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SUPPLEMENTAL REDUCED GLUTATHIONE,
VITAMIN E AND SELENIUM
FOR THE WEANLING PIG

presented by

Lora Lynn Foehr

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Animal Science

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SUPPLEMENTAL REDUCED GLUTATHIONE, VITAMIN E AND SELENIUM FOR THE WEANLING PIG

By

Lora Lynn Foehr

A THESIS

Submitted to
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ABSTRACT

SUPPLEMENTAL REDUCED GLUTATHIONE, VITAMIN E AND SELENIUM FOR THE WEANLING PIG

By

Lora Lynn Foehr

Thirty-two 28-day old weanling pigs were used to determine the influence of supplemental reduced glutathione, vitamin E and selenium on their biological antioxidant status as indicated by plasma concentrations of selenium, α-tocopherol, glutathione peroxidase, aspartate aminotransferase, alanine aminotransferase, and the concentration of reduced glutathione in whole blood. Pigs were weighed and blood samples taken initially and weekly throughout a 4-week study. Plasma α-tocopherol concentration was elevated in pigs fed supplemental vitamin E. Plasma selenium, glutathione peroxidase and whole blood reduced glutathione were elevated in pigs receiving supplemental selenium. Plasma alanine aminotransferase was slightly lower in pigs fed supplemental reduced glutathione. There is no firm evidence that reduced glutathione is a limiting factor in the function of the glutathione peroxidase system of the weanling pig.

ACKNOWLEDGEMENTS

Upon completion of my Masters of Science Degree I wish to express my deepest thanks to Dr. E.R. Miller. Without his patience and understanding I would not have finished this part of my education.

I would also like to thank the rest of my committee members, Dr. D.E. Ullrey, Dr. M.G. Hogberg and Dr. H.D. Stowe. I appreciate the time and advise each has given to me.

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Lastly, but perhaps most importantly, I wish to express my most sincere appreciation and love to my parents who have always been my biggest supporters. Thank-you!!

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I. INTRODUCTION

The swine industry is becoming increasingly more competitive. It is economically vital for producers to raise pigs at the least possible cost. A source of expense is pig mortality, with death loss at weaning constituting a major portion. Physiological factors affect pig viability at weaning (Kornegay, 1980). Three changes occuring at weaning which affect the pigs physiological status are environmental, immuno-competent and nutritional.

At weaning pigs are removed from their dams, possibly intermixed with other litters, and placed in a new pen. Weaning necessitates that pigs adapt to entirely new physical surroundings. A weaned pig is also no longer receiving antibodies from it's dam and, in addition, it's own immune system is functioning only minimally. Weaning also introduces a different diet. Prior to weaning, milk has been the major feed consumed by the pig, and the gastrointestinal environment is developed to digest milk quite efficiently. Cereal-based diets require different enzymes for efficient digestion, and these are not yet in great concentration at weaning. These major changes at weaning introduce stresses to the pig.

Newly weaned pigs characteristically respond to these stresses by going "off feed." Nutrient deficiencies

are therefore more likely to develop during this period. Mahan (1986) suggested that serum tocopherol and selenium concentrations are significantly lower in the early post weaning period. Michel et al. (1969) and Trapp et al. (1970) diagnosed and characterized vitamin E selenium deficiency in swine herds. Some of the characteristic deficiency signs were sudden death of weanling pigs, liver necrosis, skeletal and cardiac muscle degeneration and edema.

Researchers in Japan observed similar signs in yellowtail fish (Imada and Suga, 1986). Elevated serum enzymes, indicative of cellular damage, and hepatic disorder were characteristic of yellowtail fish with nutrient deficiency. In severely deficient fish, death resulted. Researchers at Kyowa Hakko, the parent company of Biokyowa, experimented with various compounds in an attempt to prevent the deficiency problem. Vitamin E was found to give some protection; however, another compound, reduced glutathione, was found to significantly lower certain serum enzyme activities and increase survival rate in yellowtail fish.

The purpose of the following study was to determine the effects of supplementing reduced glutathione, vitamin E and selenium to the diet of the weaning pig.

II. LITERATURE REVIEW

A. Biological Need for Antioxidants

1. Definition

Living organisms exist in a dynamic state. Within organisms the biochemical status is a continuum of anabolic and catabolic processes. In order for these processes to continue, energy is required. Highly regulated oxidative reactions are the ultimate source of energy for living organisms. The reduction of molecular oxygen to water is the final step in the process producing energy in the form of ATP. Intimately linked with biological oxidations is the process of electron flow and transfer. In addition to oxidation reactions being required to sustain life, they can generate products which are potentially harmful to the organism. Some of these products are free radicals (Table 1).

2. Free Radicals

Molecules which contain an unpaired electron in their outer orbital are considered to be free radicals (Slater et al., 1987). These molecules may also be produced by the impact and/or absorption of radiation. Metabolism of xenobiotics can also generate free radical products. The metabolism of t-butyl hydroperoxide by erythrocytes produces

TABLE 1. FORMATION OF FREE RADICALS

Formation	
(1)	By the impact or absorption of radiation, or both:
	(a) high energy or ionizing radiation
	(b) ultra-violet radiation
	(c) visible light with photosensitizers
	(d) thermal degradation of organic material
(2)	By electron transfer ('redox') reactions:
	(a) catalysed by transition metal ions
	(b) catalysed by enzymes

Modified from Slater et al. (1987)

t-butyl radicals (Trotta et al., 1983). The liver metabolizes carbon tetrachloride to trichloromethyl radical (Dianzani, 1987). Free radicals are usually very reactive, and are able to participate in numerous reactions such as: (1) electron donation, (2) electron acceptance, (3) hydrogen abstraction,(4) addition reactions, (5) self-annihilation reactions, and (6) disproportionations (Slater, 1984). A free radical's versatile and highly reactive nature make macromolecules such as proteins, lipids, carbohydrates and nucleotides susceptible to their action (Figure 1).

Molecular oxygen is a free radical containing not one but two unpaired electrons in its outer orbital. (Figure 2). Oxygen is ubiquitous and required for aerobic life; however, it is also potentially toxic (Haugaard, 1968) Both electrons in oxygen's outer orbital have parallel spins, therefore, for oxygen to combine with another molecule in a two-electron reduction, there must be two corresponding electrons with antiparallel spins. This is in accordance with the Pauli principle. Consequently, oxygen is slow to react with non-radical molecules, and is more apt to undergo one-electron reduction reactions. In a review by Halliwell and Gutteridge (1984), it was stated that molecular oxygen can become more reactive if its spin restriction is removed. Singlet state oxygen has had the spin restriction removed by a rearrangement of the electrons. Some biological pigments have the ability to capture the energy necessary to raise oxygen to a 'singlet state'. Singlet oxygen is electrophilic and capable of reacting directly with surrounding molecules,

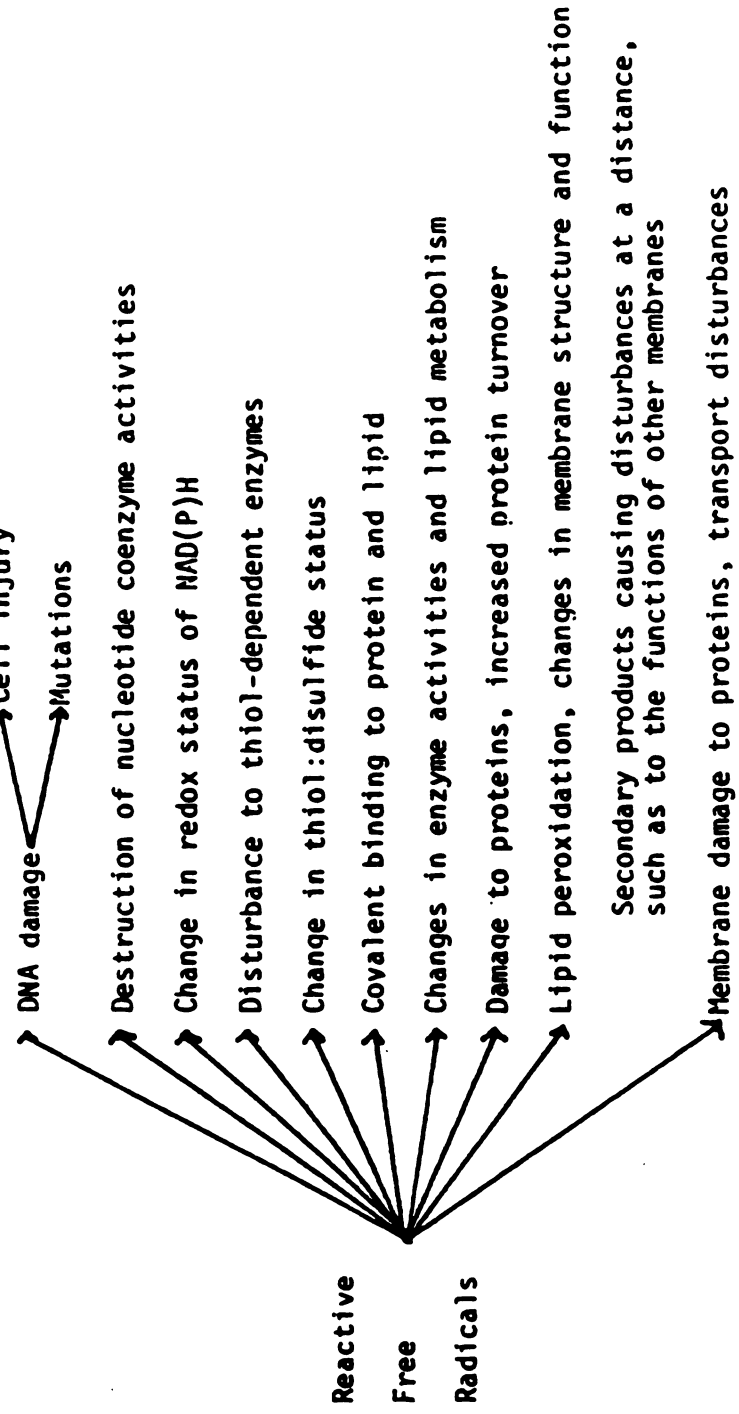


Figure 1. Possible damaging reactions of free radicals
Adapted from Slater et al. (1987).

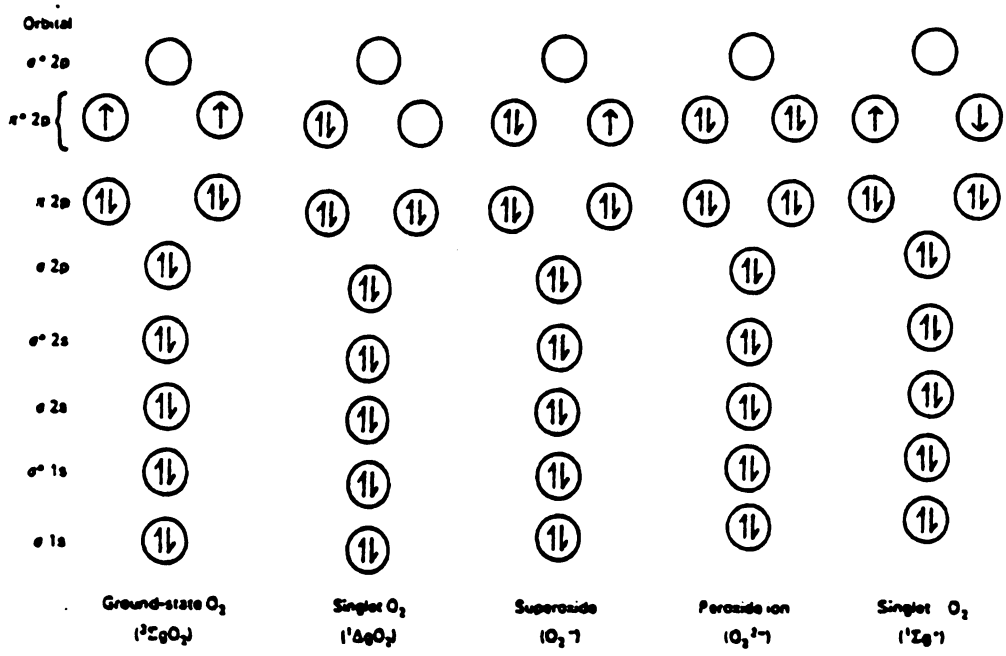


Figure 2. The diatomic oxygen molecule.
Taken from Halliwell and Gutteridge (1984).

or it may transfer its energy to another molecule causing it to become excited. Molecular oxygen's complete reduction requires the addition of four electrons. The intermediate reduction species in this process are known to be reactive and capable of damaging cellular processes. The first intermediate formed by the addition of a single electron is the superoxide radical (O_2-). According to Kanner (1987), activation of granulocytes, subcellular organelles and certain enzymes, the autoxidation of some molecules, and the oxidation of reduced metals are all reactions in which O_2- may be produced. Most body tissues and fluids have a pH in the range of 6.4-7.5, therefore, most O_2- remains in the unprotonated form. The protonated form (HO_2-) is a more powerful reducing and oxidizing agent than O_2-. The superoxide radical acts mainly as a nucleophile, and is therefore able to reduce surrounding compounds. The destruction of O_2- is usually through a dismutation reaction, where both protons and an enzyme catalyst are required. Superoxide dismutase (SOD) is the enzyme. The general dismutation reaction is as shown:

$$O_2^- + O_2^- + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

An electron addition to superoxide produces the peroxide ion, (O_2^{-2}),which has no unpaired electrons. Any O_2^{-2} formed at a physiological pH will protonate to yield hydrogen peroxide (H_2O_2). There are also other reactions and enzyme systems which produce H_2O_2 as a product without having the

O_2^{-2} as an intermediate (such as D-amino acid oxidase in the peroxisome). Human granulocytes are yet another source of H_2O_2, which is produced during the phagocytic function (Root et al., 1975). Hydrogen peroxide, like O_2^{2-}, is not a radical. Usually H_2O_2 acts as a weak oxidizing agent, and is able to penetrate membranes fairly rapidly due to its nonpolar properties. Its precursor, O_2^{-2}, is not as capable of penetrating membranes. Another toxic product of intermediary oxygen metabolism is the hydroxyl radical (OH·). Homolytic fission of H_2O_2 will produce two OH· radicals. Metals such as iron (Fe) and copper (Cu) are also able to generate the OH· radical from H_2O_2, as shown below:

(1) $Fe^{2+} + H_2O_2 \xrightarrow[Fe^{3+}]{} Fe^{3+} + OH^- + OH\cdot$ (Fenton)

(2) $O_2^- + H_2O_2 \longrightarrow OH^- + OH\cdot + O_2$ (Haber Weiss)

(3) $Cu^+ + H_2O \longrightarrow Cu^{2+} + OH^- + OH\cdot$

The reaction involving ferrous iron (Fe^{+2}) has been termed the Fenton Reaction, and that involving ferric, the Haber-Weiss Reaction. The superoxide radical and H_2O_2 are also thought to be capable of interacting without Fe^{3+} involvement to form water and the superoxide radical (Halliwell, 1985). The OH· radical is extremely reactive and undergoes three primary reactions: (1) hydrogen abstraction, (2) addition, and (3) electron transfer. Due to the highly reactive nature of the OH· it will affect molecules in the immediate vicinity of its production site.

Oxygen intermediates have been associated with various

cellular injuries and diseases. In a series of papers by Davies (Davies, 1987; Davies et al.,1987a,b; and Davies and Delsignore, 1987; and Davies et al., 1987) the disruption of various proteins by oxygen radicals was studied. These authors suggest, that proteins have an increased proteolytic susceptibility due to oxygen radicals (specifically $OH^{\cdot}$), causing an initial alteration of the primary protein structure. Alterations at the primary level lead to modifications of secondary and tertiary structures causing the peptide to unfold. Unfolding exposes previously "hidden" peptide bonds to protease hydrolysis.

The hydroxyl radical was produced when oxyhemoglobin and methemoglobin were in the presence of H_2O_2 (Puppo and Halliwell, 1988) as well as other uncharacterized radical species. The authors suggest that H_2O_2 decomposed the heme moiety of methemoglobin causing the release of iron. The $OH^{\cdot}$ was then produced via an iron-H_2O_2 interaction, as in the Fenton reaction. Oxyhemoglobin, in the presence of H_2O_2, also produced a reactive species, however, it did not appear to be $OH^{\cdot}$. The ability of free hemoglobin to produce radicals in the presence of H_2O_2 may provide an explanation of hemoglobin toxicity.

Nohl and Jordan (1987) performed an <u>in vitro</u> experiment incubating semiquinones with H_2O_2. They found that these radicals were capable of combining with H_2O_2 in a manner similar to the Haber-Weiss reaction. From this information, it appears that quinones normally involved in physiological processes may also play a role in catalyzing

the production of free radicals. Hydrogen peroxide is thought to act on the adenylate cyclase system of the β-adrenoceptor system, as well as to initiate lipid peroxidation. β-adrenoceptor hyperstimulation and excessive radical formation have been linked with cardiotoxicity. Haenen et al. (1988) incubated calf heart membranes with H_2O_2 to monitor its influence on the β-adrenoceptor system. Hydrogen peroxide caused and increase in lipid peroxidation and a reduction in adenylate cyclase activity. Catecholamines, capable of autoxidation, may induce their myocardial toxicity through the production of H_2O_2.

3. Lipid Peroxidation

Lipid peroxidation, according to Pole and coworkers (1987), is a degradative process which is a consequence of the production and propagation of free radical reactions, primarily involving membrane polyunsaturated fatty acids (PUFAs). Girotti (1985) defined lipid peroxidation as the process of formation and breakdown of lipid hydroperoxides which are dioxygen adducts of unsaturated lipids.

Lipid peroxidation has been associated with various cellular disorders, however; lipid peroxidation's role as a cause or effect of cell damage is uncertain. Some of the cellular disorders with which lipid peroxidation has been associated are: (1) membrane bilayer structural derangement and altered fluidity, (2) increased permeability of cytosolic constituents, (3) lysosomal enzyme release, (4) inactivation of intrinsic enzymes and transporters, (5) cross-linking of

lipids and proteins, (6) polypeptide strand scission, (7) DNA damage and mutagenesis, and (8) depletion of NADPH due to antioxidant activity. Ivanov (1985) characterized some of the effects observed with lipid peroxidation as changes in the essential properties of membranes, such as permeability, viscocity, phase transitions, or as a change in lipid-protein interactions resulting in a potential loss of the enzyme activities of the membrane-bound and soluble systems. Haenen et al. (1988) found lipid peroxidation to occur in the calf heart membranes used in their experiment. Increased lipid peroxidation has been observed with some oxidatively stressful conditions and injuries. Davies et al. (1982) found a greater concentration of lipid peroxides in exercised rats than in sedentary controls. An earlier study by Brady and coworkers (1979) also showed an increase in lipid peroxidation products in exercised rats.

Initiation of lipid peroxidation may or may not be enzymatically catalyzed. In a study using rat lysosomes, Fong et al. (1973) looked at how flavin enzymes affected membrane lipids. They observed increased lipid peroxidation of the membrane. They concluded that lysozyme enzymes generated O_2^{-2}, which became protonated to H_2O_2. Hydrogen peroxide, in the presence of iron, produced the free radical $OH^{\cdot}$ in a Fenton reaction. The initiating event of lipid peroxidation is the interaction of a free radical with an unsaturated fatty acid causing a hydrogen to be abstracted from the lipid. The resulting alkyl radical ($L^{\cdot}$) of the

fatty acid undergoes an intramolecular rearrangement forming a more stable conjugated diene. Molecular oxygen is able to add on to this alkyl radical forming the peroxyl radical (LOO·). The LOO· is then able to abstract a hydrogen from another PUFA, generating lipid hydroperoxide (LOOH) and another alkyl radical. It is obvious that the effects of a single free radical initiation reaction may be greatly amplified, especially in oxygen and unsaturated lipid environments. Several termination reactions of lipid peroxidation are known to occur, some of which are listed below:

(1) 2 L· ------> LL
(2) 2 LOO· ------> LOOL + O_2
(3) L· + LOO· ---> LOOL
(4) RH + L· -----> R· + LH
(5) RH + LOO· ---> R· + LOOH

4. Antioxidation

In order for organisms to survive they must have defense mechanisms to regulate the production and destruction of free radicals. Some molecules which are thought to have a role in antioxidation reactions are ascorbic acid, β-carotene, α-tocopherol, and glutathione (Slater et al., 1987; Kanner et al., 1987). Usually, a good antioxidant must be an effective hydrogen or electron donor. Enzymes are also involved with antioxidation. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-px) are the three main antioxidant enzymes. Two forms of SOD exist, a cytosolic form containing copper and zinc, and a mitochondrial form containing manganese. SOD catalyzes O_2^{-2}

dismutation. As shown below, CAT is involved in the destruction of H_2O_2, and is located within the peroxisomes.

$$H_2O_2 + H_2O_2 \xrightarrow{CAT} O_2 + 2\ H_2O$$

Glutathione peroxidase, located both in the cytosol and mitochonddria, also catalyzes the destruction of H_2O_2, but is capable of destroying other peroxides as well. According to Simmons and Jamall (1988), GSH-px has a more important role than CAT in controlling peroxidation products. In its reduction reaction with peroxides, GSH-px utilizes reduced glutathione (GSH) as its electron donor substrate. Reed et al. (1986) found GSH-px activity to be critical in the prevention of damaging effects caused by cellular bioreduction reactions.

B. Glutathione

1. Definition

The substrate, glutathione (GSH), is widely distributed in animal and plant cells as well as in microorganisms. It is probably the most abundant low-molecular-weight thiol, as well as perhaps the most important nonprotein thiol in living systems. Reduced gluthathione is composed of three amino acids: glutamate, cysteine and glycine (Figure 3). Reduced gluthathione has two characteristic structural features: a sulfhydryl (SH) group on the cysteine moiety, and a δ-glutamyl linkage between the glutamate and cysteine residues (Meister and Tate, 1976).

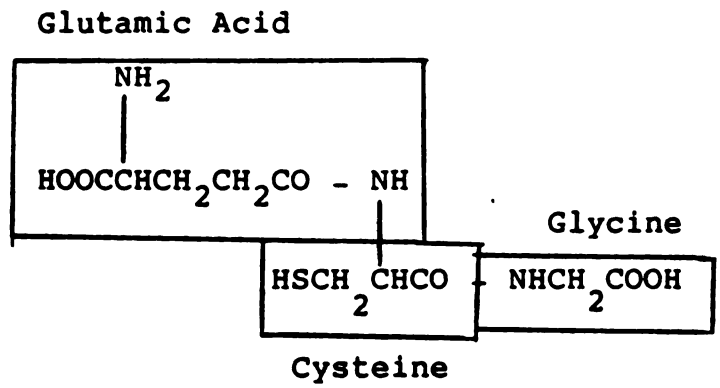


Figure 3. Reduced Glutathione.

The glycine moiety protects GSH from the degradative action of δ-glutamyl cyclotransferase, and the δ-glutamyl linkage of GSH renders it nonsusceptible to peptidases which cleave α-bonds. The δ-glutamyl bond may be degraded by the enzyme δ-glutamyl transpeptidase (δ-GT). δ-GT appears to be mainly a membrane bound enzyme, and it hydrolyzes GSH into δ-glutamyl and cysteinyl-glycine. Once this occurs, peptidase enzymes are able to break the cysteine-glycine bond.

2. δ-Glutamyl Cycle

Gamma-glutamyl transpeptidase action is the first step in GSH breakdown. The biodegradation and biosynthesis of GSH is cyclical in nature and involves enzyme-catatyzed reactions. This "cycle," described by Meister (1981) and Meister and Tate (1976), is referred to as the δ-Glutamyl cycle (Figure 4). GSH concentration determines the activity of δ-GC by nonallosteric feedback inhibition, therefore GSH acts as its own regulator. The intracellular concentration of GSH ranges from $1\text{-}50\times10^{-4}$ M (Kosower and Kosower, 1976), and it may exist in heterogeneous pools (Tateishi et al., 1977). Reduced glutathione, through the action of GSH-px, becomes oxidized to yield oxidized glutathione (GSSG). Oxidized glutathione can be found in cells, but it is usually at a much lower concentration than GSH ($6\text{-}200\times10^{-6}$M). The ratio of GSH to GSSG has been used as an indicator of oxidant stress (McCoy et al., 1988). In a study utilizing exercised verses sedentary control rats, Lew and coworkers (1985) observed decreased GSH and increased GSSG in exercised rat

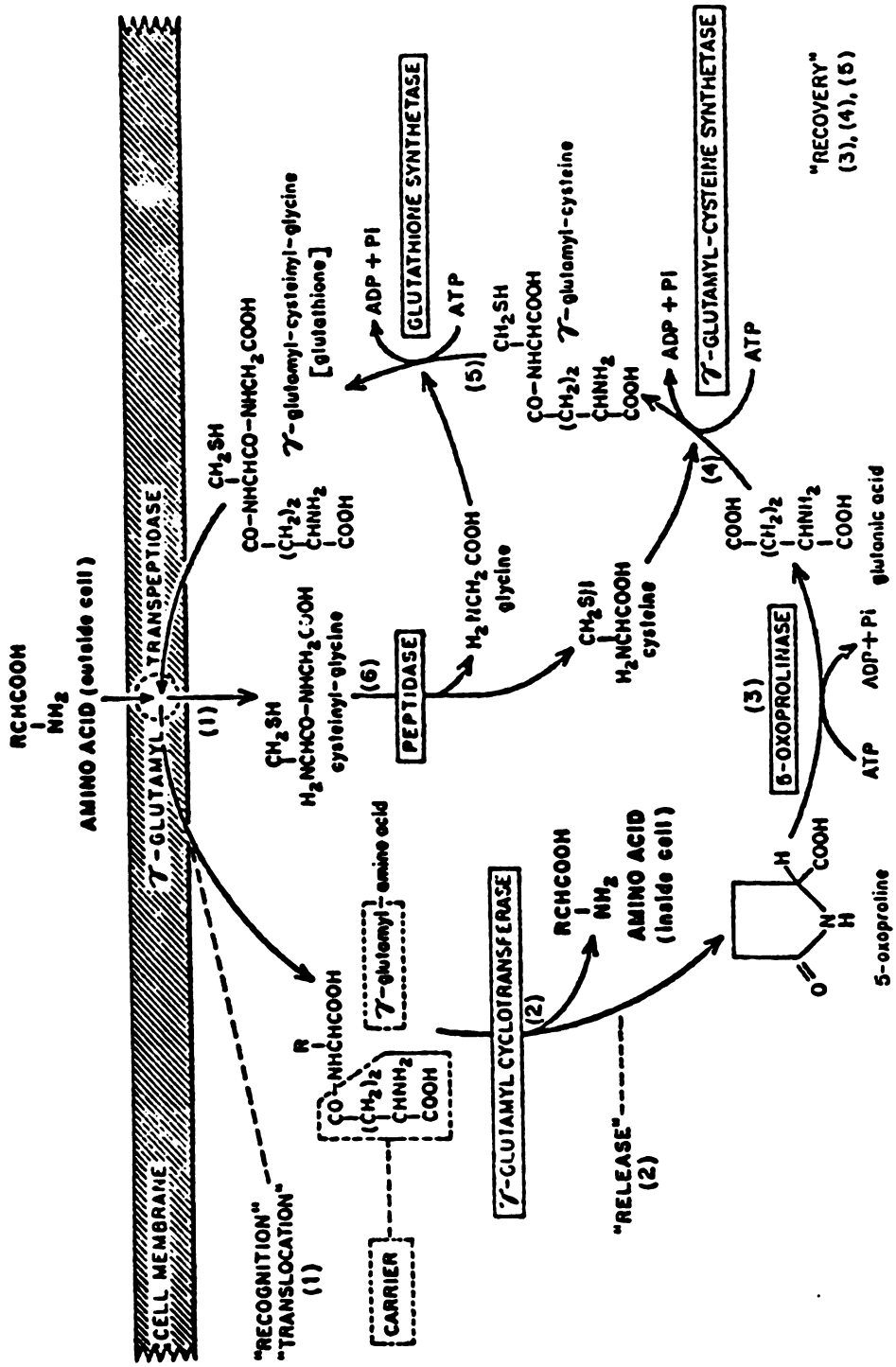


Figure 4. The gamma-Glutamyl cycle.
Taken from Meister (1973).

tissues. This effect on the redox status of glutathione is associated with oxidative stress (Figure 5).

The enzyme glutathione reductase (GR) catalyzes the NADPH-dependent reduction of GSSG to GSH (Beutler, 1975) (Figure 6).

$$GSSG + NADPH \xrightarrow{GR} 2\ GSH + NADP^{+}$$

GSH may also form mixed disulfides with cellular proteins. The structural integrity and maintenance of the functional processes of cell membranes and organelles are very important. According to Kosower and Kosower (1978), GSH, GSSG and GSS-protein are associated with numerous aspects of normal cellular structural maintenance and functional processes.

3. Redox Importance

Olafsdottir and Reed (1987) observed that rat liver mitochondrial oxygen consumption leads to H_2O_2 production, and this in turn may cause lipid hydroperoxide development. Paller and Sikora (1988) estimated that from 1 to 10% of the oxygen in the respiratory chain is not entirely reduced. Reduced glutathione is used by GSH-px to reduce this oxygen, however, the concurrent oxidation of GSH has been shown to coincide with an increase in Ca^{+2} permeability of the inner mitochondrial membrane. GSH oxidation may therefore decrease a cell's ability to reduce important protein thiol groups involved with membrane function. Prasad et al. (1986) suggested that any toxic process which increases the free

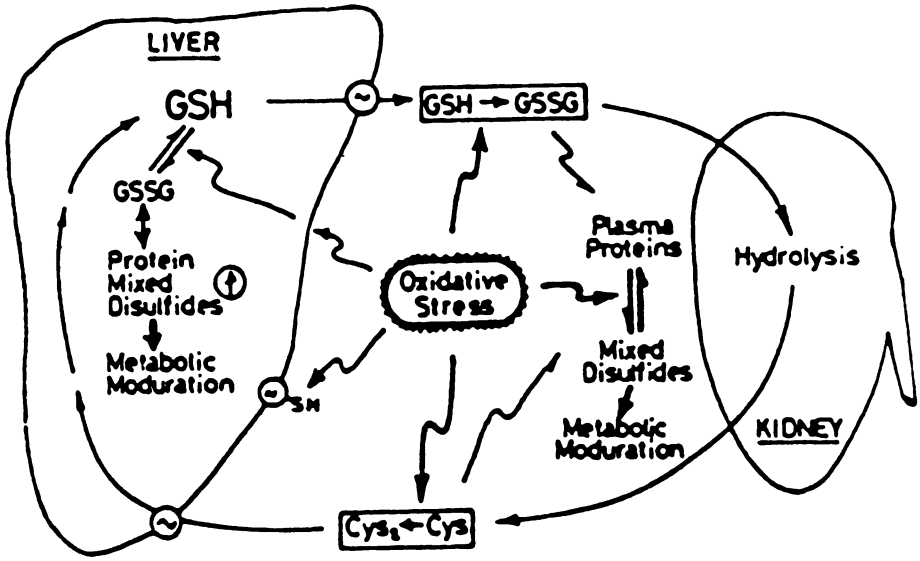


Figure 5. Metabolic moduration of redox states in proteins. Taken from Inoue et al. (1987).

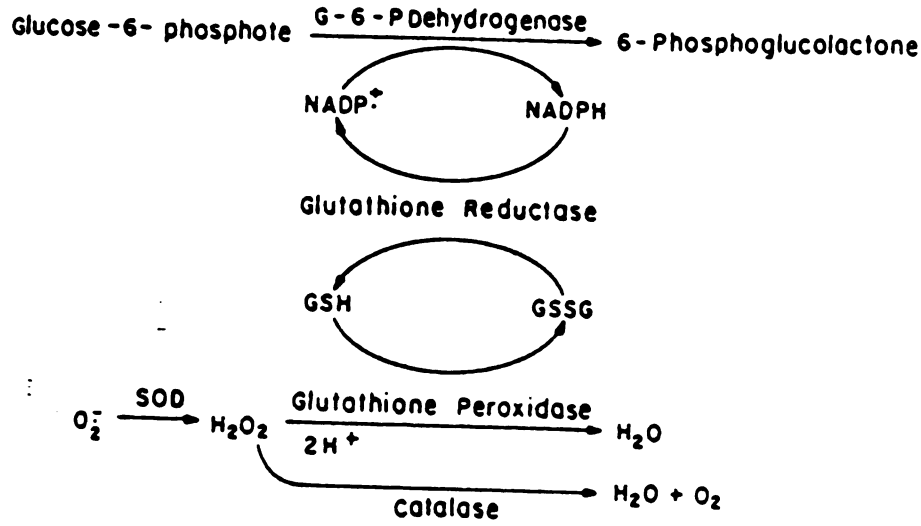


Figure 6. Action of glutathione reductase.
Taken from Kanner et al. (1987).

calcium concentration by inactivating either microsomal or mitochondrial pumps for Ca^{+2} may lead to significant cellular damage. Inactivation is usually via oxidation of protein sulfhydryls resulting in disulfide formation. GSH appears to reverse these effects (Erickson et al., 1987; Reed et al., 1987). Jones et al. (1983) also reported that thiols (GSH) may protect the Ca^{2+} sequestering system of the endoplasmic reticulum.

Comporti (1987) observed that certain xenobiotics exert their toxicity by causing decreases in GSH, resulting in the subsequent disulfide formation of vital sulfhydryls. Teaf and coworkers (1987) looked at male reproductive tissue in rats, and observed that protein alkylation, particularly of the cysteine-rich protamines, resulted in toxic effects on spermatozoa. Disulfide crosslinks within protamines may be one reason spermatozoa have an extraordinary resistance to both chemical and mechanical insult. Reduced glutathione status appears to be involved in the resistance. Reduced glutathione status may also play an important role in the process of fertilization. It was shown, in a study by Perreault et al. (1987), that disulfide bonds in sperm have to be reduced in order for the nucleus to become reactivated. They concluded that mature hamster oocytes have more GSH than their immature precursors or those oocytes already fertilized (Figure 7). Mature oocytes have the capability to reduce the protamine disulfides via GSH, thereby initiating nuclear decondensing activity. Kuross and associates (1988) hypothesized that thiol oxidation was a potential explanation

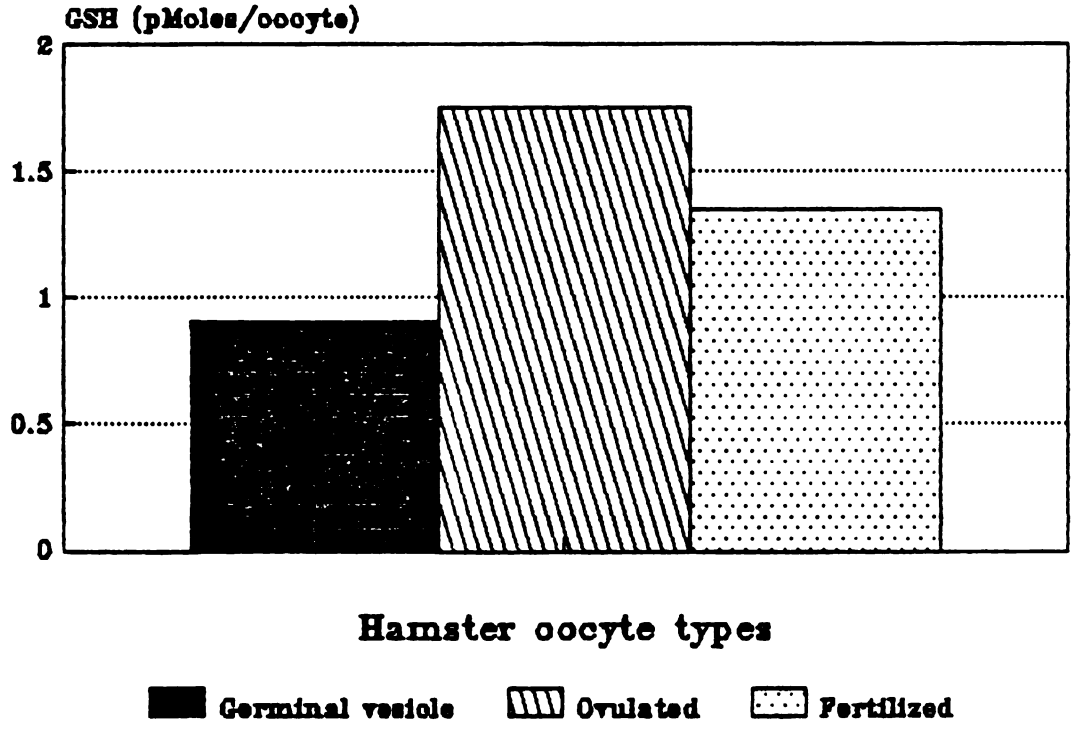


Figure 7. Levels of GSH in hamster oocytes. Adapted from Perreault et al. (1988).

for some of the defects seen in sickle red blood cells (RBC). Garel et al. (1986) also worked with sickle RBC's, and they found GSH to be beneficial in reducing the severity of sickling symptoms. Reduced glutathione combined with hemoglobin to form a mixed disulfide; this resulted in increased oxygen affinity and a decrease in the incidence of sickled RBC's.

Increasing the intracellular GSH concentration may have beneficial effects on cells. Mitchell (1988) suggested that the amount of intracellular GSH influences the cytotoxicity of cancer treatment modalities. Reduced glutathione has such functional diversity and assumes such a pivotal role in numerous bioreductive reactions that GSH modulation could obviously have an impact on the entire system (Figs. 8, 9 and 10).

Arrick and coworkers (1982) observed an increased sensitivity of GSH-depleted tumor cells to oxidative cytolysis. Modulation of GSH is known to affect other processes as well (Figure 10). Fidelus et al. (1987) suggested that GSH plays an important role in lymphocyte activation and proliferation. Honda and Matsuo (1988) found that increasing the cellular GSH level in human diploid fibroblasts extended their in vitro life span. These cells are known to have a finite, replicative life span, and are used as a model system to study aging at the cellular level. Jensen and Meister (1983) found human lymphoid cells depleted of GSH to be more radiosensitive. In looking at the lens of

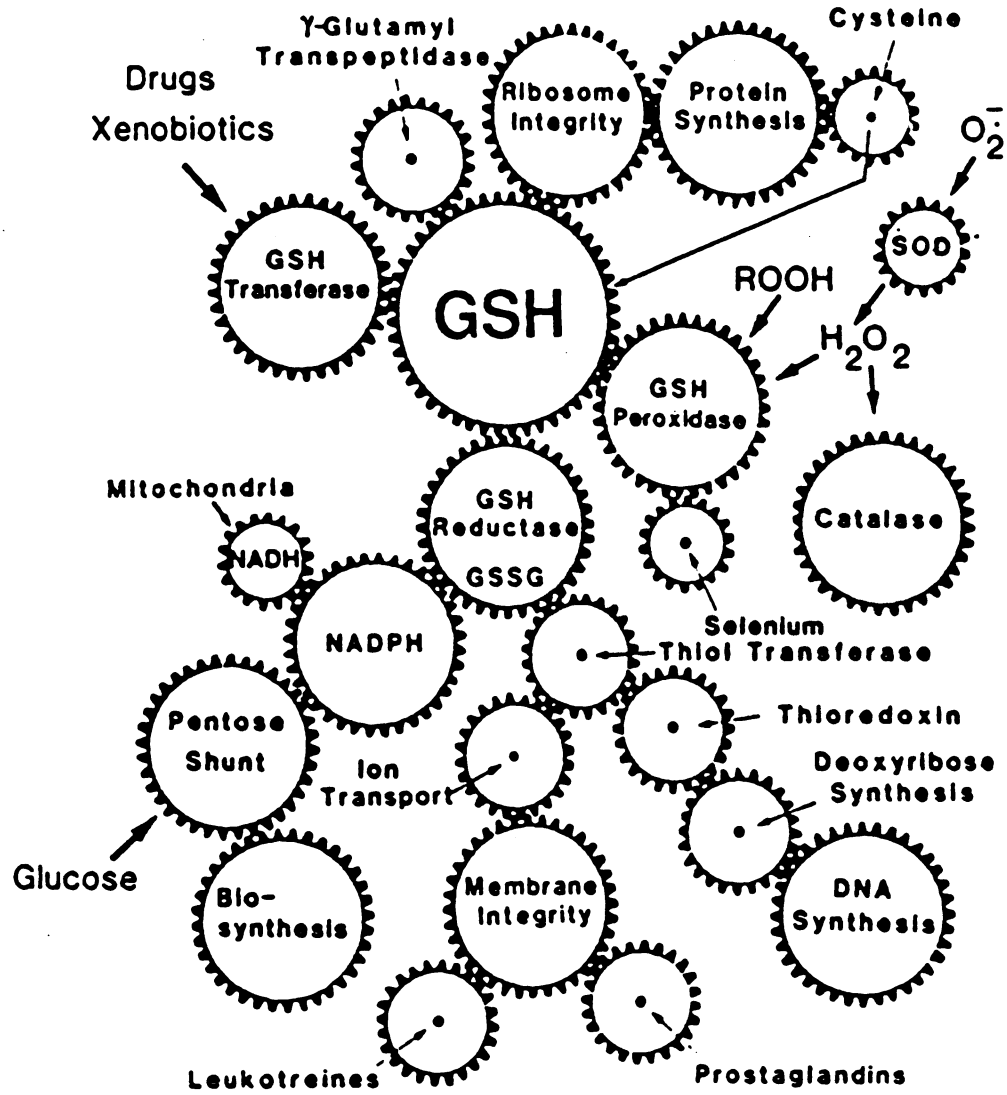


Figure 8. The interrelationships of GSH.
Taken from Mitchell (1988).

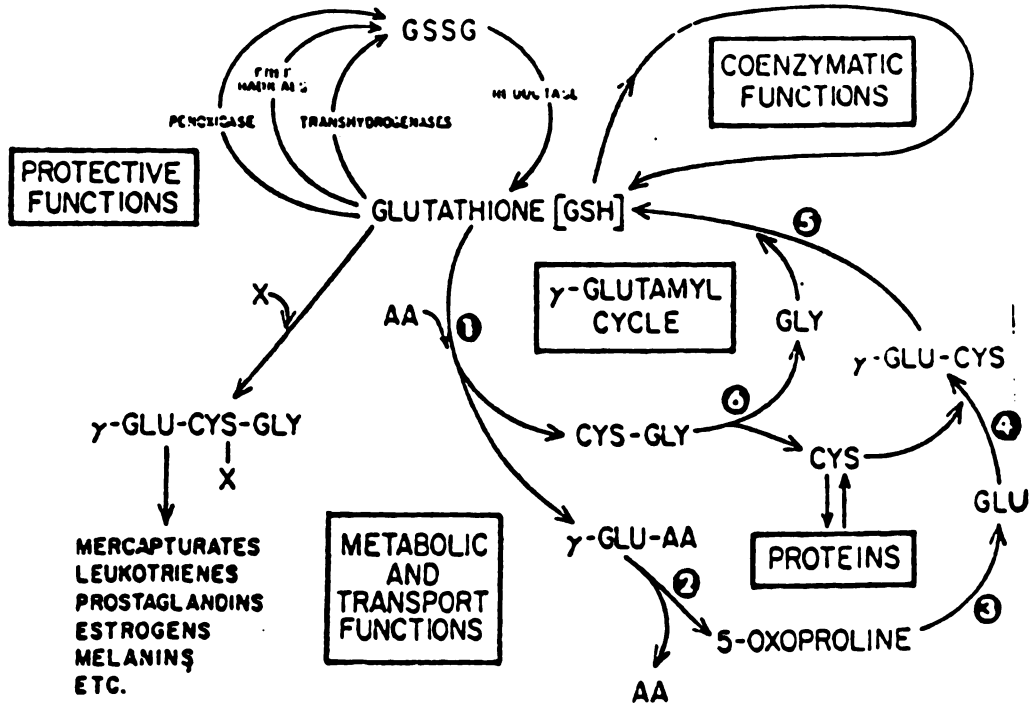


Figure 9. GSH metabolism and function. 1=gamma-glutamy transaminase, 2=gamma-glutamyl cyclotransferase, 3=5-oxoprolinase, 4=gamma-glutamyl cysteine synthetase, 5=GSH synthetase, 6=dipeptidase. Taken from Meister (1981).

Level	Class of effect		
	Structural	Functional	Pathological
Organism	Development and growth[b] Membrane and cellular integrity[c]	Feeding response[e] Management of drugs and other endogenous compounds[f]	Anemia[d] Neurological disease[e] Cataract[h]
Physiological		Muscle contraction[i] Neurotransmitter release[e] Detoxification[f]	Radiation syndromes[i] Hemolysis[d] Lens opacity[h] Altered cell proliferation[i] Cell necrosis
Biochemical and molecular	DNA and RNA synthesis[i] Protein synthesis[c] Unsaturated lipids[c]	Actomyosin[i] Neurosteroin[e] Conjugation reactions[f]	Lipid peroxidation and denaturation of protein[c,d] Ion transport[e] Macromolecular reactions with free radicals[i]

Figure 10. Possible effects of GSH-GSSG disturbance. Taken from Kosower and Kosower (1976).

diabetic or galactosemic rats, Lou et al. (1988) found a substantial depletion in GSH concentration. A major factor involved in cataract formation is oxidative insult whereby GSH may play a key reducing role. In another study, Spector et al. (1987) found that increasing the GSH level above normal did not enhance the cell's (lens) overall ability to withstand or recover from oxidative damage induced by H_2O_2.

4. Metabolism

Prior to manipulating GSH content, its' metabolism should be understood. Cornell and Meister (1975) looked at crypt and villus tip cells of rat jejunal mucosa. Villus tip cells had a much higher δ-GT activity and a lower GSH content than crypt cells. Linder and coworkers (1984) used brush border membranes from pig jejunum to study GSH uptake. Their data appear to indicate that GSH is primarily transported intact into an intravesicular space across pig intestinal brush border membrane. This process functions against a concentration gradient and is Na^+-dependent. Lash et al. (1986) have found a Na^+-dependent GSH uptake system present on the basolateral membrane of rat intestinal epithelial cells. Intestinal cells therefore have the ability to utilize plasma GSH. This transport system has characteristics similar to that found in the kidney (Lash and Jones, 1983; Lash and Jones, 1984) (Figure 11).

Reduced glutathione is continually turning over within the body. Reduced glutathione is synthesized intracellularly, but it can be translocated. According to

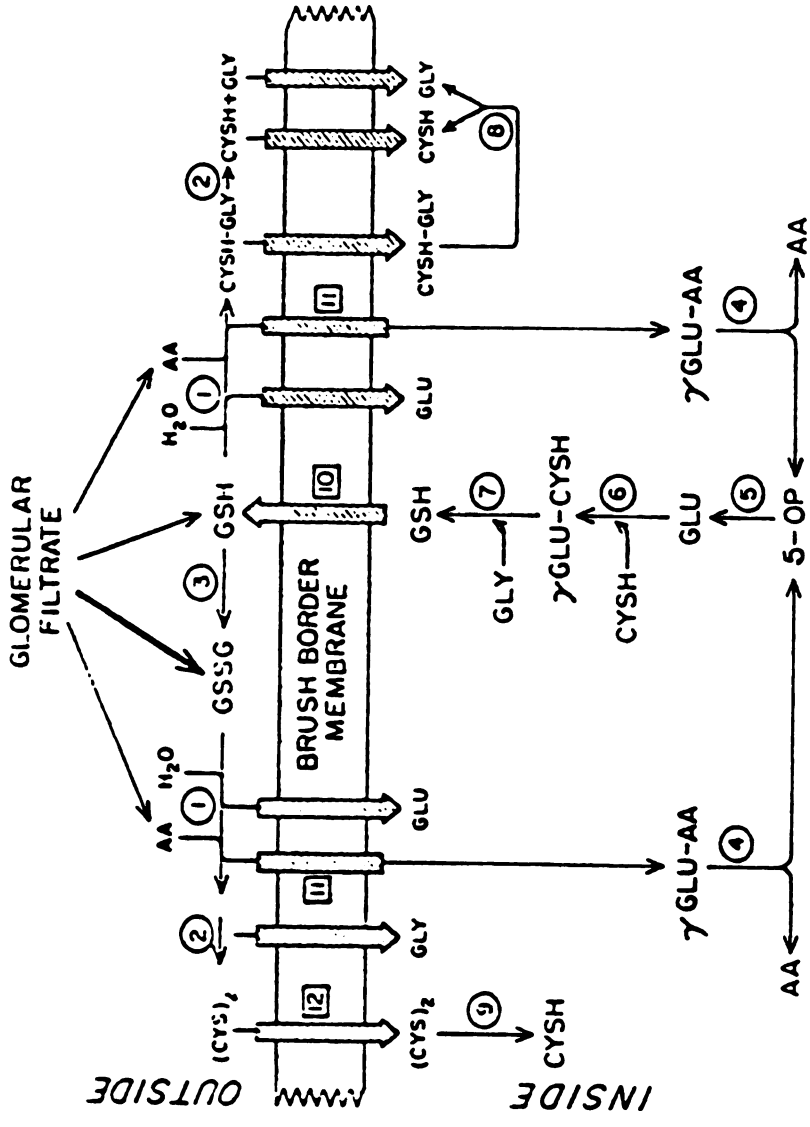


Figure 11. GSH metabolism. 1 = gamma-GT, 2 = dipeptidase, 3 = GSH oxidase, 4 = gamma-glutamyl cyclotransferase, 5 = 5-oxoprolinase, 6 = gamma-glutamylcysteine synthetase, 7 = GSH synthetase, 8 = dipeptidase. Taken from Griffith and Meister (1979).

Griffith and Meister (1979), the highest GSH turnover is in kidney, followed by liver, pancreas, and skeletal muscle (Figure 12). Cells with appreciable δ-GT activity tend to utilize plasma GSH while cells with low δ-GT tend to be suppliers of plasma GSH. The kidney has very high δ-GT activity, and it removes approximately 67% of the plasma GSH. Most of the plasma GSH is synthesized by the liver (Lauterburg and Smith, 1986). Plasma GSH itself has a rapid turnover rate; however, red blood cells have a low GSH turnover rate. Oxidized glutathione also appears to be translocated out of cells (Srivastava and Beutler, 1969; Prchal et al., 1975; Lunn et al., 1979; and Kondo et al., 1987).

The turnover of GSH may be involved in the transport of amino acids. Evidence supporting this hypothesis include: (1) GSH is synthesized intracellularly, (2) δ-GT, the initial degradative enzyme, is found on the exterior membrane surface, (3) cleavage of GSH by δ-GT yields cysteinyl-glycine and δ-glutamyl. Gamma-glutamyl is combined with an extracellular free amino acid through the continuing catalytic action of δ-GT, and the δ-glutamyl-amino acid becomes immediately interiorized. Gamma-glutamyl transpeptidase activity tends to be very high at sites of amino acid absorption. Looking at rat placenta, Baumrucker and Stover (1987) observed that δ-GT activity increased coincidentally with the period of greatest tissue growth. This increase may be in response to greater protein synthesis occurring at this time. Ross et al. (1973)

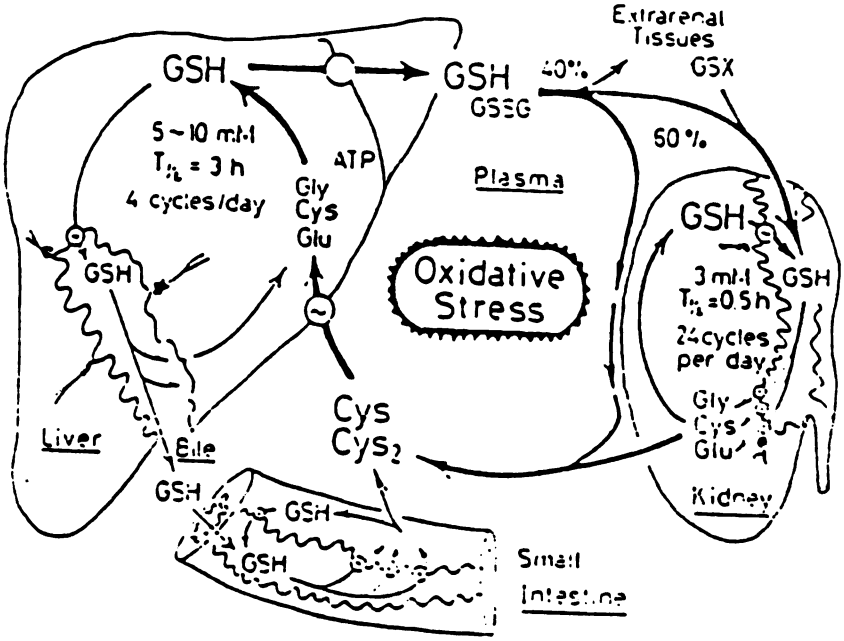


Figure 12. Inter- and intraorgan metabolism and transport of GSH. Taken from Inoue et al. (1986).

concluded that high δ-GT activity on the basal portion of the epithelial cells of the ciliary body might be the amino acid transporter to the aqueous humor. Meister (1973) and Orlowski and Meister (1970) support this theory of amino acid transport as well.

Reduced glutathione appears to be involved with numerous biological processes, such as: (1) maintaining the proper redox status, (2) maintaining proper thiol status, (3) drug metabolism, (4) protein and DNA synthesis, (5) acting as a coenzyme for glutathione S-transferase, and (6) serving as a substrate for the enzyme GSH-px. Glutathione peroxidase is a selenoenzyme (Rotruck et al., 1973). Flohe et al. (1973) found GSH-px to contain about four gram atoms of selenium per mole. Glutathione peroxidase is comprised of four identical subunits, and has an approximate molecular weight of 80,000. This weight differs from species to species and tissue to tissue (Sunde et al., 1980). Glutathione peroxidase is located in the cytosol and mitochondrial matrix, and it catalyzes the reduction of peroxides (Figure 13). Glutathione peroxidase functions occur in a series of bimolecular steps. The first step involves oxidation of the enzyme's active site by the peroxide substrate with a release of the corresponding alcohol. This step is followed by two GSH additions and the release of GSSG:

$$2\ GSH + ROOH \xrightarrow{GSH\text{-}px} GSSG + ROH + H_2O$$

Jorgensen and Wegger (1979), as well as Jorgensen et al.

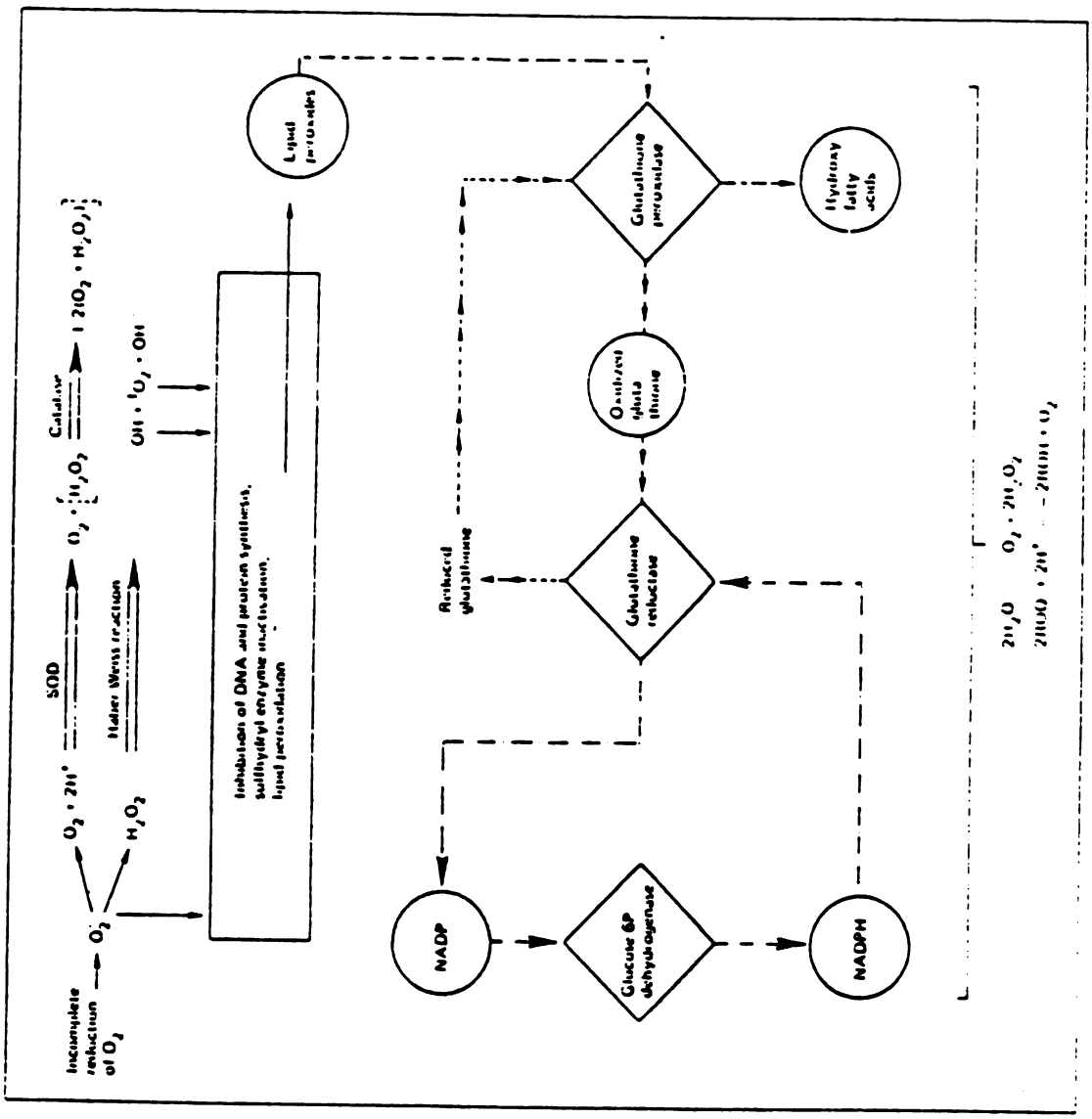


Figure 13. Mechanisms for antioxidative defense. Taken from Das et al. (1987).

(1977), observed that pigs with higher GSH-px activity were less susceptible to disease than pigs having low GSH-px activity. Friendship and Wilson (1985) found a high degree of variability in the blood GSH-px levels among pig litters. They also found that GSH-px concentrations in one-day old pigs were not associated with body weight, but were, however, weakly correlated with piglet viability. Neonatal pigs with higher GSH-px concentrations may have a greater survivability rate. Mills (1959) suggested that GSH-px protects cells from the deleterous effects of H_2O_2. Flohe and Zimmermann (1970) found GSH-px to be an essential factor in preventing lipid peroxide accumulation and lysis of rat mitochondrial membranes in vitro. McCay (1976) proposed that GSH-px exerted its affect by preventing the initial attack on polyunsaturated membrane lipids by free radicals. When GSH becomes oxidized by GSH-px activity it may be regenerated through the reducing action of glutathione reductase (GR). GR is a riboflavin-containing flavin adenine dinucleotide enzyme. Pigs on a riboflavin-deficient diet exhibited a reduced amount of active GR within red blood cells (Brady et al., 1979). Glutathione reductase activity in muscle and liver was unaltered by riboflavin deficiency. Reduced glutathione within these cells was not found to be significantly affected. Paniker et al. (1970) also looked at GR in riboflavin deficient red blood cells. Their results were similar to Brady and colleagues (1979) in that GR did not seem to be a limiting factor in GSH production.

C. Vitamin E

1. Definition

Vitamin E is a fat-soluble vitamin. Compounds which exhibit vitamin E activity have a 6-chromonal ring structure with an associated side chain. Side chain structure separates vitamin E into two main types, the tocols and the trienols. Tocols have phytol as a side chain whereas trienols contain three double bonds in the side chain. Methyl groups on the chromonol ring differentiate various tocol and trienol isomers. Alpha-Tocopherol (all rac) is the most active form of vitamin E, and it has methyl groups at carbons 5, 7 and 8 (Figure 14). Alpha-tocopherol is practically insoluble in water; however, it is highly soluble in organic solvents. Orally ingested tocopherol or its esters appear to be absorbed only at low levels (20-40%) (Machlin, 1984). The NRC (1988) recommends approximately 15 IU/kg feed for weanling swine to prevent deficiency signs. Vitamin E is absorbed as part of the lipid-bile micelle along with free fatty acids, monoglycerides, and other fat-soluble vitamins. From the intestine, chylomicrons are transported to the lymphatic capillaries. The vitamin E is then transported throughout the circulatory system. Erythrocytes also transport vitamin E, with all of it being located in the membrane. Most tissue vitamin E is associated with membranes. There is no primary tissue storage site for vitamin E; however adipose, liver and muscle tissue have significant concentrations (Bieri, 1987). Mobilization of vitamin E can be either rapid or slow depending upon the

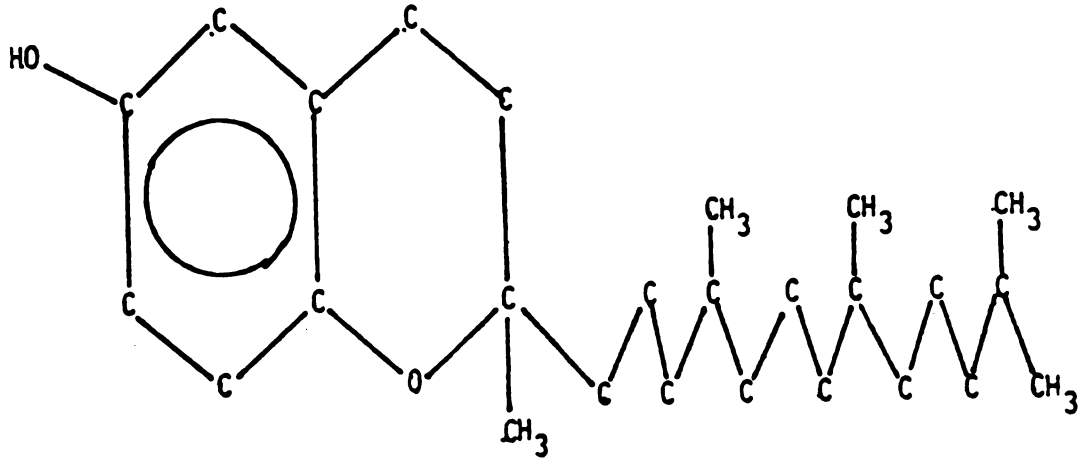


Figure 14. Alpha-tocopherol (5,7,8-trimethyltocol).

storage site involved.

2. General Functions

Vitamin E has been shown to have a role in immune function. Nikbin and coworkers (1986) looked at the splenocyte response in 3- and 24-month old mice supplemented with vitamin E for six weeks. They observed a mitogenic response of the splenocytes which may implicate vitamin E as being immuno-stimulatory. Prostaglandin (PGE_2) was also measured in this study due to its known inhibitory effect on lymphocyte proliferation. Prostaglandin levels were decreased in the 24-month old supplemented mice versus 24-month old controls. Vitamin E has also been found to decrease PGE_2 synthesis, and this most likely explains the results observed. In a study by Peplowski et al. (1981) the effects of dietary vitamin E were examined in weanling swine antigenically challenged with sheep red blood cells. Vitamin E elevated hemagglutination titers which implicated it as an immuno-stimulant.

3. Antioxidation

Vitamin E is thought to play a critical role in antioxidation functioning as a scavenger of reactive molecular species. Coquette et al. (1986) studied macrophages <u>in vitro</u> and showed that oxidative stress to the cells resulted in a 40% decrease in the intracellular tocopherol level. Though increasing the oxidative insult, these workers were unable to oxidize the tocopherol

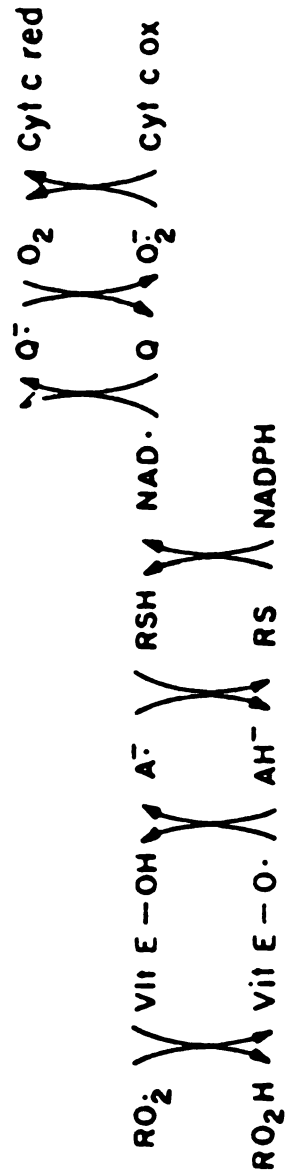


Figure 15. Vitamin E reduction.
Taken from Kanner et al. (1987).

completely. A redox cycle may exist to maintain tocopherol in its active form (Figure 15).

Yamamoto and coworkers (1986) looked at vitamin E-deficient and sufficient red blood cells exposed to an oxidative stress. A greater percentage of vitamin E-deficient cells were seen to undergo hemolysis. Hemolysis appears to occur when oxidative reactions overwhelm the cell's ability to counterbalance them. Vitamin E, in those cells having a sufficient concentration, was utilized to scavenge peroxy radicals ($AO_2^{\bullet}$) and lipid peroxy radicals ($LO^{\bullet}$) which were the primary oxidation products. Lipid and protein oxidation, though significantly less, still occurred in cells with 'sufficient' vitamin E. Meydani and associates (1988) studied the effects of vitamin E and selenium deficiency in brain tissue. Chronic vitamin E deficiency signs in neurological disorders include axonal swelling and dystrophy, demyelination of nerves, increased oxidative enzyme activity, and neuronal pigment accumulation. Brain tissue is particularly susceptible to the activity of free radicals due to its high rate of oxygen consumption and high phospholipid content with PUFAs. Results of the Meydani study showed that brain regions respond to dietary vitamin E uniquely. Regions with greater α-tocopherol concentration had decreased lipid peroxidation. Amemiya (1987) investigated muscular changes in response to vitamin E deficiency,and observed that vitamin E deficiency caused both endothelial and mitochondrial degeneration. The degeneration may be a result of peroxidation due to insufficient vitamin E

protection.

D. Selenium

1. Definition

Selenium (Se) is a metalloid, and shows a close relation in its chemical properties to sulfur. Se is an essential trace element for animals (Schwartz and Foltz, 1957; Thompson and Scott, 1969). The NRC (1988) suggests that the dietary Se requirement of swine is between 0.1 and 0.3 ppm, and presently the U.S. Food and Drug Administration allows the addition of 0.3 ppm Se to all swine diets. Meyer et al.(1981) indicated that weanling swine may require an even greater amount of Se early in the post-weaning period.

Se may exist in various organic and inorganic forms, and the bioavailabilities of each form differ (Ku et al., 1972, 1973). Rats given Se as either selenite, selenocysteine or selenomethionine exhibited no significant differences in GSH-px activity; however, the tissue Se content tended to be greater with selenomethionine supplementation (Deagen et al., 1987). Glutathione peroxidase is considered an accurate diagnostic marker in determining Se status (Rotruck et al., 1973). Hassan (1986) studied the efficacy of various Se forms on the prevention of exudative diathesis (ED) in chicks. Sodium selenite was most efficient in preventing ED, and increasing plasma GSH-px activity compared to wheat, barley and fish meal Se. Cardiac muscle Se concentrations were elevated more by organic Se from feed ingredients. Wheat and barley Se appears to be in

the form of selenomethionine, but the form in fish meal remains uncertain.

2. Metabolism

Selenium is absorbed mainly in the duodenum. Absorbed Se is immediately taken up by red blood cells and then becomes translocated into the plasma. Plasma proteins then bind Se and deliver it throughout the circulation. Lee et al. (1969) showed that in order for plasma proteins to bind Se, the Se must have been within an erythrocyte. Red blood cells themselves may be carriers of Se, but it is only as a component of the enzyme GSH-px (Rotruck et al., 1973). Tissues retain Se at differing concentrations. The kidney has the highest concentration followed by the liver. Skeletal muscle has the highest absolute amount of Se (Ullrey, 1983). Both the dietary amount and form affect tissue Se status.

3.Functions

Se appears to play a role in the modulation of immune responses (Kiremidjian-Schumacher and Stotzky, 1987). Petrie et al. (1986) found <u>in vitro</u> lymphocyte proliferation could be affected by Se. These researchers suggested that Se may have a role in regulating lymphocyte response to antigen challenges. In swine, a syndrome termed vitamin E and selenium deficiency (VESD) by Hakkarainen and coworkers (1978) was protected against by 5 mg DL-α-tocopheryl acetate and 135 ug Se/kg of feed (as sodium selenite). Vitamin E selenium deficiency is described as a myopathy characterized

by muscle degeneration, weakness, and the sudden death of pigs. Exudative diathesis may also occur in swine, which results in a pooling of fluids in the muscle and subcutaneous regions. Bengtsson et al. (1978a) found VESD signs in Se-deficient pigs supplemented with vitamin E. Alpha-Tocopherol was shown to delay the onset of VESD signs, but some Se is required for prevention. In a second study by Bengtsson and coworkers (1978b) when α-tocopherol was deficient and Se was adequate in pig diets, pigs also developed VESD.

Selenium most likely exerts its protective effects through the enzyme GSH-px of which it is a part. Hafeman and coworkers (1974) found rats supplemented with Se had higher plasma and red blood cell GSH-px activity than deficient controls. Control animals had lost most GSH-px activity (activity < 1% of weaning amount) by day 24 postweaning. Death usually occurred by day 28 in these rats, and hepatic necrosis was diagnostically determined in all cases. Glutathione peroxidase does appear to plateau at a certain Se supplemental level (Hafeman et al., 1974; Meyer et al., 1981). Noguchi et al. (1973) studied the effects of Se on ED in chickens. Glutathione peroxidase activity in the red blood cells of Se-deficient chicks was high at the onset of ED and remained high throughout the experimental period. Glutathione peroxidase in the livers of these chicks fell to approximately 50%, and in plasma the level was nearly zero. Even though plasma GSH-px was almost zero by day five, signs of ED did not appear until day eight. Glutathione peroxidase may be the first line of defense against peroxidation of the

unsaturated lipids of the capillary plasma membrane. In an experiment involving rat erythrocytes by Rotruck and coworkers (1972) glucose addition was capable of preventing oxidative damage in Se-sufficient RBC's. Glucose most likely provides the NADPH necessary for the reduction of GSSG by GR. Combs et al. (1975), in an article on the mechanisms of action of Se in the protection of membranes, relates the biological activity of Se to that of GSH-px. As a component of GSH-px, Se is involved in the protection against damaging effects of both lipid peroxides and hydrogen peroxide.

E. Summary

It is critical for living organisms to maintain antioxidant defense mechanisms in adequate condition. Reduced glutathione, vitamin E, and Se all have an association and function with biological antioxidation, and therefore are likely to be interrelated in their activity.

III. MATAERIALS AND METHODS

A. Experimental Design

A split-block, repeat-measure with 2 x 2 x 2 factorial in the whole plot (over 5 weeks in the sub-plots) design was constructed to involve two levels of supplemental Se (0.0 or 0.3 ppm), two levels of supplemental vitamin E (0.0 or 50 IU/kg), and two levels of supplemental GSH (0.0 or 20 ppm). Selenium was supplemented as sodium selenite, vitamin E as dl-α-tocopheryl acetate, and GSH in its reduced form (Biokyowa). Supplements were added to the control diet at the expense of corn starch. Diet compositions are shown in Table 2. The composition of the vitamin-trace mineral premix used in all diets is listed in Table 3. Table 4 shows the calculated nutrient and energy density of the control weanling starter diet.

Thirty-two pigs, weaned at 4 weeks of age, were blocked into treatments from 4 litters (one pig/litter) of eight pigs. Blocking was also according to sex and initial weight. Pigs were housed in the north, enviromentally-controlled nursery trailer at the MSU Swine Research and Teaching Center. Pens were 1.2 x 2.4 meters in dimension. The pens had a two gutter, gravity flow system, and the gutters were covered by woven wire. Separating the gutters

TABLE 2. COMPOSITION OF THE DIET.

Ingredients %	Diets							
	1	2	3	4	5	6	7	8
Corn starch	.15	.14	.09	.10	.15	.14	.09	.10
Ground shelled corn	71	71	71	71	70.5	70.5	70.5	70.5
Soybean meal (44%)	25	25	25	25	25	25	25	25
Mono-dicalcium phosphate	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Calcium carbonate	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Salt	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
L-lysine hydrochloride premix[a]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
VTM premix[b]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Aureo-SP 250[c]	.25	.25	.25	.25	.25	.25	.25	.25
Reduced glutathione[d]	00	00	00	00	0.5	0.5	0.5	0.5
Sodium selenite	00	00	.15	.15	00	00	.15	.15
dl-alpha-tocopherol acetate	00	.01	.01	00	00	.01	.01	00

[a]Containing 78% lysine.

[b]See Table 3 for composition of vitamin-trace mineral premix.

[c]Containing 110 mg/kg chlortetracycline, 110 mg/kg sulfamethazine, and 55 mg/kg penicillin.

[d]Premix containing 0.40 % reduced glutathione.

TABLE 3. COMPOSITION OF VITAMIN-TRACE MINERAL PREMIX

Nutrient	Amount supplied per kg diet
Vitamin A	1500 IU
Vitamin D	300 IU
Menadione	2 mg
Riboflavin	3 mg
Niacin	18 mg
Panthothenic acid	16 mg
Choline	110 mg
Vitamin B_{12}	20 ug
Zinc	75 mg
Iron	60 mg
Manganese	10 mg
Copper	10 mg
Iodine	0.25 mg

TABLE 4. CALCULATED ENERGY AND NUTRIENT DENSITY

Item	NRC[a]	Control diet
Metabolizable energy, kcal/kg	3160	3175
Crude protein, %	18.0	17.2
Lysine, %	0.79	1.10
Calcium, %	0.65	0.73
Phosphorus, %	0.55	0.64

[a]NRC (1979)

was a concrete sleeping area which had hot water heat within the concrete. Each pen contained two automatic nipple waterers and one stainless steel feeder with five feeder spaces. Feeders were checked daily and feed added as necessary.

Dietary treatments were prepared in 45.5 kg batches as needed in order to maintain feed freshness. A 0.4% GSH premix, using ground corn as the carrier, was utilized to deliver 20 mg/kg by adding 0.227 kg. The premix was stored in the freezer at the Research Center to help maintain its activity. Feed was mixed in the small, stainless steel, horizontal mixer located in the MSU Swine Farm feed preparation facility.

B. Sample Collection and Handling

Pigs were weighed initially and weekly thereafter for 4 weeks. Pigs were also bled initially, and then weekly for the subsequent 4 weeks. Analyses performed were whole blood GSH and plasma Se, α-tocopherol, GSH-px, aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT). Pigs were restrained during bleeding in a V-trough, and blood was removed from the anterior vena cava. 18 gauge x 1 1/2 inch needles were used with 10 ml Sarstedt Monovette Li-Heparin syringes. From the 10 ml whole blood sample, 0.5 ml was immediately pipetted into a 15 ml. corex test tube containing a 3.5 ml solution of 0.003 M disodium dihydrogen ethylenediaminetetraacetate and 25% meta-phosphoric acid (2.5 to 1.0, respectively). This caused a

lysing of the erythrocytes, thereby releasing their cellular GSH into the solution,and maintaining it in the reduced form. Duplicate assays for whole blood GSH concentration were performed for each animal. Blood samples obtained from each animal were immediately held in an iced cooler to limit oxidation. When all bleeding had been accomplished, samples were then transported to the nutrition laboratory in room 202 of Anthony Hall. Lysed blood samples for GSH were centrifuged (Sorvall[1]) at 5000 x g for 10 minutes. The supernatant was pipetted off and, placed into smaller, plastic tubes which were then capped. These tubes were placed in racks and stored at 4°C. Blood sample tubes were centrifuged[2] at approximately 1215 x g for 15 minutes. The supernatant (plasma) was pipetted into three corresponding smaller, plastic tubes. N_2 was used to displace all air prior to being capped. Two tubes from each sample were placed in a rack and stored at -20.0°C for later α-tocopherol analysis and GSH-px analysis. Glutathione peroxidase analyses were performed within 24 hours of sampling (Zhang et al., 1986). The remaining tubes were kept at 4°C for later Se, AST and ALT analyses.

C. Analyses

Reduced glutathione concentration was determined by a fluorometric method using an AMINCO-BOWMAN[3] spectrophotofluorometer with excitation at 350 nm and emission at 420 nm. Reduced glutathione stock solution was made by dissolving 50 mg GSH (EC 1.6.4.2) in 0.005 M EDTA to

[1]Model RC2-B, Ivan Sorvall, Inc., Norwalk, Conn.
[2]Model PR-6000,Damon/IEC Division, Needham Hts., Mass.
[3]American Instrument Co., Inc., Silver Spring, Md.

100 ml volumn. Less concentrated GSH working standards were prepared, ranging from approximately 0.5 to 10 ug GSH/ml, from the stock solution. Test tubes used in the GSH analyses were acid washed to insure accurate analysis readings. To each test tube the following was added: 0.2 ml of either the working standard or sample, 3.6 ml of 0.1 M sodium phosphate buffer and 0.2 ml of 0.1% o-Phthaladehyde (OPT). These were then gently inverted several times for thorough mixing followed by a 15-20 minute reaction period at room temperature. Each tube was then placed in the spectrophotofluorometer and the transmission (T%) recorded. Transmissions (T%) of the standards were regressed against micrograms (ug) of GSH, using a linear program. Reduced glutathione concentrations in the samples were calculated by inserting the T% of the sample into the linear regression equation.

In analyzing for GSH-px, a buffer mixture solution was prepared just prior to the actual analysis. This buffer mixture contained 0.2 M potassium phosphate buffer, NaN_3, NADPH (Sigma, Type III)[1], glutathione reductase (NAD(P)H:oxidized-glutathione oxidoreductase EC 1.6.4.2), and water. A 30% H_2O_2 and a reduced GSH (U.S. Biochenical Corp.) solution were also made. From these, the following were added to four cuvettes (Starna, 10 mm path); 0.915 ml of the buffer solution, 0.50 ml of the GSH solution, and 0.025 ml of the thawed plasma sample (cuvettes 2, 3 and 4 only). Cuvette 1 received 0.025 ml of water to be used as a blank. All cuvettes were incubated for two minutes at 25 °C prior to the

[1]SIGMA Chemical Co., St.Louis, MO.
[2]Beckman Instruments, Inc., Fullerton, CA.

addition of 0.01 ml of 0.124 umole H_2O_2 solution to begin the reaction. A Beckman DU spectrophotometer[2] at 340 nm was used to measure change in NADPH activity. Changes were recorded on a chart recorder. Change in NADPH activity was reflective of the amount of GSH-px present in the sample. Glutathione peroxidase activity was reported in enzyme units (EU) which were expressed as moles of GSH oxidized per minute. The EU for a plasma sample was calculated by: (the change in A_{340} of plasma/min. - the change in A_{340} of blank/min.) x 8.0386. The factor 8.0386 is determined by sample size, the molar extinction coefficient of 6.22 x 10^3 for NADPH and the stoichiometry for the reaction, 2 moles GSH per mole NADPH oxidized. This assay was first used by Paglia and Valentine (1967), and modified by Lawrence et al. (1974).

Alpha-tocopherol was determined in plasma samples by a fluorometric procedure developed by Whetter and Ku (1982) from a tissue α-tocopherol procedure (Taylor, 1976). Alpha-tocopherol in the sample can be dramatically reduced by oxidation. Therefore, careful precautions were taken to minimize the oxidation process. This was accomplished by extracting the samples in acid-washed glassware, keeping the samples on ice throughout the extraction process and displacing the air in the test tubes with nitrogen (N_2) prior to vortexing.

Duplicate standards were prepared from a stock standard solution (2.0 mg α-tocopherol/ml hexane) in absolute ethanol (AR grade) to obtain standards of 0.0, 1.0, 2.0 and 4.0 ug α-

tocopherol/ml. Two milliliters of absolute ethanol were added to the test tubes prepared for plasma samples followed by 1.0 ml of plasma. One milliliter of deionized, distilled water (DD H_2O) was added to each of the standards to bring them to equal volume with the sample and all tubes were vortexed for 5 seconds to precipitate the protein.

Cyclohexane (2 ml, Eastman Kodak, AR grade) was added to each preparation followed by 20 seconds of vortexing to extract the α-tocopherol from the sample. Centrifugation of the samples in a Damon/IEC Model PR-6000 refrigerated centrifuge at 2070 x g for 15 min. provided complete sedimentation of the sample debris from the cyclohexane X α-tocopherol layer. The α-tocopherol in the cyclohexane fluoresced at excitation wavelength of 296 nm and an emission wavelength of 330 nm. This layer was carefully transferred to 2 dram vials, and read in the Aminco-Bowman spectrophotofluorometer set at the appropriate wavelengths. The reading from the spectrophotofluorometer was in percent transmission (%T). To calculate the α-tocopherol concentration in the plasma, the known concentration of α-tocopherol in the standards and the %T for the standards were used to formulate a curvilinear regression line. The %T's for the samples were then read from this line to determine plasma α-tocopherol in ug/ml.

Plasma Se concentrations were determined by a spectrofluorometric procedure (Whetter and Ullrey, 1978). Duplicate plasma samples (1.0 ml) and standards of 0.0, 0.05, 0.10, 0.20 ug of Se/ml were digested in 3.0 ml of nitric acid

(HNO_3) and 2.0 ml of perchloric acid ($HClO_4$) and the HNO_3 was driven off. Nine milliliters of ethylene diamino tetraacetic acid (EDTA) were used to wash down the sides of the digestion flask and prevent contamination of the sample with other metals. Approximately 1.0 ml of concentrated ammonium hydroxide (NH_4OH) was added to each sample to neutralize the remaining $HClO_4$. Cresol red was added to indicate the proper acid:base balance of the sample. Any adjustment in pH was made by adding drops of NH_4OH to HCl (1:9).

Five milliliters of 2,3-diaminonaphthalene (DAN) added to each sample complexed with the Se to form diazoselenol, a light-sensitive complex. This complex was then extracted into cyclohexane (5.0 ml), and transferred to a test tube to allow its %T to be read in the Aminco-Bowman spectrophotofluorometer (excitation 376 nm and emission 510 nm). Plasma Se concentration (ug/ml) were read from a curvilinear regression line calculated from similarly processed standards.

Aspartate aminotransferase and ALT activities were determined by a spectrophotometric procedure developed by SIGMA Diagnostics[1]. Aspartate aminotransferase and ALT reagents were reconstituted with 50.0 ml DD-H_2O. Nine-tenths of a milliliter enzyme reagent and 0.1 ml of sample were added to a cuvette and allowed to incubate for 2 min. at 30°C. Samples were then read at 340 nm. Duplicates were performed for each sample.

The decrease in absorbance rate at 340 nm was directly

[1]SIGMA Chemical Co., St.Louis, MO.

proportional to enzyme activity. Aspartate aminotransferase and ALT activity were reported in EU's which were the amount of enzyme producing 1.0 umole of NAD per minute. The EU's for a plasma sample were calculated by multiplying the change in absorbance/min. X 1688. The factor 1688 was determined by total volume (1.05 ml), sample volume (0.15 ml), light path (1.0 cm), millimolar absorptivity of NADH (6.22), and a conversion of units per ml to units per liter (1000).

D.Statistical Analyses

The pig data were analyzed in a split-plot design, testing the effects of treatment and time on each variable measured. In the event of a significant ($P<0.05$) interaction between treatment and time the Bonferroni t statistic was used to compare treatments with periods and periods within treatments (Gill, 1978a,b,and c.). All statistics were produced using the analysis of variance of contrast variables program which is part of the Statistical Analysis System (SAS).

IV. RESULTS AND DISCUSSION

A. Glutathione

Supplemental GSH does not appear to significantly affect any of the blood measures analyzed in this study. Reduced glutathione also did not significantly alter pig gains or feed efficiencies (Table 5 and Table 6). Alanine aminotransferase concentration in week 3 and 4 is somewhat lower in GSH-supplemented pigs ($P<0.05$) (Figure 16). Aspartate aminotransferase and ALT serum levels can be used as general indices of cellular damage. Moss (1979) noted that pigs subjected to various stresses exhibited increases in serum enzyme activities. Cornelius et al.(1959) found AST levels within the horse, cow, pig and dog to be greatly elevated after having been given carbontetrachloride. Hepatic necrosis accompanied the higher AST levels, however liver ALT was found to be low. Wilson and coworkers (1972) found AST and ALT to be good non-specific indices of tissue damage, but ALT is a poor indicator within pig tissues due to its lower concentration. In light of this, the lower ALT at weeks 3 and 4 in the present study may not necessarily be associated with less cellular damage. Aspartate aminotransferase, a more sensitive index of cell damage in pigs, was not significantly affected.

TABLE 5. BODY WEIGHT[a] OF PIGS AS INFLUENCED BY DIET AND TIME ON TRIAL.

Time on trial	Dietary Treatments[b]							
	Control[c]	GSH[c]	Se[c]	E[c]	G+Se[c]	G+E[c]	Se+E[c]	G+Se+E[c]
Initial[d]	7.08	6.96	7.16	6.48	6.84	7.21	7.49	7.30
Week 1[d]	8.16	8.06	8.26	7.64	7.97	8.39	8.47	8.77
Week 2[d]	10.28	10.31	10.25	9.98	9.89	10.92	10.66	11.11
Week 3[d]	13.87	14.29	13.64	13.30	12.99	14.61	13.64	14.63
Week 4[d]	17.82	18.47	17.62	17.71	16.21	18.34	17.93	18.82

[a]Average weight of 4 pigs per treatment, kg.

[b]GSH-and G=supplemental reduced glutathione (20 mg/kg diet); Se=supplemental selenium (0.3 mg/kg kiet as sodium selenite); E=supplemental vitamin E (50 IU/kg diet as dl-alpha-tocopheryl acetate).

[c]Standard error across weeks=6.95.

[d]Standard error within weeks=1.63.

TABLE 6. FEED EFFICIENCIES[a]

Item	Dietary Treatments[b]							
	Control	GSH	Se	E	G+Se	G+E	Se+E	G+Se+E
Week 1[c]	1.57	1.63	2.04	2.05	1.64	1.69	2.05	1.57
Week 2[c]	2.70	2.14	2.37	2.22	2.14	2.08	2.26	1.99
Week 3[c]	1.67	1.50	1.82	1.75	1.65	1.59	1.78	1.71
Week 4[c]	1.99	1.99	1.89	1.87	1.84	2.12	1.81	1.98

[a]Average of 7 pigs per pen (4 pigs of which were on study).

[b]See footnote of Table 5.

[c]Feed to gain ratios for each week, F/G.

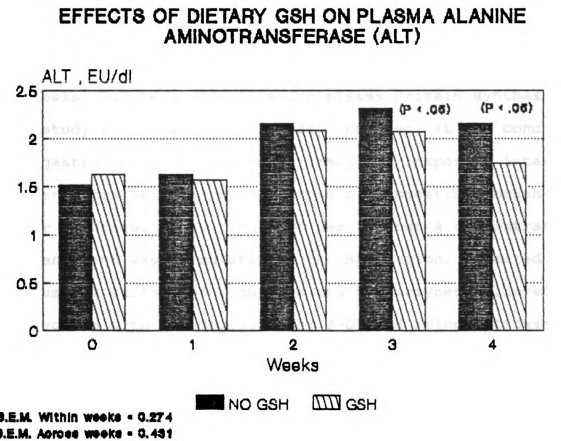


Figure 16. Effects of supplemental GSH on plasma ALT.

One possible interpretation as to why supplemental GSH showed no significant effects within weanling pigs is that GSH could be hydrolyzed into its constituent amino acids before being absorbed, and upon absorption these amino acids may be used for protein synthesis. Tateishi and coworkers (1982) found dietary GSH had a methionine-sparing effect in protein synthesis of rats. The methionine-sparing ability of GSH was similar to that of cysteine for liver protein synthesis, but less effective for plasma protein synthesis. In a study by Linder et al. (1984), however, it was concluded that gastrointestinal GSH was primarily transported intact into an intravesicular space across pig intestinal brush border membrane. This transport was against a concentration gradient, and was stimulated by the Na+ cation. Located on the luminal surface of membranes are two enzymes believed to be involved with GSH degradation; δ-GT and aminopeptidase M which hydrolyzes L-cysteinyl-glycine. Once GSH enters the enterocyte, these enzymes may break it down. Villus tip cells exhibit high δ-GT activity (Cornell and Meister, 1976), and perhaps absorbed GSH becomes the primary, immediate substrate for this enzyme, reduced glutathione's role being that of a δ-glutamyl donor in which δ-GT could link with intestinal free amino acids in the process of carrier-mediated absorption. The enterocyte might also use GSH in a protective, nucleophilic role against harmful agents which may have been ingested (Lash et al., 1986). This study by Lash and coworkers (1986) did, however, involve the utilization of plasma GSH rather than dietary GSH. Assuming

GSH is absorbed as the tripeptide and not altered by the enterocyte, entry into the circulation may result in it becoming rapidly oxidized to GSSG and other products such as mixed disulfides (Anderson et al., 1980). This may be another reason that supplemental GSH seemingly has no effects. Circulating GSH might also affect other factors not quantified in this study. Dietary GSH was found by Sugiyama et al. (1987) to lower plasma and liver cholesterol in rats fed a high cholesterol diet. Also in a study by Elwyn et al. (1968), with unanesthetized dogs, GSH was thought to be a major transport molecule delivering glutamate, cysteine and glycine to various tissues. This would support the findings of Tateishi et al. (1982) as stated earlier. Erythrocytes are known to have an approximate life span of 120 days in humans, but 60 to 70 days in pigs (Tao et al., 1981); therefore, the experimental time period may have been too short to quantify a GSH response in whole blood. This should not have made a difference in the other factors measured. Excretion of GSH might possibly be another explanation. Due to the δ-glutamyl linkage, the tripeptide may be incapable of gastrointestinal enzyme hydrolysis.

B. Vitamin E

Vitamin E (α-tocopherol) supplementation at 50 IU/kg clearly increased the serum α-tocopherol concentration within weanling pigs. The increase was significant at weeks 2,3 and 4 ($P<0.01$) (Figure 17). Bieri (1987) stated that dietary vitamin E is absorbed at approximately 25%, and that a 10-

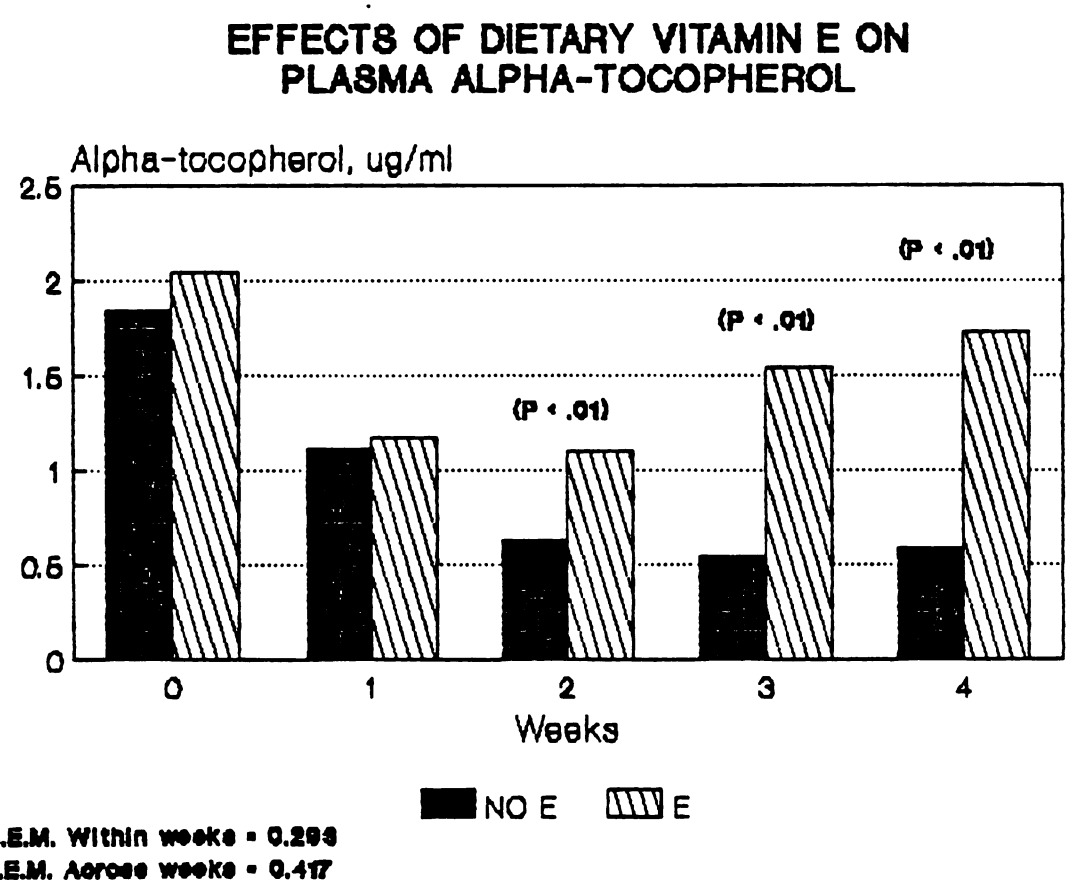


Figure 17. Effects of supplemental vitamin E on plasma vitamin E.

fold intake of α-tocopherol is required to double the plasma concentration in humans. Plasma α-tocopherol levels can be used as an indicator of vitamin E status (Ullrey, 1981). Chow and coworkers (1973) performed an experiment involving rats on corn-oil diets either with or without supplemental vitamin E (0 or 45 mg dl-α-tocopheryl acetate/kg). Glutathione peroxidase activity was found to increase in the perirenal adipose, paraepididymal adipose and muscle tissue of unsupplemented rats. The increase observed in GSH-px activity was probably a result of increased lipid peroxidation in these tissues due to vitamin E deficiency. The GSH-px activities of liver, lung, kidney and testes were not significantly different between treatments. Results of the present study show no increase in plasma GSH-px activity in the vitamin E-deficient pigs. Plasma α-tocopherol is more closely associated with liver tocopherol concentrations, and this may perhaps explain why there was no significant increase in GSH-px activity observed. Also, in the Chow et al. (1973) study, fluorescent products, which are closely related to the extent of lipid peroxidation in mitochondria and microsomes, were increased in unsupplemented rats. Increased lipid peroxidation of cell membranes may cause cells to leak their contents, thereby increasing serum AST/ALT levels. In the present experiment, no increases were detected in the plasma of unsupplemented pigs for either of these marker enzymes. Vitamin E has been shown to interact with Se, and this will be discussed in the following section.

C. Selenium

Selenium supplementation as sodium selenite at 0.3 ppm was shown to elevate plasma Se concentrations in pigs ($P<0.01$) (Figure 18). These elevations were observed at weeks 2,3 and 4. It has been shown that Se supplementation increased the serum Se concentration in weanling pigs (Mahan and Moxon, 1978). Selenium status may also be affected by or interact with vitamin E. Bengtsson and coworkers (1978a, 1978b) and Hakkarainen et al. (1978) performed three studies with pigs on various dietary treatments. In their second study (Bengtsson et al., 1978b), the dietary Se content was manipulated in α-tocopherol -deficient diets. Pigs which received 0-,5- or 15 ug of Se/kg of feed all exhibited signs of vitamin E-selenium deficiency (VESD) and all died. Cutaneous microangiopathy, hepatosis dietetica, skeletal muscle degeneration, mulberry heart and edema were characteristic signs of VESD. Aspartate aminotransferase levels were intermittently increased in these pigs. Pigs receiving 45- and 135 ug of Se/kg of feed also showed signs of VESD, however, they survived. Supplementation at these levels (45 and 135 ug/kg) allowed blood Se concentrations to rise. In the first study by Bengtsson et al. (1978a), Se-deficient diets resulted in decreased blood Se, independent of α-tocopherol supply. Michel et al. (1969) and Trapp et al. (1970) observed similar deficiency signs in pigs with VESD. VESD may best be prevented by supplementing with a combination of Se and vitamin E (Hakkarainen et al., 1978).

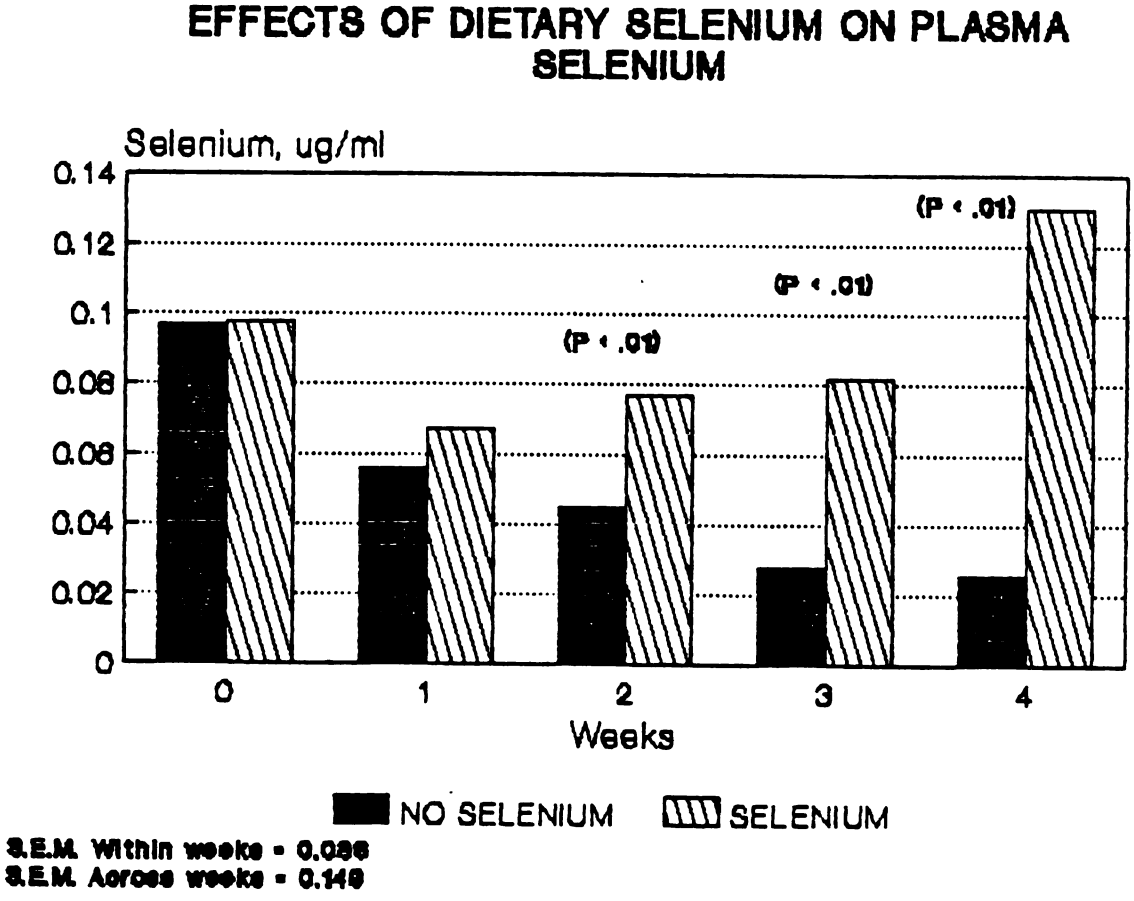


Figure 18. Effects of supplemental Se on plasma Se levels.

The second effect of supplementing with Se in the present experiment was an increase in plasma GSH-px activity. This increase was significant ($P<0.01$) at weeks 2, 3 and 4. (Figure 19). In a study by Serfass and Ganther (1976), GSH-px activity in peritoneal exudate polymorphonuclear neutrophils, pulmonary alveolar macrophages and peritoneal exudate macrophages was markedly lower in rats depleted of dietary Se. Other studies involving Se deficiency and its effects on GSH-px activity have also been performed (Chow and Tappel, 1974; Hafeman et al., 1974; and Hill et al., 1987). It appears that animals deficient in Se have lower GSH-px activity, and animals totally devoid of Se lose practically all GSH-px activity within 3 to 4 weeks. Providing Se in the diet causes GSH-px activity to increase, but it does appear to plateau. Jensen and coworkers (1979) suggested that GSH-px activity in pigs to be influenced by Se supplementation, but they also found it to be highly correlated with litters. They concluded that GSH-px can be used as an indicator of a pig's Se status. Jorgensen et al. (1977) also suggested that GSH-px could be used as a Se-status index in pigs, but that physiological factors should not be overlooked (litter, sex, age.). The genetic influence on GSH-px appears to be very great. Friendship and Wilson (1985) found GSH-px levels in newborn pigs to be weakly associated with the viability of these pigs ($P<0.01$).

The third result of selenium supplementation in the present experiment was elevated whole blood GSH ($P<0.06$) (Figure 20). Hill and Burk (1982) found Se-deficient rat

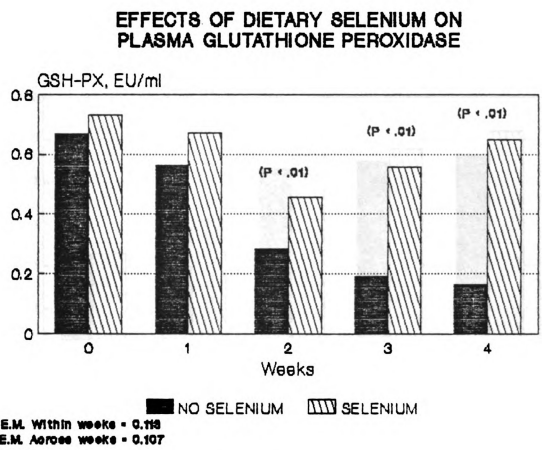
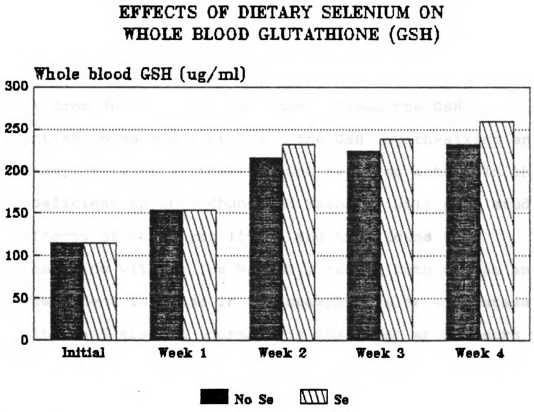


Figure 19. Effects of supplemental Se on plasma GSH-px.



(P < 0.06)

Figure 20. Effects of supplemental Se on whole blood GSH.

hepatocytes *in vitro* had 1.4 times as much GSH as controls, and they also released twice as much GSH (not GSSG) into the incubation medium. Control hepatocytes received 0.5 mg/kg of Se as sodium selenite. Extending their observations to the intact liver, isolated perfused livers were used to measure GSH release in vivo. Four times as much GSH was released into the blood from the Se-deficient liver as from Se-adequate liver. GSH was mainly in its reduced form. The plasma from Se-deficient rats had 3 times the GSH concentration as did controls. The GSH- synthesizing enzyme, δ-glutamylcysteine synthetase, was twice as active in the rats deficient in Se. Chung and Maines (1981) also studied the effects of Se on rat liver, and they found that supplementing with Se (as Na_2SeO_3) caused both a time and dose-dependent increase in δ-glutamylcysteine synthetase activity, and also an increase in GSH level at 24 hours post treatment. Control rats (0 ug/kg feed) did not exhibit these effects. Results of the present study are supported by these findings, but are in conflict with the later findings of Hill and Burk (1982) as mentioned earlier. LeBoeuf and Hoekstra (1983) found Se supplementation as Na_2SeO_3 at 6 ppm for six weeks in rats, caused an increase in hepatic nonprotein sulfhydryls (NPSH). The NPSH elevation was preceded by an increase in GSSG levels. Dietary Se at this level appears to have caused a shift in the glutathione status (GSSG:GSH) of the liver cell. The NPSH was approximately 94% GSH according to this group of researchers, therefore, perhaps more GSH was

being synthesized as proposed by Chung and Maines (1981). The increased GSH may be a cellular response to redox ratios such as, GSSG:GSH, which must be maintained for cells to continue functioning properly.

TABLE 7. PLASMA REDUCED GLUTATHIONE CONCENTRATIONS[a] OF PIGS.

Time on trial	Dietary Treatments[b]							
	Control[c]	GSH[c]	Se[c]	E[c]	G+Se[c]	G+E[c]	Se+E[c]	G+Se+E[c]
Initial[d]	115	111	114	112	120	120	111	115
Week 1[d]	155	162	148	152	151	149	154	164
Week 2[d]	234	200	222	219	243	207	241	220
Week 3[d]	241	212	220	225	243	217	257	232
Week 4[d]	233	218	251	246	276	277	261	251

[a] Average of 4 pigs per treatment (ug/ml).

[b] See footnote of Table 5.

[c] Standard error across weeks=32.9.

[d] Standard error within weeks=16.6.

TABLE 8. PLASMA GLUTATHIONE PEROXIDASE ACTIVITY[a] OF PIGS.

Time on trial	Dietary Treatments[b]							
	Control[c]	GSH[c]	Se[c]	E[c]	G+Se[c]	G+E[c]	Se+E[c]	G+Se+E[c]
Initial[d]	.64	.80	.74	.65	.64	.58	.67	.69
Week 1[d]	.59	.59	.63	.58	.64	.51	.74	.68
Week 2[d]	.27	.38	.46	.26	.44	.24	.43	.51
Week 3[d]	.19	.18	.51	.20	.56	.19	.56	.61
Week 4[d]	.14	.17	.80	.17	.67	.17	.46	.67

[a] Average of 4 pigs per treatment (EU/ml).

[b] See footnote of Table 5.

[c] Standard error across weeks=0.12.

[d] Standard error within weeks=0.11.

TABLE 9. PLASMA TOCOPHEROL CONCENTRATION[a] OF PIGS.

Time on trial	Dietary Treatments[b]							
	Control[c]	GSH[c]	Se[c]	E[c]	G+Se[c]	G+E[c]	Se+E[c]	G+Se+E[c]
Initial[d]	1.86	1.73	2.01	2.53	1.77	1.87	1.76	2.0
Week 1[d]	1.08	1.16	1.00	1.11	1.11	1.22	1.22	1.15
Week 2[d]	.62	.57	.49	1.09	.84	.94	.94	1.45
Week 3[d]	.71	.62	.16	1.57	.71	1.66	1.26	1.68
Week 4[d]	.72	.59	.51	1.81	.55	1.79	1.86	1.47

[a]Average of 4 pigs per treatment (ug/ml).

[b]See footnote of Table 5.

[c]Standard error across weeks=0.42.

[d]Standard error within weeks=0.29.

TABLE 10. PLASMA SELENIUM CONCENTRATION[a] OF PIGS.

Time on trial	Dietary Treatments[b]							
	Control[c]	GSH[c]	Se[c]	E[c]	G+Se[c]	G+E[c]	Se+E[c]	G+Se+E[c]
Initial[d]	.10	.10	.10	.10	.09	.08	.09	.10
Week 1[d]	.05	.06	.07	.05	.07	.06	.07	.07
Week 2[d]	.04	.05	.08	.05	.08	.05	.08	.07
Week 3[d]	.03	.03	.08	.03	.08	.03	.09	.08
Week 4[d]	.02	.02	.13	.03	.13	.03	.13	.14

[a] Average of 4 pigs per treatment (ug/ml).

[b] See footnote of Table 5.

[c] Standard error across weeks=0.15.

[d] Standard error within weeks=0.09.

TABLE 11. PLASMA ASPARTATE AMINOTRANSFERASE ACTIVITY[a] OF PIGS.

Time on trial	Dietary Treatments[b]							
	Control[c]	GSH[c]	Se[c]	E[c]	G+Se[c]	G+E[c]	Se+E[c]	G+Se+E[c]
Initial[d]								
Week 1[d]	1.64	1.61	1.69	1.93	1.62	2.85	2.00	1.70
Week 2[d]	2.55	2.83	2.81	2.68	2.19	2.38	2.14	2.54
Week 3[d]	2.63	2.67	2.56	2.69	2.46	2.17	3.20	2.83
Week 4[d]	1.94	2.15	2.46	2.29	2.35	2.42	2.28	2.26

[a] Average of 4 pigs per treatment (EU/dl).

[b] See footnote of Table 5.

[c] Standard error across weeks=0.69.

[d] Standard error within weeks=0.55.

TABLE 12. PLASMA ALANINE AMINOTRANSFERASE ACTIVITY[a] OF PIGS.

Time on trial	Dietary Treatments[b]							
	Control[c]	GSH[c]	Se[c]	E[c]	G+Se[c]	G+E[c]	Se+E[c]	G+Se+E[c]
Initial[d]	1.38	1.40	1.44	1.54	1.62	1.72	1.80	1.76
Week 1[d]	1.42	1.66	1.96	1.51	1.57	1.61	1.71	1.47
Week 2[d]	1.97	2.12	2.39	2.20	1.84	2.23	2.06	2.12
Wee k 3[d]	2.22	2.35	2.35	2.54	1.80	1.88	2.16	2.23
Week 4[d]	2.06	1.80	2.17	2.21	1.42	1.85	2.20	1.94

[a]Average of 4 pigs per treatment (EU/dl).

[b]See footnote of Table 5.

[c]Standard error across weeks=.43.

[d]Standard error within weeks=.27.

TABLE 13. ANALYSIS OF VARIANCE SIGNIFICANCE LEVELS.

Effects	Measures[a]						
	E	Se	GSH	ALT	AST	GSH-px	Weight
Weeks X GSH	.04	NS	NS	.01	NS	NS	NS
Weeks X Se	NS	.0001	.06	NS	NS	.0001	NS
Weeks X E	.0001	NS	NS	NS	NS	NS	NS
W[b]X GSH X Se	.01	NS	NS	NS	NS	NS	NS
W X GSH X E	NS	NS	NS	NS	NS	NS	NS
W X Se X E	NS	NS	NS	NS	.01	NS	NS
W X GSH X Se X E	NS	NS	NS	.08	NS	NS	NS
GSH	NS	NS	NS	.04	NS	NS	NS
E	.001	NS	NS	NS	NS	NS	NS
Se	NS	.001	.06	NS	NS	.001	NS
GSH X Se	NS	NS	NS	NS	NS	NS	NS
GSH X E	NS	NS	NS	NS	NS	NS	NS
Se X E	NS	NS	NS	NS	NS	NS	NS
GSH X Se X E	NS	NS	NS	.06	NS	.01	NS

[a] Vitamin E=E, selenium=Se, glutathione=GSH, alanine aminotransferase=ALT, aspartate aminotransferase=AST, glutathione peroxidase=GSH-px.

[b] Weeks=W

V. CONCLUSIONS

1) Glutathione supplementation of the diet of weanling pigs lowered plasma ALT activity slightly ($P<0.05$) in weeks 3 and 4 of the 4 week trial period. Glutathione supplementation had no significant influence on whole blood GSH, plasma GSH-px, plasma α-tocopherol, plasma Se, or plasma AST.

2) Vitamin E supplementation significantly elevated plasma α-tocopherol in weeks 2, 3 and 4 ($P<0.01$). Whole blood GSH, plasma GSH-px, Se, ALT or AST were not significantly affected by dietary vitamin E.

3) Selenium supplementation significantly elevated plasma Se levels in weeks 2, 3 and 4 ($P<0.01$). Supplemental Se also significantly elevated plasma GSH-px activity in weeks 2, 3 and 4 ($P<0.01$). Selenium elevated whole blood GSH slightly ($P<0.06$) Alanine aminotransferase and AST were not affected by supplemental Se.

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