lanes), Se-adequate rat liver cytosol (B lanes), and Se-deficient rat liver cytosols (C lanes) were subjected to gradient SDS-PAGE. Lanes A (1 ug), B (24 ug) and C (24 ug) were stained with coomassie blue. Lanes A1 (1 ug), A2 (0.5 ug), B1 (47 ug), B2 (24 ug), C1 (47 ug), and C2 (24 ug) were electrophoretically transferred to nitrocellulose. Anti-GSH-Px antibodies that bound to polypeptides on the nitrocellulose were detected using anti-GSH-Px-IgG and anti-rabbit-IgG linked to HRP. Bands were stained with HRP substrate (Bio-Rad). The arrows indicate the position of GSH-Px subunits.
coomassie blue-stained doublet, and immunoblotting against 1 and 0.5 ug purified GSH-Px (lanes A1, A2) showed a darkly stained doublet at the position of GSH-Px subunits. A low molecular weight antibody reactive polypeptide was observed in these lanes which may have been a degraded polypeptide from the purified GSH-Px. A distinct doublet of antibody binding was observed with 47 and 24 ug of Se-adequate rat liver cytosol (lanes B1 and B2) at the position of the GSH-Px subunits, although the GSH-Px doublet in Se-adequate liver cytosol had a slightly reduced mobility as compared to purified GSH-Px. No corresponding bands were observed with Se deficient rat liver cytosol lanes (C1 and C2). There were some faintly stained low affinity cross-reactive polypeptides with higher molecular weight in both the Se-adequate and Se-deficient lanes. However, the faintness of their stain implies that this is a background reaction of the assay, rather than a specific immunoreaction. Thus the purified IgG contains precipitating anti-GSH-Px antibodies that are highly-specific for rat liver GSH-Px.

To verify that the anti-GSH-Px IgG could be used to quantitate GSH-Px protein in a matrix of liver cytosol, various ratios of Se-adequate and long-term Se-deficient liver cytosol were mixed and assayed (Fig. 3). The ELISA showed a positive correlation (r = 0.987) against GSH-Px activity indicating that the ELISA was suitable to assay liver GSH-Px protein levels.

Liver cytosol ELISA. Rats that had been fed the Se-adequate diet exhibited a graded increase in GSH-Px activity of 66% and a similar
Figure 3. Correlation of anti-GSH-Px antibody binding (ELISA) with GSH-Px activity. Liver cytosol from a Se-adequate rat was diluted with liver cytosol from a long-term Se-deficient rat to provide various levels of GSH-Px in a liver cytosol matrix. GSH-Px activity (EU/mg protein) was assayed using $\text{H}_2\text{O}_2$. Anti-GSH-Px antibody binding ($A_{405}$) was assayed using ELISA analysis as described in the text. Each point indicates the GSH-Px activity and ELISA reactivity of one dilution. A correlation coefficient of 0.987 was obtained.
increase in GSH-Px protein of 50% over the 28 day period (Fig. 4). In contrast, the liver cytosol of rats fed the Se-deficient diet showed an exponential decline in GSH-Px activity and GSH-Px protein (Fig. 4). The GSH-Px activity declined to zero by day 21, with a half life over the first 14 days of 2.8 days (Fig. 4, inset). GSH-Px protein decreased with a significantly slower (p < 0.001) half life of 5.2 days during the first 14 days of Se deficiency. Furthermore, the GSH-Px protein did not decrease to zero, but leveled off at an A405 of 0.19 which was different significantly from 0 (p < 0.001).

When GSH-Px protein was plotted against GSH-Px activity for both the Se-adequate and the Se-deficient groups, a positive correlation of 0.917 was observed (Fig. 5). The correlation coefficient for the Se-deficient rats alone was 0.939, whereas a weaker correlation (r = 0.629) was observed for the Se-adequate rats alone. This weaker correlation in Se-adequate rats may have been due to the greater variability of both GSH-Px activity and GSH-Px protein values in Se-adequate as compared to Se-deficient rats (Fig. 4).

**DISCUSSION**

Liver GSH-Px activity decreases to undetectable levels within 24 d in rats fed a Se-deficient diet when the livers are perfused to remove contaminating erythrocytes (2). In the present study, antibodies to purified rat liver GSH-Px were produced in rabbits challenged three times with antigen. The antisera co-precipitated 75Se-labelled GSH-Px
Figure 4. Effect of dietary Se on GSH-Px protein and GSH-Px activity. Liver cytosol from rats fed the Se-deficient (○, △) or Se-supplemented (●, ▲) diet was assayed for GSH-Px protein (A405) using ELISA analysis (△, ▲), and for GSH-Px activity (EU/mg protein) (○, ●). The results are plotted versus days of dietary treatment. Error bars represent the mean ± SEM for 5 rats. INSET: Exponential replot of data for day 0 through day 14 showing the exponential decrease of GSH-Px protein (△, r=0.941) and GSH-Px activity (○, r = 0.897). The decrease in GSH-Px activity (t1/2 = 2.8 d) was significantly faster (p<0.001) than that of GSH-Px protein (t1/2 = 5.2 d).
Figure 5. Correlation of GSH-Px protein and GSH-Px activity in rat liver. Liver GSH-Px protein data shown in fig. 4 was plotted versus GSH-Px activity for Se-deficient (○), Se-adequate (●) and day 0 rats (□). A correlation coefficient of $r = 0.917$ was obtained.
and GSH-Px activity, showing the presence of antibodies for GSH-Px. Immunoblot analysis showed that the antibodies were highly-specific for GSH-Px in Se-adequate rat liver cytosol, but did not show strong reaction with liver cytosol from long-term Se-deficient rats. IgG purified from the antisera was used in an ELISA analysis to determine the effect of progressive Se-deficiency on the level of GSH-Px protein in rat liver. In Se-supplemented controls, liver GSH-Px activity and GSH-Px protein exhibited small, parallel increases over the 28 d experimental period. In rats fed a Se-deficient diet, liver GSH-Px activity declined to 0 in 21 days with an exponential half-life of 2.8 d. GSH-Px protein also declined exponentially with a half-life of 5.2 d, but GSH-Px protein did not decline to zero within this period.

The small increase in liver GSH-Px activity observed in this experiment when weanling rats were supplemented with 0.2 ppm Se agrees with the results of Hafeman, Sunde and Hoekstra (2), Chow and Tappel (3), Lawrence et al. (82), and other later reports. Thus, the small rise in GSH-Px protein during Se supplementation, parallel to the increase in GSH-Px activity, was expected in the present study. A decline in GSH-Px activity during progressive Se deficiency is easily explained by decreasing liver concentrations of Se, the moiety essential for activity, but the observed decrease in GSH-Px protein during Se deficiency is less easily explained. This decrease could be due to a reduced rate of GSH-Px synthesis or to an increased rate of GSH-Px protein degradation during Se deficiency. Proteins that bind metals are
often protected against degradation when the metal is present (iron stabilization of ferritin, for example (64)); a similar accelerated degradation of the Se-free form of GSH-Px could explain the decline in GSH-Px protein during Se deficiency. The continued synthesis of an enzyme without activity would waste energy, however, so additional mechanisms for controlling the level of GSH-Px protein are also likely to be in effect.

The codon for the [Se]Cys moiety in both mouse GSH-Px (5) and in bacterial formate dehydrogenase (34) has recently been reported to be the nonsense or termination codon, UGA. The UGA codon can be read by suppressor tRNAs, including a seryl-tRNA that occurs naturally in higher animals (87). The serine is converted by a kinase to phosphoserine while esterified to this tRNA, and we have suggested (33) that further metabolism of the phosphoserine to [Se]Cys would permit the co-translational insertion of [Se]Cys into GSH-Px using a selenocysteyinyl-tRNA, similar to the mechanism proposed by Hawkes and Tappel (31). This would also explain our results showing that inhibition of protein synthesis by cycloheximide blocks Se incorporation into GSH-Px in both Se-deficient and Se-adequate rats (86), that Se from inorganic forms is more readily incorporated into GSH-Px than is Se from [Se]Cys (32), and that the carbon skeleton of [Se]Cys is derived from serine (33). During Se deficiency in our experiment, a reduced level of such a selenocysteyinyl-tRNA may have caused early termination of GSH-Px synthesis, thus causing the decrease in both GSH-Px activity and GSH-Px
protein. The polypeptide resulting from early termination would have a molecular weight of 4000 Da, and thus may not have been detected by immunoblotting (Fig. 2). If this polypeptide was recognized by the anti-GSH-Px antibodies, however, it may have elicited the observed slower decline in GSH-Px protein as compared to GSH-Px activity, and it may have been the cause of the apparent inactive GSH-Px protein that was observed 14, 21 and 28 days after the start of the Se-deficient period.

A second possible cause of the slower loss of GSH-Px protein as compared to GSH-Px activity during Se deficiency may have been the incorporation of some other amino acid into the GSH-Px protein in place of [Se]Cys, resulting in Se-free protein that cross-reacted with the anti-GSH-Px antibodies during the ELISA. A third possible explanation for the longer half-life of GSH-Px protein as compared to activity is that the detected inactive GSH-Px was a proteolytic break-down product of GSH-Px. This hypothesis is not substantiated by the immunoblot of Se-deficient rat liver cytosol, which did not show low-molecular weight antibody-reactive species. Breakdown products of GSH-Px would presumably have been localized in the lysosomes which would have been separated from the cytosol during subcellular fractionation. A fourth possibility is that other proteins with faint cross-reactivity may have contributed to the apparent GSH-Px protein. These species, however, are not likely to have resulted in a significant contribution, as the affinity of the anti-GSH-Px antibodies for these species was far lower than that for GSH-Px (Fig. 2).
The level of a number of metalloproteins are regulated by the metal. For example, ferritin synthesis as well as degradation is regulated by iron (68,89) copper administration to rats leads to an increase in liver copper-zinc-superoxide dismutase (62), manganese induces the distinct manganese superoxide dismutase in E. coli (90), and zinc induces metallothionein in numerous tissues (91). Transcription of eukaryotic genes appears to be modulated by the binding of factors to specific nucleotide sequences (regulatory elements) that are located upstream (before) the protein-coding regions of the gene. The exact mechanism whereby metals regulate the level of metalloproteins has not been established, but recent experiments with fusion genes suggest that upstream regulatory elements of the copperthionein gene in yeast are modulated either by additional transcriptional factors activated by copper (92) or by the gene product itself (66). Chambers and coworkers (5) have detected upstream regulatory elements in their cloned mouse GSH-Px gene, so similar mechanisms may control the level of GSH-Px synthesis.

In conclusion, we have observed an exponential decrease in GSH-Px protein in rat liver during progressive Se deficiency, indicating that Se status regulates the level of GSH-Px protein as well as activity. This loss of GSH-Px protein occurred at a slower rate than the loss of GSH-Px activity, suggesting the presence of an inactive GSH-Px polypeptide during short-term Se deficiency.
CHAPTER III

EFFECT OF SELENIUM REPLETION ON
GLUTATHIONE PEROXIDASE PROTEIN LEVEL
IN RAT LIVER.\(^1\)

SIMON A.B. KNIGHT and ROGER A. SUNDE

\(^1\)Published in Journal of Nutrition, 118: 853–858, 1988
INTRODUCTION

In chapter II we showed that progressive Se deficiency caused an exponential decrease in GSH-Px protein level when weanling rats were fed a Se-deficient diet for 28 d. Takahashi, Newburger and Cohen (7), using anti-GSH-Px antibodies produced against human erythrocyte GSH-Px, reported that GSH-Px protein level and GSH-Px activity increased in parallel during Se repletion of HL-60 cells and of a Se-deficient human, indicating that Se repletion restores the synthesis of GSH-Px along with restoring GSH-Px activity.

The purpose of these experiments was to determine the response of rat liver GSH-Px protein level and GSH-Px activity to dietary Se repletion and to intravenous injection of physiological levels of Se.

MATERIALS AND METHODS

Animals and diets. Male 21-d-old weanling rats (Holtzman Co., Madison, WI) were housed in hanging wire mesh cages and maintained on a 12-h light and dark cycle (lights on at 0730 h). During the preliminary Se depletion period rats were housed two to a cage, and during Se repletion the rats were housed individually. Food and deionized water were provided ad libitum. The basal diet (Table 1), described previously by Knight and Sunde (93), was a 30% torula yeast-based diet that was supplemented with 0.4% D,L-methionine (U.S. Biochemical Corp., Cleveland, OH) and 100 IU/Kg all-rac-α-tocopheryl acetate (Sigma Chemical Co., St. Louis, MO) to allow adequate growth and to prevent
liver necrosis, respectively. Fluorometric analysis (94) showed that the basal diet contained 0.023 mg Se/kg diet. Se-adequate control rats were fed the basal diet supplemented with 0.2 mg Se/kg diet as Na$_2$SeO$_3$ (Sigma) since weanling.

**Experiment 1.** To determine the changes in liver cytosolic GSH-Px protein level and GSH-Px activity during dietary Se repletion, 70 weanling rats were fed the Se-deficient diet and 2 groups of 5 Se-adequate control rats were fed the 0.2 mg Se/kg diet. After 124 d the Se-deficient rats (420 g average weight) were randomly divided by weight into 14 groups, 5 rats per group. One group was fed the Se-deficient diet, six groups were fed the basal diet supplemented with 0.1 mg Se/kg diet as Na$_2$SeO$_3$, six groups were supplemented with 0.5 mg Se/kg diet and one Se-deficient group and one Se-adequate group were killed to obtain initial GSH-Px measurements. At 1, 2, 3, 5, 7 and 14 d after the start of resupplementation, one 0.1 mg Se/kg diet group and one 0.5 mg Se/kg diet group were killed. On day 14 the second Se-deficient and Se-adequate control groups were killed.

Rats were anesthetized with ether and blood was drawn by cardiac puncture with a heparinized syringe. The livers were perfused in situ with ice-cold 0.15 M KCl to flush out erythrocytes, rinsed in distilled water, lightly blotted and weighed. Approximately 0.5 g of each large left liver lobe was homogenized in 9 vol of 0.25 M sucrose containing 0.25 mM EDTA. The homogenate was centrifuged (7800 x g, 20 min, Model J-21C, JA-20 rotor, Beckman Instruments, Palo Alto, CA) and the
supernatant was recentrifuged (105,000 x g, 60 min. Model L5-50, 50Ti rotor, Beckman Instruments). GSH-Px activity of the supernatant was assayed using the coupled assay procedure (82) with H2O2 so that only Se-dependent GSH-Px was measured. Protein was determined as described by Lowry et al. (83). After analysis, the tissues were stored at -85°C until GSH-Px protein was assayed using anti-GSH-Px antibodies. The anti-GSH-Px antibodies used in experiment 1 were antibodies purified as an immunoglobulin G (IgG) fraction from rabbit antisera (rabbit 1 in our nomenclature), and were characterized previously and used in our Se-depletion study (93). GSH-Px protein level in the liver cytosol was assayed using an ELISA modified from the method of Engvall and Perlman (73) and described by Knight and Sunde (93). The final colorimetric reaction, however, was stopped at 8 min in these experiments rather than at 15 min used earlier (93).

**Experiment 2.** To study the early response of liver cytosolic GSH-Px protein level and GSH-Px activity to Se repletion, 60 weanling rats were fed the basal diet and 2 groups of 5 rats were fed the 0.2 mg Se/kg diet. After 61 days the Se-deficient rats (405 g average weight) were randomly divided by weight into 12 groups, 5 rats per group. Five groups of rats were anesthetized with ether, injected with 15 ug of Se as Na2SeO3 in phosphate-buffered saline (PBS) into the right femoral vein, and allowed to recover from anesthesia. Five groups were injected in a similar fashion with 60 ug Se. One Se-deficient and one Se-adequate group were injected with PBS and one Se-deficient and one Se-
adequate group were killed to provide initial GSH-Px levels. The 15 and 60 ug Se doses corresponded to the amount of Se these rats would have consumed if fed a 0.5 mg Se/kg diet or a 2.0 mg Se/kg diet, respectively, and if the rats daily diet consumption was equal to 7.5% of their body weight. At 1, 3, 6, 12 & 24 h post-injection, one 15 ug Se group and one 60 ug Se group were killed as described for experiment 1. At 24 h the Se-deficient and Se-adequate groups injected with PBS were also killed. To match the Se injection schedule with the feeding habits of the rats, all injections were given 1.5 h (2100 h) after the start of the dark period of the light/dark cycle.

Tissue preparation, GSH-Px activity assays and GSH-Px protein ELISA were identical to those described for experiment 1. Because we had an insufficient amount of the anti-GSH-Px antibody used in the previous study (93) and in experiment 1, anti-GSH-Px antibodies were produced in a different rabbit (rabbit 7). GSH-Px (0.25 mg) purified from rat liver (33) was emulsified in Ribi Adjuvant System (RIBI, ImmunoChem Research Inc., Hamilton, MT) and injected into the hind leg lymph nodes of an anesthetized 6-mo old rabbit. It should be noted that we have observed little differences between Freund's complete adjuvant and the primary challenge vehicle used for production of this antibody in terms of titer. Secondary and tertiary challenges of 0.15 mg GSH-Px in incomplete Freund's adjuvant were given 2 wk and 4 wk later. The rabbit was bled 2 wk after the third injection and thereafter weekly. Antisera was collected by centrifugation (3000 x g, 20 min, Acuspin, Beckman
Instruments) and assayed against a crude preparation of GSH-Px (83 EU/mg) in an ELISA as described previously by Knight and Sunde (93). Antisera was stored frozen at -85°C and later pooled for purification of IgG by precipitation with 35% saturated ammonium sulfate followed by DEAE-cellulose chromatography (84). The IgG was shown to be specific for GSH-Px using the procedure of immunoblotting (75) as described previously (93). The ELISA protocol was the same as used in experiment 1 except that the liver cytosol was diluted 1/100 and the final color development time was 10 min. These conditions gave the most graded response when various ratios of Se-deficient and Se-adequate liver cytosols were mixed and GSH-Px protein level was measured using the ELISA (Fig. 6.). The absorbance ($A_{405nm}$) showed a positive linear correlation against GSH-Px activity ($r = 0.976$), indicating that these new anti-GSH-Px antibodies were suitable to assay the GSH-Px protein level in liver cytosol. Furthermore, the lowest point on this curve, equivalent to 0.000345 EU/150 ul well, indicated that as little as 0.4 pg GSH-Px protein could be detected, as calculated using a specific activity of 853 EU/mg for GSH-Px purified from rat liver (31).

**Statistical analysis.** One-way ANOVA and Duncan's multiple range test were used to compare levels of GSH-Px activity and GSH-Px protein (95). Linear regression and Student's t-test were used to compare the rates of GSH-Px protein increase verses GSH-Px activity increase during Se repletion (95). Correlation coefficients were calculated to evaluate
**Figure 6.** Correlation of anti-GSH-Px antibody binding (ELISA) with GSH-Px activity, using the antibodies used in experiment 2. Liver cytosol from a Se-adequate rat was diluted with liver cytosol from a Se-deficient rat to provide the indicated levels of GSH-Px activity in a liver cytosol matrix. GSH-Px protein was assayed by an ELISA ($A_{405}$) as described in text. GSH-Px activity (EU/mg) was assayed with $H_2O_2$. A linear correlation coefficient of $r = 0.976$ was obtained.
the hyperbolic relationship between GSH-Px protein and GSH-Px activity (95).

RESULTS

Experiment 1. Dietary Se repletion of Se-deficient rats with 0.5 mg Se/kg diet resulted in initial linear increases in liver cytosolic GSH-Px protein level and GSH-Px activity (Fig. 7.). Both GSH-Px protein level and GSH-Px activity increased significantly (p<0.05) after 1 d in rats repleted with 0.5 mg Se/kg diet as compared to unsupplemented rats. After 5 d for GSH-Px protein and 7 d for GSH-Px activity, the rate of increase began to plateau in a hyperbolic-type manner (Fig. 7., r=0.816 and r=0.943 for hyperbolic least-squares fits of protein and activity values, respectively) such that at d 7 for GSH-Px protein and d 14 for GSH-Px activity the values were no longer significantly different from those of Se-adequate controls. When the values of GSH-Px protein level and GSH-Px activity were expressed as a percentage of the mean values of the Se-adequate control rats, the rate of increase of GSH-Px protein over the first 5 d of repletion, was significantly higher (p<0.005) than that of GSH-Px activity. GSH-Px protein and GSH-Px activity were restored to 50% of the Se-adequate control level 3.8 d and 6.6 d, respectively, after the start of dietary repletion with 0.5 mg Se/kg diet.

In contrast to the effect of Se repletion at a level of 0.5 mg Se/kg diet, resupplementation of Se-deficient rats with 0.1 mg Se/kg diet
Figure 7. Effect of dietary Se repletion on GSH-Px protein level and GSH-Px activity. Liver cytosol from rats fed diets containing 0.1 mg Se/kg diet (○, △) or 0.5 mg Se/kg diet (●, ▲) was assayed for GSH-Px protein level (A405) using ELISA (△, ▲) and for GSH-Px activity (EU/mg) (○, ●). The GSH-Px protein and GSH-Px activity scales were adjusted so that the horizontal dashed line represents the level of both Se-adequate GSH-Px protein (■) and GSH-Px activity (■) in rats fed 0.2 mg Se/kg diet since weaning. Results are plotted vs. days of dietary treatment. Error bars represent mean ± SEM for five rats. GSH-Px protein level and GSH-Px activity were significantly greater (p<0.05) than that in Se-deficient rats at 1 d and all other days for rats fed 0.5 mg Se/kg diet, but only at 14 d for rats fed 0.1 mg Se/kg diet.
did not increase GSH-Px protein or GSH-Px activity in the liver cytosol significantly (p>0.05) above the levels in Se-deficient rats until 14 d after the start of Se repletion (Fig. 7.). As observed previously in perfused Se-deficient rat livers prior to Se repletion (93), GSH-Px protein level was significantly greater than zero, whereas GSH-Px activity was not significantly different from zero. When the individual GSH-Px protein values were plotted against the GSH-Px activity values for all Se-repleted rats and Se-deficient rats in experiment 1, a positive correlation of r = 0.923 was observed (Fig. 8.). This relationship further substantiates that these anti-GSH-Px antibodies are specific for GSH-Px.

Experiment 2. When the short-term response of Se-deficient rats to injected Se was investigated, liver cytosolic GSH-Px protein levels and GSH-Px activities did not change significantly over the 24 h period in rats injected with 15 μg Se (Fig. 9.). Rats injected with 60 μg Se also did not have significant increases in GSH-Px protein or GSH-Px activity within the first 12 h after injection. There was, however, a significant decrease in GSH-Px activity but not GSH-Px protein at 6 h post-injection (Fig. 9.). At 24 h after the injection of 60 μg Se, analysis of variance indicated that liver cytosolic GSH-Px activity had increased significantly (p<0.001) above the activity of Se-deficient controls, but that the GSH-Px protein level at 24 h post-injection had not increased significantly above the protein level of the Se-deficient controls and the other groups injected with 60 μg Se (p=0.12) (Fig. 9.).
Figure. 8. Relationship of GSH-Px protein level and GSH-Px activity in rat liver during dietary Se repletion. Liver GSH-Px protein levels shown in Fig. 2. were plotted vs. GSH-Px activity for Se-deficient rats (□), 0.1 mg Se/kg diet repleted rats (○) and 0.5 mg Se/kg diet repleted rats (●). A correlation coefficient of \( r = 0.923 \) was obtained.
Figure 9. Effect of Se injection on GSH–Px protein level and GSH–Px activity. Rats were injected with 15 µg (○, △) or 60 µg (●, ▲) of Se, and liver cytosol was assayed for GSH–Px protein level using ELISA (A405) (▲, △) and for GSH–Px activity (EU/mg) (〇, ●). Results are plotted vs hours after injection. Liver cytosol of Se-deficient rats, killed at the beginning and end of the experiment was assayed for GSH–Px protein (▲) and GSH–Px activity (〇) to provide baselines. Error bars represent mean ± SEM for five rats. At 24 h liver GSH–Px activity in rats injected with 60 µg Se was significantly higher (ANOVA, p<0.05) than that of Se-deficient rats, and GSH–Px protein level was marginally higher (Student's one-tailed t-test, p<0.05).
A one-tailed Student's t-test that compared GSH-Px protein level in rats injected with 60 ug Se at 24 h with only Se-deficient controls, however, indicated that this difference was significant (p<0.05). No relationship (r = 0.118) was observed when GSH-Px protein level was plotted against GSH-Px activity for the Se-injected rats and the Se-deficient rats in experiment 2 (data not shown).

**DISCUSSION**

We have previously used anti-GSH-Px antibodies to show that GSH-Px protein level decreases exponentially with a half life of 5.2 d in the liver cytosol of rats fed a Se-deficient diet, and that GSH-Px activity decreases exponentially with a significantly shorter half life of 2.8 d (93). In this present experiment we used the same technique to examine the reversal of these changes following dietary Se repletion and Se injection. When rats were repleted with 0.5 mg Se/kg diet liver GSH-Px protein and GSH-Px activity were significantly elevated after 1 d of dietary repletion, and after 7 d and 14 d, respectively, these levels were no longer significantly different from levels found in Se-adequate rats. These response curves for GSH-Px protein and GSH-Px activity during Se repletion were hyperbolic, suggesting that during the later stages of Se repletion, homeostatic processes control the level of GSH-Px. This implies that the level of Se is not the primary factor that regulates the level of liver GSH-Px in Se-adequate animals. When rats were repleted with 0.1 mg Se/kg diet neither GSH-Px protein level nor
GSH-Px activity increased significantly above the values for Se-deficient controls until 14 d after the start of Se repletion. Clearly the level of Se required to maintain GSH-Px activity (2) only slowly restored GSH-Px activity and GSH-Px protein in Se-deficient rats.

In experiment 1, the rate of increase of GSH-Px protein during repletion with 0.5 mg Se/kg diet was nearly twice that of GSH-Px activity when each parameter was expressed as a percentage of the levels found in Se-adequate controls. The reason for this is not clear, but it may be that synthesis of GSH-Px polypeptides occurs at a faster rate than the appearance of active GSH-Px. This observation is different from that reported by Takahashi, Newburger and Cohen (7). Using a radioimmune assay for human erythrocyte GSH-Px, these workers showed that GSH-Px protein and GSH-Px activity were restored in a parallel manner when a Se deficient individual was supplemented with Se for 16 weeks and when HL-60 cells were transferred from a Se-deficient medium to a Se-supplemented medium for 7 d. The results of Takahashi, Newburger and Cohen (7), however, were not expressed as a percentage of Se-adequate control values, so the results of these two experiments may not be directly comparable.

It is well established that dietary Se repletion causes an increase in GSH-Px activity (2,3,96). Our response curves for restoration of GSH-Px activity are in good agreement with those recently reported by Hill, Burk and Lane (97) who used similar diets and who observed that GSH-Px activity in rats repleted with 0.5 mg Se/kg diet was not
significantly different from Se-adequate rats (supplemented with 0.5 mg Se/kg diet) after 14 d of repletion. Liver GSH-Px activity of rats repleted with 0.1 mg Se/kg diet in that study also increased at a slower rate, such that after 14 days the GSH-Px activity was still less than 50% of the GSH-Px activity in the Se-adequate rats (97).

There was no significant increase in liver GSH-Px protein or GSH-Px activity of rats injected intravenously with either 15 or 60 ug Se until 24 h after the injection, and then only with the 60 ug Se dose. These doses of Se were numerically equivalent to the daily Se ingestion of rats fed 0.5 and 2.0 mg Se/kg diet, respectively. Thus a gradual increase in GSH-Px would have been expected over the entire 24 h period if GSH-Px synthesis started as soon as the supplemented Se reached the liver. When 0.5 ug or 3.9 ug Se as \(^{75}\text{Se}\)selenite are injected iv into Se-deficient rats, the maximum liver \(^{75}\text{Se}\) content is observed 1 h after injection (48,88). When we spiked the 15 ug Se dose with 10 uCi \(^{75}\text{Se}\)selenite and injected this dose into a Se-deficient rat using the same protocol, \(^{75}\text{Se}\) counting indicated that liver contained 4.02 ug Se (27% of the dose) at 1 h (data not shown). Thus uptake of Se by the liver following the Se injection should have rapidly increased the liver Se concentration in experiment 2. In contrast to Se administration via the diet, injection of 15 or 60 ug Se in a single intravenous dose may have resulted in altered Se metabolism similar to metabolism of toxic levels of Se, such that less of the Se in the liver was in a form that could elicit an increase in GSH-Px synthesis. The lack of an increase in
GSH-Px within the first 12 h after Se injection, however, does indicate that an increased concentration of cellular Se may not lead to an immediate acceleration in the rate of GSH-Px synthesis.

Delays in the induction of GSH-Px have been reported previously. Pierce and Tappel (96) did not detect a significant increase in GSH-Px activity until 48 h after a 300 ug dose of Se as selenite was administered to rats by stomach tube. A lag of 20 h prior to the increase in GSH-Px protein level and GSH-Px activity was also observed when HL-60 cells grown in a Se-deficient medium were transferred to a Se-supplemented medium (7). Sunde and Hoekstra (88) reported that $^{75}$Se incorporation into GSH-Px was delayed in Se-deficient rats as compared to Se-adequate rats. Furthermore, while $^{75}$Se incorporation into GSH-Px was detected using Sephadex G-150 chromatography as early as 3 h after the injection of 3.9 ug Se as $[^{75}$Se]selenite, an increase in GSH-Px activity in the chromatograms was not detected up to 72 h post-injection (88). More recently, analysis of liver cytosol from Se-deficient rats injected iv with 0.5 ug $^{75}$Se, using SDS-PAGE showed that $^{75}$Se incorporation into GSH-Px subunits was 1/5 that observed in Se-adequate controls 1 h post-injection, and that the quantity of $^{75}$Se incorporated into GSH-Px subunits did not increase by 24 h whereas it increased 3-fold in Se-adequate rats (48).

The lag in Se-deficient rats between administration of Se and detection of increased GSH-Px protein or activity may occur because Se must first induce an increase in the level of mRNA for GSH-Px before the
rate of GSH-Px synthesis is increased (88). A second possible cause for the observed delay is that enzymes and other hypothetical proteins necessary for the synthesis of [Se]Cys and the co-translational incorporation of Se into GSH-Px must be induced in Se-deficient rat liver prior to detectable synthesis of GSH-Px (88). Lastly, this lag in a detectable increase in liver GSH-Px may occur if one of the other selenoproteins, such as selenoprotein P (47), plasma GSH-Px (97), or the 65 kDa liver selenoprotein (48), has first priority for Se in Se-deficient rat liver.

Examination of the 24 h response curves (Fig. 9.) may suggest that GSH-Px activity in rat liver changes in a circadian rhythm. The observed decrease in GSH-Px activity (expressed as EU/mg liver protein) 6 h after the Se injection, however, was apparently caused by the significant increase in total liver protein we detected at 6 h (data not shown). Scornik (98) has reported a similar increase in liver protein that occurs at this time during the 24 h feeding cycle of the rat.

The results obtained in this study confirmed the presence of a low level of anti-GSH-Px antibody reactive protein in Se-deficient rats (93), and illustrate that anti-GSH-Px antibodies can be used to assess Se status. The dietary Se repletion of deficient rats resulted in hyperbolic-type response curves for both GSH-Px protein level and GSH-Px activity, suggesting that homeostatic processes control the level of GSH-Px. The delayed appearance of a significant increase in liver
GSH-Px prior to 24 h implies that more than an increase in cellular Se is necessary to increase synthesis of liver GSH-Px in the Se-deficient rat.
CHAPTER IV

CROSS-REACTIVITY OF ANTI-RAT LIVER GLUTATHIONE PEROXIDASE ANTIBODIES WITH GLUTATHIONE PEROXIDASE IN OTHER SPECIES AND TISSUES.
INTRODUCTION

Se-dependent GSH-Px activity has been detected in a large number of tissues, such as: liver, lung, erythrocytes, lens, and placenta, from a variety of different species: rat, hamster, cow, sheep, human (listed in 99). There have recently been reports that some distinct differences may exist between the Se-dependent GSH-Px present in plasma and in heart compared to GSH-Px present in liver. Takahashi, Newburger and Cohen (7) reported that human plasma GSH-Px was a glycoprotein and was not recognized by antibodies produced against human RBC GSH-Px. There has also been a report of a phospholipid hydroperoxide GSH-Px present in pig heart (52) which differs in its activity from liver GSH-Px. The size of this protein suggests that phospholipid hydroperoxide GSH-Px may be an active monomer of classical GSH-Px present in liver.

In the previous two chapters we used the anti-GSH-Px antibodies, produced against GSH-Px purified from rat liver, to quantitate the level of GSH-Px protein in rat liver. The purpose of the experiments in this chapter was to examine the immunoreactivity of our anti-GSH-Px antibodies against GSH-Px present in different tissues and species. In addition we had previously observed in the immunoblots, a difference in the mobility of GSH-Px in liver cytosol compared to purified GSH-Px (93). We thus investigated the cause of this difference in mobility.
MATERIALS AND METHODS

Animals and diets. Two male 21-d-old weanling rats (Holtzman Co., Madison, WI) were housed individually in hanging wire mesh cages and fed diet and water ad libitum. The basal diet (Table 1), described previously by Knight and Sunde (93), was a 30% torula yeast-based diet that was supplemented with 0.4% D,L-methionine (U.S. Biochemical Corp., Cleveland, OH) and 100 IU/kg all-rac-α-tocopherol acetate (Sigma Chemical Co., St Louis, MO) to allow adequate growth and to prevent liver necrosis, respectively. Fluorometric analysis (94) showed that the basal diet contained 0.023 mg Se/kg diet. One rat was fed the basal diet and the second rat was fed the basal diet supplemented with 0.2 mg Se/kg diet as Na2SeO3 (Sigma). After 88 d of feeding, the Se-deficient rat weighed 440 g and the Se-adequate rat weighed 455 g.

A male guinea pig (Hartley; Harlan, Sprague Dawley, Madison WI, donated by Dr. D.J. McNamara) was housed in a plastic guinea pig cage with sawdust bedding. It was fed guinea pig chow (Wayne Research Animal Diets) containing 0.2 mg Se/kg diet (specified by manufacturer) and tap water ad libitum for 240 d and weighed 647 g. Male mice (Charles River CD1, donated by Dr. C. Weber) were housed together in plastic mice cages with sawdust bedding. They were fed mouse chow (Wayne Research Animal Diets) containing 0.35 mg Se/kg diet (specified by manufacturer) and tap water ad libitum for 28 d and weighed 16.5 and 17.5 g. A Single Comb White Leghorn chicken (Dekalb XL, donated by Dr. B.L. Reid) was housed in an indirect calorimeter and was fed a 65% ground milo based diet
containing 0.049 mg Se/kg diet (100) and tap water ad libitum and weighed 1550 g.

**Tissue preparation.** The rats, guinea pig and mice were anesthetized with ether and blood was drawn by cardiac puncture with a heparinized syringe. Blood from the chicken was drawn from a wing vein into a heparinized tube, then the chicken was killed by anesthesia with ether. The livers were perfused in situ with ice-cold 0.15 M KCl to flush out erythrocytes, rinsed in distilled water, lightly blotted and weighed. Liver cytosolic fractions were prepared as described previously (Chapter II). Plasma was separated from erythrocytes by centrifugation (1000 x g, 15 min, Beckman Acuspin, Beckman Instruments, Palo Alto, CA). The white cells were removed by aspiration and the erythrocytes were restored to the original blood volume with phosphate buffered saline (PBS, pH 7.4). Rat kidney was removed, rinsed in distilled water, blotted and weighed. A kidney cytosolic fraction was prepared in the same manner as for liver cytosol. Rat heart was removed, rinsed in distilled water, blotted and weighed. Approximately 1 g of heart was homogenized in 9 vols of cold 10 mM tris buffer pH 7.4 containing 1 % (w/v) SDS and 10 mM B-mercaptoethanol, by using a Polytron (Model PT 10/35, Brinkman Instrument Co., Westbury, N.Y.). The heart homogenate was centrifuged (105,000 x g, 60 min) and the supernatant saved. GSH-Px activity in liver cytosol, RBC and plasma from rat, guinea pig, mice and chicken was assayed by the coupled assay procedure (82). Rat kidney cytosol and heart supernatant were also
assayed for GSH-Px activity. Protein was determined in all these tissue fractions using the method of Lowry et al (83). After these assays the tissues were stored at -85°C until immunoblot analysis with anti-GSH-Px antibodies. A human blood sample (from the author) and a pathological sample of human heart from a 75 year old male (kindly provided by Dr. Sobonya) were also prepared, as described above, for immunoblot analysis.

The anti-GSH-Px antibodies used in these experiments were produced in two rabbits; rabbit 2 and rabbit 9 in our nomenclature, that had been challenged with GSH-Px. Anti-GSH-Px antibodies from rabbit 2 were produced as described previously (93) and the anti-GSH-Px antibodies from rabbit 9 were produced as described in chapter III using Freund's adjuvant in place of the Ribi adjuvant system. The rabbits were bled from an ear vein at weekly intervals. Antiserum was collected by centrifugation (3000 x g, 20 min, Beckman Acuspin) and stored frozen at -85°C. The IgG fraction was purified by precipitation with 35% saturated ammonium sulfate followed by DEAE-cellulose chromatography (84).

Immunoblot analysis. To determine the sensitivity of the anti-GSH-Px antibodies in detecting GSH-Px, liver cytosol from the Se-adequate rat was diluted with liver cytosol from the Se-deficient rat to provide a range of GSH-Px levels in a liver cytosol matrix. The liver cytosol mixes contained 0.075, 0.056, 0.037, 0.006, 0.001, 0.000 and <0.000 EU/100 ug protein. They were subjected to gradient (7.5 - 20%) SDS-PAGE (85). Then the polypeptides were electrophoretically transferred
(Transphor TE52, Hoeffer, San Francisco, CA) to nitrocellulose (NC) membranes (Hoeffer) at 1 amp for 2 h at 10°C (75). The NC membranes were immediately washed in 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)-buffered saline (TBS) for 5 min and incubated with 3% gelatin in TBS containing 0.05% Tween (TBST) for 30 min. (These and all subsequent steps were carried out at 23°C with gentle agitation). After washing three times with TBST, the NC membranes were incubated overnight with an IgG fraction (purified from rabbit 9 in our nomenclature) containing the anti-GSH-Px antibodies diluted 1:50 in TBST containing 1% gelatin. After washing three times in TBST, the NC membranes were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (GAR-HRP, Sigma) diluted 1:1000 in 1% gelatin TBST for 4 h. After washing twice with TBST, then three times with TBS, the membrane was incubated with HRP Color Development Solution (Bio-Rad) until immunoreactive bands were observed. The membrane was finally rinsed in TBS, dried and photographed.

To determine if the anti-GSH-Px antibodies were immunoreactive against GSH-Px present in other species and tissues, the prepared tissue fractions were diluted to give 0.05 EU of GSH-Px per lane on the polyacrylamide slab gel. The tissue fractions were subjected to immunoblotting as described above, using anti-GSH-Px antibodies from rabbit 9.

We had noted previously (93) that GSH-Px present in liver cytosol had less mobility on a SDS-polyacrylamide gel than purified GSH-Px. To
confirm this, 4 ug of purified GSH-Px was added to 100 ug of liver cytosol from a Se-adequate and Se-deficient rat. The liver cytosols with purified GSH-Px added, liver cytosols from Se-adequate and Se-adequate rats, and purified GSH-Px were subjected to immunoblotting as described above using anti-GSH-Px antibodies from rabbit 2.

To investigate where in the purification procedure (31) the change in mobility of GSH-Px occurred, liver homogenate, liver cytosol, dialysed liver cytosol, GSH-Px purified by carboxymethylcellulose (CMC) chromatography and GSH-Px purified by sephadex G-200 chromatography, was immunoblotted by the procedure described above using anti-GSH-Px antibodies from rabbit 2. Each of the above fractions were adjusted to contain 0.2 EU of GSH-Px for the immunoblot.

RESULTS

The anti-GSH-Px antibodies could readily detect as little as 0.037 EU of GSH-Px and faintly detect 0.006 EU of GSH-Px (Fig. 10). These enzyme units are equivalent to 43 and 7 pg of GSH-Px protein respectively, as calculated by using a specific activity of 853 EU/mg for GSH-Px purified from rat liver (31).

When the anti-GSH-Px antibodies were examined for immunoreactivity with GSH-Px present in extra-hepatic tissue, there was both an effect of tissue type and Se-status. Positive immunoreaction occurred with proteins of equal molecular weight to the GSH-Px subunits in erythrocytes and kidney cytosol from the Se-adequate rat (Fig. 11).
Figure 10. Immunoblot of decreasing levels of GSH-Px in rat liver cytosol. Liver cytosols from an Se-adequate rat was diluted with liver cytosol from an Se-deficient rat, to provide various levels of GSH-Px in a liver cytosol matrix. The preparations were immunoblotted using IgG 9. Lane 1, 0.075 EU GSH-Px; Lane 2, 0.056 EU GSH-Px; Lane 3, 0.037 EU GSH-Px; Lane 4, 0.006 EU GSH-Px; Lane 5, 0.001 EU GSH-Px; Lane 6, 0.000 EU GSH-Px; Lane 7, <0.000 EU GSH-Px. The arrow indicates the position of the GSH-Px subunits.
There was no immunoreaction towards proteins of this molecular weight in the corresponding tissue from the Se-deficient rat (Fig. 11). There was no immunoreaction with plasma proteins from either Se-adequate or Se-deficient rats (Fig. 11). In heart supernatant from both Se-adequate and Se-deficient rats there was faint immunoreactivity against a protein of similar size to the GSH-Px subunits (Fig. 11). However because there was no evidence of an effect of Se status on the presence of this immunoreactive protein, it may not be Se-dependent GSH-Px.

Of the mouse, guinea pig and chicken tissues examined for immunoreactivity with the anti-GSH-Px antibodies, only mouse liver cytosol and erythrocytes had an immunoreactive protein of the size of the GSH-Px subunit (Fig. 12). A 48 kDa cross-reactive band was observed in guinea pig liver cytosol (results not shown). To determine whether this was a selenoprotein a second guinea pig was injected iv with 50 uCi $^{75}$Se as selenite and killed 72 h later. Using the technique described by Evenson & Sunde (48), liver cytosolic proteins were separated by SDS-PAGE and $^{75}$Se in gel slices was counted using a gamma counter (Packard Instruments). No $^{75}$Se counts were recorded at the 49 kDa position indicating that this 49 kDa cross-reactive species was not a selenoprotein. In none of the species examined did the anti-GSH-Px antibodies react with the GSH-Px present in plasma. Nor was there immunoreactivity with liver cytosol or red blood cell proteins from the chicken (results not shown).

To investigate the difference in mobility of purified GSH-Px to
Figure 11. Immunoblot of rat tissues from Se-adequate and Se-deficient rats. IgG 9 was used in the immunoblot. Lanes 1+,1-, liver cytosol; Lanes 2+,2-, kidney cytosol; Lanes 3+,3-, heart supernatant; Lanes 4+,4-,RBC; Lanes 5+,5-, plasma. The + signifies tissue from an Se-adequate rat and a - signifies tissue from a Se-deficient rat. The arrow indicates the position of the GSH-Px subunits.
GSH-Px present in liver cytosol, purified GSH-Px was added to liver cytosol from a Se-adequate rat. When this preparation was immunoblotted two distinct immunoreactive bands were observed (Fig. 13a). The more mobile band, corresponding to purified GSH-Px, had a molecular weight of 20 kDa (calculated from myofibrilar protein molecular weight standards). The non-purified GSH-Px present in Se-adequate rat liver cytosol had a molecular weight of 23 kDa. When purified GSH-Px was added to Se-deficient rat liver cytosol only one immunoreactive band was observed, corresponding, as expected, to the purified GSH-Px (Fig. 13a). Se-deficient liver cytosol did not show any immunoreactive bands except for a 72 kDa contaminant, discussed later. This apparent change in GSH-Px molecular weight occurred during overnight dialysis of liver cytosol prior to carboxymethylcellulose chromatography (Fig. 13b). There were no further molecular weight changes during subsequent steps of the purification procedure (Fig. 13b).

DISCUSSION

The two anti-GSH-Px antibodies (IgG 2 and IgG 9) used in these studies were produced in different rabbits injected with different GSH-Px preparations. Both were immunoreactive against purified GSH-Px and GSH-Px present in Se-adequate liver cytosol. When IgG 2 was used in immunoblots a strong immunoreactive contaminant of 72 kDa was always observed, however the presence of this contaminant was not affected by Se status and nor did it interfere with analysis of GSH-Px subunits.
Figure 12. Immunoblot of mouse liver cytosol, RBC and plasma. IgG 9 was used in the immunoblot. Lane 1, rat liver cytosol; Lane 2, mouse liver cytosol; Lane 3, mouse RBC; Lane 4, mouse plasma. The arrow indicates the position of GSH-Px subunits.
The cause of this contamination is not known, though possibly it could originate from a contaminating protein in the purified GSH-Px injected into the rabbit. The fact that IgG 1, used in chapter II and produced against the same GSH-Px preparation as IgG 2, did not show such a cross reaction implies that immune responses are not identical in all rabbits. IgG 9 also did not show a cross reaction with a 72 kDa protein, occasionally a diffuse cross-reactive band was seen with a plasma protein of molecular weight 26 to 30 kDa. This cross-reactivity was not affected by Se status and because the band was so faint no conclusions could be drawn from it.

These experiments have shown that the recognition of GSH-Px by anti-GSH-Px antibodies, that have been produced against rat liver GSH-Px is dependent on tissue, species and Se status. Of the rat rat tissues studied the anti-GSH-Px antibodies recognized GSH-Px in liver, RBC and kidney from Se-adequate, but not from Se-deficient rats. There was an ambiguous faint immunoreaction with a protein of similar size to GSH-Px in heart supernatant. However the presence of this protein was not effected by Se status of the rat, implying that this was not the same protein as Se-dependent GSH-Px present in the liver cytosol. Ursini, Maiorino and Gregolin (54) reported on a Se containing phospholipid hydroperoxide GSH-Px. It is possible that the anti-rat liver GSH-Px antibodies cross reacted with this species. Though as this is also a selenoenzyme it does not explain the equal degree of immunoreactive stain present in the heart tissue from Se-deficient and Se-adequate
Figure 13a. Immunoblot comparison of purified GSH-Px and GSH-Px present in liver cytosol. IgG 2 was used in the immunoblot. Lane 1, 4 ug purified GSH-Px; Lane 2, 100 ug liver cytosolic proteins from an Se-adequate rat; Lane 3, 4 ug purified GSH-Px added to 100 ug liver cytosolic proteins from a Se-adequate rat; Lane 4, 100 ug liver cytosolic proteins from an Se-deficient rat; Lane 5, 4 ug purified GSH-Px added to 100 ug liver cytosolic proteins from a Se-deficient rat. The upper arrow indicates position of GSH-Px subunits present in liver cytosol (23 kDa) and the lower arrow indicates position of purified GSH-Px subunits (20 kDa).

Figure 13b. Immunoblot of fractions from steps in the GSH-Px purification procedure. IgG 2 was used in the immunoblot. Each fraction immunoblotted contained 0.2 EU GSH-Px. Lane 1, liver cytosol; Lane 2, dialysed liver cytosol; Lane 3, carboxymethyl-cellulose chromatography pool; Lane 4, sephadex G-200 pool. Arrows indicate change in molecular weight of the GSH-Px subunits from 23 to 20 kDa.
rats, unless this selenoprotein has high priority for any available Se or that it is not regulated in the same manner as liver GSH-Px. The anti-GSH-Px antibodies did not recognize plasma GSH-Px in the rat or the other species examined. It is known that plasma GSH-Px is a glycoprotein and immunologically different from RBC GSH-Px (7). Thus it is perhaps not surprising that our anti-GSH-Px antibodies which recognize RBC GSH-Px did not show any immunoreaction against plasma GSH-Px. It is quite likely that the oligosaccharides attached to the plasma GSH-Px sterically prevent the binding of the anti-GSH-Px antibodies to the plasma GSH-Px epitopes. Recent evidence indicates that the plasma GSH-Px is synthesized in the liver (54), thus the lack of immunoreaction against plasma GSH-Px could imply that there is compartmentalization within the liver, separating newly synthesized plasma GSH-Px from liver cytosolic GSH-Px.

GSH-Px from mouse liver and RBC possessed similar epitopes to rat liver GSH-Px. There was however, no cross reactivity of the anti-GSH-Px antibodies towards guinea pig or chicken GSH-Px from liver, RBC or plasma. Nor was there cross reactivity against human GSH-Px in RBC, plasma or heart supernatant (data not shown). Comparison of the amino acid sequence for mouse reticulocyte GSH-Px, derived from the cDNA (5) with rat liver GSH-Px (101), for the first 46 amino acids showed 91% homology. Only the first 46 amino acids were compared as the complete amino acid sequence for rat GSH-Px has not yet been determined. A similar comparison of the rat GSH-Px amino acid sequence with the amino
acid sequence from bovine erythrocyte GSH-Px showed 82% homology and with human RBC (102) showed 87% homology. Thus although GSH-Px appears to be highly conserved from species to species, there are greater differences between more distantly related species. It is perhaps these small differences in the amino acid sequence that account for the observed differences of the antigenicity of GSH-Px from different species.

Using the anti GSH-Px antibodies in the immunoblotting procedure it is possible to specifically detect GSH-Px protein amongst the many other liver cytosolic proteins. This sensitivity is illustrated in that GSH-Px protein comprises approximately 0.1% of the total liver cytosolic proteins, calculated from using a specific activity of purified GSH-Px of 853 EU/mg (31) and a specific activity of GSH-Px in Se-adequate rat liver cytosol of 1 EU/mg. As a consequence of this sensitivity we observed that GSH-Px purified using our procedure had an apparent molecular weight of 20 kDa that was about 3000 Da smaller than that of GSH-Px present in liver cytosol. This change in molecular weight occurred at a single step in the purification, namely during dialysis of liver cytosol prior to carboxymethylcellulose chromatography. It is probable that this apparent change in molecular weight is due to proteolytic cleavage, though it does not appear to effect the enzyme activity of the GSH-Px.

We have previously seen on commassie blue stained gels, immunoblots (93) and $^{75}$Se labelling of proteins in conjunction with SDS-PAGE (48).
that GSH-Px, either purified or in liver cytosol, appears as doublet. This doublet does not directly correspond to the the 3000 Da difference seen between purified GSH-Px and GSH-Px present in liver cytosol. For example on coomassie blue stained gels the upper band of purified GSH-Px has an apparent molecular weight of 21 kDa and the lower band 20 kDa. For clarification the molecular weight of GSH-Px calculated from the sum of the amino acids of human RBC GSH-Px, is 21.9 kDa (102). Both the upper and lower band of this GSH-Px doublet have $^{75}$Se incorporated, though the upper (higher molecular weight band) has substantially greater $^{75}$Se incorporation (48). It is possible that the lower molecular weight band of the doublet is the result of in vivo post-translational modification of the higher molecular weight band. Voight and Autor (103) have reported the existence of a 28 kDa precursor to the GSH-Px subunit that is processed into the 23 kDa species. Alternatively the lower band of the doublet may be an artifact of proteolytic cleavage during tissue preparation. However, in a recent immunoblot of liver cytosols from progressively Se-deficient rats using a more sensitive biotin-avidin detection system (69), it was observed that the lower band of the doublet decreased in stain intensity at a faster rate than the upper band. This suggests that the rate of synthesis and/or degradation of the lower band of the doublet is different to that of the upper band. These studies have shown that between closely related species such as rat and mouse the GSH-Px protein is antigenically highly conserved. Between more distant related species such as human or guinea pig and rat
this antigenic conservation of the GSH-Px protein has been lost. The lack of cross-reactivity against plasma GSH-Px implies that within the liver there is separation of GSH-Px synthesized for "export" and that synthesized for function in the liver cytosol.
CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS
These studies have demonstrated that the anti-GSH-Px antibodies, produced against rat liver GSH-Px, can be used to quantitate the levels of GSH-Px present in the rat liver. We observed that the immunoreactivity of the anti-GSH-Px antibodies towards GSH-Px was affected by both the tissue and the species in which the GSH-Px was present as well as the Se status of the animal. For example anti-GSH-Px antibodies used in the immunoblotting in this thesis were not immunoreactive against plasma GSH-Px in any of the species examined. It should be mentioned, however, that IgG 1, used in earlier experiments, did show strong cross-reactivity against a rat plasma protein of molecular weight 20 kDa. Furthermore this cross-reactivity was not affected by Se status. As we were not able to precipitate plasma GSH-Px activity using this particular antibody, we were unable to confirm whether this 20 kDa protein was GSH-Px or not. Plasma GSH-Px has been shown to be a glycoprotein (7) that is synthesized in liver cells (54) and presumably exported via the endoplasmic reticulum and Golgi apparatus. Such an intracellular compartmentalization of newly synthesized plasma GSH-Px within the liver suggests the existence of two pools of liver GSH-Px; one for export (plasma GSH-Px) and the other for function in the liver cytosol.

The main objective of our studies was to use the anti-GSH-Px antibodies to study the regulation of GSH-Px protein level by Se. During Se deficiency we observed a decrease in GSH-Px protein level and GSH-Px activity and during Se repletion of Se-deficient rats GSH-Px
protein level and GSH-Px activity increased. Although the responses of GSH-Px protein and GSH-Px were coordinated, they were not parallel. GSH-Px activity was lost more readily and restored more slowly than GSH-Px protein. The reason for these differences is not known. We suggested that during Se deficiency early termination of the GSH-Px polypeptide, at the [Se]Cys codon, would generate a 4000 kDa polypeptide that could be potentially immunoreactive, but not possess catalytic activity. This species, as yet, has not been detected on immunoblots. The faster rate of appearance of GSH-Px protein than GSH-Px activity, during Se repletion, implies that synthesis of GSH-Px polypeptides occurs at a faster rate than the appearance of active GSH-Px.

When we studied the short term effects of Se repletion on GSH-Px, we observed a 24 h delay between Se administration and increase in GSH-Px protein and GSH-Px activity. This may have occurred because other selenoproteins, such as selenoprotein P (47) or plasma GSH-Px (97), have priority for Se in Se-deficient rat liver. Also Se may first have to induce an increase in the level of mRNA for GSH-Px (88), and possibly induce synthesis of other hypothetical proteins necessary for the cotranslational insertion of Se into GSH-Px.

There are a number of cellular events where Se could regulate the level of GSH-Px. Saedi and coworkers (104) have demonstrated that the level of mRNA for GSH-Px in the Se-deficient rat liver is only 7 to 17% of that in the Se-adequate rat liver. Furthermore progressive Se deficiency reduced the GSH-Px mRNA levels in a similar manner to the
decrease seen in GSH-Px protein and GSH-Px activity (69). This suggests that Se regulates the level of mRNA for GSH-Px, which in turn could regulate the level of GSH-Px protein.

The hyperbolic response curves for GSH-Px protein and GSH-Px activity suggest that when Se is no longer a limiting factor, other homeostatic processes control the level of GSH-Px. If it is assumed that at the steady state rate of synthesis equals rate of degradation then using the equation for exponential decay:

\[ N = N_0 e^{-0.693 \times \frac{1}{\text{half-life}}} \quad (\text{for } t = 1 \text{ d}) \]

\( N_0 = \text{initial number of molecules} \)
\( N = \text{number of molecules remaining} \)

the level of GSH-Px protein present at the steady state can be determined as follows:

\( \text{half-life} = 5.2 \text{ d} \)
(calculated from data in Fig. 4)

\( \text{rate of synthesis} = 0.0842 A_{405}/\text{d} \)
(calculated from data in Fig. 7)

\( 1 \text{ d of degradation} = N_0 - N = 0.0842 \)

thus \( 0.0842 = N_0 - N_0 e^{-0.693 \times \frac{1}{5.2}} \)
and \( N = 0.0842/(1 - e^{-0.693 \times \frac{1}{5.2}}) \)

\( N_0 = 0.675 A_{405} \)

This value is in good agreement to the experimental plateau level of GSH-Px protein (Fig. 7). Such plateaus in GSH-Px activity have also been observed by others (2,60,97). Thus in Se adequacy, Se is not
primary factor that regulates the level of GSH-Px.

How Se regulates the level of GSH-Px is not known. Saedi and coworkers (69 have recently shown that the level of GSH-Px mRNA decreases at the same rate as GSH-Px activity when rats are fed a diet deficient in Se. They suggest that GSH-Px mRNA in Se deficiency might be more susceptible to degradation if low levels of cellular Se results in premature termination of GSH-Px translation, leaving the GSH-Px mRNA unprotected by ribosomes. An experiment to compare the half-life of GSH-Px mRNA in Se-deficient rats to Se-adequate rats may answer this question. It is possible too that Se might in some way induce GSH-Px mRNA transcription, perhaps by means of positive transcription factors as have been proposed for regulation of metallothionein by copper (67).

In conclusion these studies have demonstrated that when Se is a limiting factor it controls the level of GSH-Px protein in rat liver. The mechanism by which Se does this remains to be determined.
APPENDIX A

ELISA AND IMMUNOBLOT PROCEDURES
ELISA PROCEDURE

Original reference:

Reference to cite in conjunction with original reference:

Reagents:
Carbonate buffer. (pH 9.6, 0.05 M)

\[ \text{Na}_2\text{CO}_3 \quad 1.59 \text{ g} \]
\[ \text{Na HCO}_3 \quad 2.93 \text{ g} \]

Total 1 l

pH should be 9.6 ± 0.1

Store solution at 4°C for not more than 2 weeks.
Phosphate buffered saline plus Tween (PBST) pH 7.5

NaCl 8.0 g
KH₂PO₄ 0.2 g
Na₂HPO₄ 1.15 g
KCl 0.2 g
Tween-20 0.5 ml

total 1 l

pH should be 7.5 ± 0.1

Blocking solution.
1 % (w/v) bovine serum albumin in PBST.

0.001 M ABTS-Citrate buffer.

ABTS [2-2' azino-di-(3-ethylbenzthiazoline sulfonic acid) 54.8 mg
citric acid 2.10 g

Make up to approx. 90 ml with DD H₂O.
pH to 4.2 with NaOH.
Bring to 100 ml with DD H₂O.
Store at -20°C for not more than 4 weeks.
Immediately prior to use add 10 ul 30 % H₂O₂ per 10 ml ABTS.

Stopping solution.
10 % (w/v) SDS in DD H₂O.
Procedure:

For this procedure 150 ul per well is used.

1. Dilute antigen solution in carbonate buffer, typically 1/10 to 1/100.

2. Place 150 ul of diluted antigen solution per well

3. Incubate 4°C overnight.

4. Wash once with PBST for 2 min.

5. Block for 30 min with BSA blocking solution.

6. Wash in 3 changes of PBST for 2 min each.

7. Incubate with anti-GSH-Px IgG diluted 1/100 in PBST for 2 h, room temp.

8. Wash, as step 6.

9. Incubate with anti-rabbit IgG linked horseradish peroxidase (HRP) diluted 1/1000 in PBST for 2 h, room temp.

10. Wash, as step 6.

11. Incubate with ABTS/H₂O₂ reagent for 10 to 15 min as appropriate.

12. Stop reaction with 40 ul SDS solution.

Notes. The top row of 8 wells on the ELISA plate are used as a blank as follows: at the end of the procedure 150 ul ABTS reagent without H₂O₂ is put into these wells and 40 ul SDS solution is added.

For controls the procedure is carried out as described but carbonate buffer without antigen is used in step 2.
IMMUNOBLOT PROCEDURE

Original reference:

Reference to cite in conjunction with original reference:

Reagents:

Transfer buffer

25 mM Tris-base 60.55 g
192 mM glycine 288.27 g
0.1% (w/v) SDS 20 g
20% (v/v) methanol 4 l

Total 20 l

pH should be 8.3 ± 0.1.

Tris-buffered saline (TBS) pH 7.5

20 mM Tris-base 4.84 g
500 mM NaCl 58.44 g

Bring to pH 7.5 with HCl

Total 2 l
Tris-buffered saline with Tween-20 (TBST) pH 7.5

20 mM Tris-base 4.84 g
500 mM NaCl 58.44 g
0.05 % (v/v) Tween-20 1 ml
Bring to pH 7.5 with HCl

Total 2 l

Blocking solution, 3 % (w/v) gelatin

Gelatin 3 g
TBST 100 ml, heat to about 55°C or microwave for approx 1 min until gelatin dissolves.

Antibody buffer, 1 % (w/v) gelatin

Gelatin 2 g
TBST 200 ml, heat as above.

Procedure:

Electrophoretic Transfer:

Carried out using Hoefer Transphor unit (TE 52). The unit is filled with precooled transfer buffer (approx. 4 l). Immediately after SDS-PAGE the gel is sandwiched against one nitrocellulose membrane (Hoefer TM-NC4-915, 0.45 micron pores, 9 x 15 cm) between filter paper and sponge in the cassette. The cassette is loaded into the tank with the nitrocellulose at the back, nearest the anode. Transfer is for 2 h at 1 amp (typically max voltage) with cooling at 10°C.

Usually 100 ug of liver cytosol protein is run on a gel for transfer, though this amount can be increased to improve sensitivity.
Immunostaining:

All steps are carried out at room temperature (24–25°C) with gentle agitation on a rotary shaker. The volumes given are for a 9 x 15 cm nitrocellulose membrane incubated in a 22 x 11 cm Pyrex dish. Immediately after 2 h of electrophoretic transfer the nitrocellulose membrane is treated as follows:

1. Mark position of gel on membrane.
2. Incubate in 200 ml TBS for 5 min.
3. Incubate in 100 ml blocking solution for 30 min.
4. Wash in 3 changes of 200 ml TBST for 5 min each.
5. Incubate with anti-GSH-Px IgG diluted 1/50 in 100 ml antibody buffer overnight.
6. Wash in 2 changes of 200 ml TBST for 5 min each.
7. Incubate with anti-rabbit IgG linked horseradish peroxidase (HRP) diluted 1/1000 in 100 ml antibody buffer for 4 h.
8. Wash in 2 changes of 200 ml TBST for 5 min each.
9. Wash in 3 changes of 200 ml TBS for 5 min each.
10. Incubate with HRP color development solution reagent (see Bio-Rad # 170-6534 sheet) until bands develop (no more than 45 min).
11. Rinse membrane in TBS, dry and photograph immediately.
REFERENCES


69. Saedi, M.S., Smith, C.G., Knight, S.A.B. & Sunde, R.A. Selenium deficiency rapidly decreases the mRNA, protein and activity of selenium dependent glutathione peroxidase in rat liver. (submitted for publication).


