IMPACT OF DIETARY PROTEIN
ON BRAIN GLUTATHIONE IN STROKE

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ABSTRACT

In the present study, we proposed that dietary protein deficiency would depress brain glutathione (GSH) concentration. If the hypothesis were correct, this would suggest compromised antioxidant defense in stroke patients with poor protein status. The effect of L-2-oxothiazolidine-4-carboxylate (OTC), a precursor of the limiting amino acid (cysteine) for intracellular GSH synthesis, was also investigated in this study. Male Long-Evans weanling rats were randomly assigned to one of three experimental groups fed: 1) a protein-deficient diet modified from the AIN-93G diet containing 7.5% protein, 2) the OTC-supplemented diet containing 7.5% protein and supplemented with 0.453% OTC to be equivalent in sulfur content with that in the protein-adequate diet, and 3) the protein-adequate (control) diet containing 17.9% protein. After 6 weeks of feeding, the liver, neocortex, striatum, hippocampus, thalamus, cerebellum, and brain stem were collected for analysis of GSH concentration by reverse-phase high performance liquid chromatography (HPLC). This method used precolumn derivatization with 5, 5'-dinitrobis (2-nitrobenzoic acid) (DTNB) and ultraviolet detection at 330 nm. Hepatic GSH concentration was significantly decreased by protein deficiency. OTC supplementation of protein-deficient rats increased hepatic GSH concentration to a near normal level. GSH concentration in various brain regions was not significantly lower in rats fed a protein-deficient diet than that fed the normal diet. OTC supplementation increased neocortical GSH concentration over that of the protein-deficient rats. These results suggest that brain GSH concentration was well maintained during moderate protein deficiency. This is probably due to sustained capacity of GSH synthesis, uptake mechanisms of GSH, and transport systems of sulfur amino acids to the brain when dietary protein intake is moderately low. Also,

OTC supplementation elevated cysteine concentration in the neocortex and cerebellum of the protein-deficient rats, suggesting that OTC is an effective vehicle for delivering cysteine and may provide a therapeutic intervention for stroke patients with depleted brain GSH.

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A(D)TP	Adenosine (diphosphate) triphosphate
BCA	Bicinchoninic acid
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetate
GPx	Glutathione peroxidase
GPx1	Classical cytosolic glutathione peroxidase
GPx4	Phospholipid hydroperoxide glutathione peroxidase
GSH	Reduced form of glutathione
GSSG	Oxidized form of gluathione
GSSG-R	Glutathione reductase
HEPES	N- (2-hydroxyethyl)poperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
NADH	α-Nicotinamide adenine dinucleotide
NADPH	α-Nicotinamide adenine dinucleotide phosphate
OTC	L-2-oxothiazolidine-4-carbosylate
PEM	Protein-energy malnutrition
ROS/RNS	Reactive oxygen (nitrogen) species
SOD	Superoxide dismutase

Chapter 1 Introduction

1.1 Background

Stroke is one of the leading causes of death and disability in most developed countries. Currently, primary prevention of risk factors has decreased the incidence of stroke (Petrasovits and Nair 1994), but little effective treatment for stroke patients has been established once stroke occurs. The decline in stroke mortality and incidence has been accompanied by increased intake of dietary protein according to numerous epidemiological studies (Shimamoto et al. 1989; Mizushima and Yamori 1992; Kodama 1993). In this study, we investigated one mechanism by which dietary protein may be involved in stroke outcome.

Stroke causes a disruption in the blood flow to an area of the brain (Juurlink and Sweeney 1997). Consequently, a number of events occur in the brain, including the depletion of adenosine triphosphate (ATP), the release of glutamate, the accumulation of Ca^{2+}, and an inflammatory response, which lead eventually to neuronal death (Juurlink and Sweeney 1997). Another major event that occurs during and following ischemia is the significant generation of reactive oxygen and nitrogen species (ROS/RNS) (Juurlink and Sweeney 1997). These species can cause oxidative damage to cells by donating or extracting electrons from macromolecules, such as deoxyribonucleic acid (DNA), proteins, and lipids (Halliwell 1992; 1994).

Several components in the antioxidant defense system, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH), work together

to scavenge ROS/RNS (Juurlink 1996). GSH, a tripeptide (L-γ-glutamyl-L-cysteinylglycine), plays a central role in minimizing the extent of oxidative stress-induced brain damage in several ways. These include providing electrons for the GPx reaction, directly scavenging radicals, and regenerating vitamin E (Juurlink 1997). However, several studies have demonstrated that brain GSH concentration is decreased during and following stroke (Folbergrová et al. 1979; Cooper et al. 1980; Rehncrona et al. 1980), further exacerbating the oxidative damage in the brain. The balance of the generation of ROS/RNS and antioxidant defense is one determinant of the extent of brain injury following stroke. The maintenance of brain GSH concentration is key in diminishing brain damage and improving the outcome of stroke.

1.2 Rationale

Nutritional factors are known to exert an impact on regulation of GSH synthesis in various tissues, predominantly by affecting the availability of the limiting amino acid, cysteine (Bray and Taylor 1993). GSH concentration in tissues such as the liver is responsive to dietary protein, in particular sulfur amino acids (Bauman et al. 1988a; Taylor et al. 1992; Hum et al. 1992). To a lesser extent, brain GSH concentration is depressed by sulfur amino acid deficiency (Tor-Agbidye et al. 1996; Paterson et al. 1998).

Several studies indicate that poor protein status is prevalent among elderly stroke patients (Dávalos et al. 1996; Axelsson et al. 1998; Gariballa et al. 1998), which may worsen the outcome of stroke. However, direct evidence that protein deficiency affects brain GSH concentration is lacking in the literature. In the present study, the role of moderate protein deficiency in the regulation of GSH concentration will be determined in various regions of rat brain.

1.3 Hypothesis

Moderate protein deficiency will compromise the brain antioxidant defense system by decreasing the GSH concentration. This will be concomitant with increased formation of protein carbonyl content, a marker of protein oxidation, in the brain. Oral supplementation of the cysteine precursor, L-2-oxothiazolidine-4-carboxylate (OTC), will maintain brain GSH concentration in rats fed a protein-deficient diet.

1.4 Objectives

1. To establish a rat model of moderate protein deficiency.
2. To determine the effects of protein deficiency on GSH concentration in the liver and various brain regions.
3. To study the effects of OTC in maintaining GSH concentration in the liver and brain in rats with protein deficiency.
4. To investigate the influence of dietary protein on the formation of protein carbonyl content in the liver and brain.

Chapter 2 Literature Review

2.1 Stroke

Stroke is defined as "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin" (Aho et al. 1980). Stroke can be classified as ischemic (thromboembolic) and haemorrhagic. The former, which is most common, is due to an inadequate blood supply as the result of a blood clot. The latter involves bleeding into the brain parenchyma or the subarachnoid space. Both types cause a disruption in blood flow to an area of the brain, resulting in severe ischemia in this region (Juurlink and Sweeney 1997).

Stroke is one of the major causes of death and disability in both developed and developing countries. Approximately 30% of all cases of stroke are fatal (Bogousslavsky et al. 1988). Victims who survive after stroke suffer from different degrees of mental and physical impairments. In Canada and the United States, stroke is the third leading cause of death after cardiac diseases and malignant neoplasms (Statistics Canada 1995; U.S. Bureau of Censor 1997). Stroke is also the leading cause of long-term disability in the United States (Barone and Feurerstein 1999).

The etiological factors of stroke are diverse. Age, gender, race, heredity, and geographic location have been identified as non-modifiable risk factors for stroke (Mayo 1993; Sacco et al. 1997). Age is the most important risk factor for stroke. Most follow-up studies show that increasing age, especially after age 50, is significantly

associated with the increased occurrence of stroke (Omae and Ueda 1992; Sacco et al. 1997). Among modifiable factors, hypertension is recognized as the most powerful risk factor for both haemorrhagic and ischemic stroke (Omae and Ueda 1992; Neaton et al. 1993). Other well-documented modifiable factors include various cardiac diseases, diabetes mellitus, sickle cell disease, cigarette smoking, and transient ischemic attacks (Mayo 1993; Sacco et al. 1997).

The rates of stroke incidence and mortality have declined in the past several decades (Petrasovits and Nair 1994) due to major progress made in primary prevention of risk factors such as treatment of hypertension. However, no new therapeutics for this devastating disease has been approved beyond tissue plasminogen activator, a thrombolytic agent administrated within 3 hours after stroke (Barone and Feuerstein 1999). Given the tremendous medical and social burden of stroke, the high demand for novel treatments to diminish the brain damage following stroke has been recognized.

The influence of dietary factors and nutritional status have not received enough attention. Recently, there are some investigations of dietary impact on the incidence of stroke. For example, high consumption of fruits and vegetables, which are rich sources of β-carotene and vitamin C, may protect against stroke development (Acheson and Williams 1983; Gillman et al. 1995). However, little information is available on the influence of diet and nutritional status on the outcome of stroke. The aim of our study was to investigate one mechanism by which dietary protein and protein status might affect the extent of brain damage following stroke.

2.2 Oxidative Damage in Stroke

2.2.1 Mechanisms of Brain Damage in Stroke

The mechanisms of brain damage during and following stroke are complex. A number of changes occur in brain tissue following a severe reduction in blood flow, which lead to neuronal death. These detrimental reactions are not mutually exclusive, that is, one event can lead to another one, which, in turn, exacerbates the initial event.

The primary change during and following ischemia is an initial depletion in cellular ATP as a result of both reduction in oxidative phosphorylation as oxygen is depleted and an increase in ATP utilization for maintenance of cell membrane potentials (Siesjö 1981; Raichle 1983). This then leads to the disruption of cell membrane potentials resulting in a massive release of K^+ and excitatory amino acids such as glutamate (Sweeney et al. 1995; Juurlink and Sweeney 1997). Increased extracellular K^+ aggravates the depletion of ATP by the depolarization of adjacent cells and activation of Na^+-K^+ ATPase (Sweeney et al. 1995).

The massive release of glutamate causes excitotoxicity that triggers and executes tissue damage during and following ischemia. One consequence of the release of glutamate is further depletion of ATP stores by several mechanisms (Sweeney et al. 1995). Most importantly, glutamate causes membrane depolarization and activates a number of ionotropic receptors such as N-methyl-D-asparate (NMDA) receptors, giving rise to an increase in intracellular Ca^{2+} through the influx from extracellular matrix and the release from endoplasmic reticulum and mitochondria (Siesjö and Bengtsson 1989; Choi 1995). The accumulation of intracellular Ca^{2+} can, in turn, cause more release of glutamate (Juurlink and Sweeney 1997). Furthermore, concomitant Na^+ influx with Ca^{2+} can also activate Na^+, K^+-ATPase resulting in the consumption of ATP (Juurlink and Sweeney 1997).

Increased intracellular Ca^{2+} level also activates several second messenger pathways, resulting in profound tissue damage profoundly. For example, increased

intracellular Ca^{2+} activates proteolytic enzymes that degrade cytoskeletal proteins such as actin and spectrin (Furukawa et al. 1997) and extracellular matrix proteases such as laminin (Chen and Strickland 1997). Ischemia also causes the development of lactacidosis. This will have deleterious effects on the cell such as induction of the release of Fe^{2+} from ferritin, resulting in the generation of the hydroxyl radical (Rehncrona et al. 1989).

In recent years, the inflammatory response in the brain, including neutrophil and monocyte invasion as well as microglia activation, has been recognized to play a role in tissue damage. The accumulation of intracellular Ca^{2+} during and following ischemia activates phospholipases, particularly phospholipase A_2, which interacts with membrane phospholipids to release large amounts of free fatty acids such as arachidonic acid (Farooqui and Horrocks 1994). The metabolism of arachidonic acid results in the formation of inflammatory mediators, such as leukotriene B_4 that causes the activation and invasion of neutrophils into the brain (Grace 1994). NF-κB (a protein transcription factor) that is required to induce transcription of a series of pro-inflammatory molecules including cell adhesion molecules (ICAM-1 and VCAM-1), cytokines (interleukin-1β, tumor necrosis factor-α), and pro-inflammatory enzymes (iNOS and cycloxygenase-2) (Christman et al. 1998) is activated during and following ischemia, leading to the generation of acute inflammation (Terai et al. 1996; Howard et al. 1998; Schmedtje et al. 1997; Carroll et al. 1998).

Brain cells can die by necrosis or undergo delayed cell death (apoptosis) as a result of excitotoxicity, Ca^{2+} overload, oxidative stress, and DNA damage after the ischemic insult (Saunders et al. 1995). Necrosis is the predominant mechanism that follows acute, permanent vascular occlusion; whereas delayed neuronal death occurs particularly within the penumbra, a region of ischemically threatened tissue adjacent to

a core zone of infarction (Dirnagl et al. 1999). There is supportive evidence that neurons at the border of the infarction area can survive for many hours or even days following ischemia (Dereski et al. 1993), providing the opportunity for treatment to restore brain function within the penumbra. It has been suggested that this treatment may provide neuroprotective effects if administrated within 4-6 hours after the ischemic insult (Shuaib and Kanthan 1997). It may be of importance for nutritional intervention to be given during this period to maximize antioxidant defense.

2.2.2 The Generation of ROS/RNS

ROS/RNS generated as one of the consequences of ischemic insult aggravate ischemia-induced brain damage. ROS/RNS is a collective term for a group of oxygen-containing and nitrogen-containing chemicals that are easily involved in redox reactions by donating or extracting electrons from other molecules (Halliwell 1996; Juurlink 1997).

ROS/RNS are grouped into two categories: free radicals and non-radical species. A free radical is defined as "any species capable of independent existence that contains one or more unpaired electrons, i.e., electrons alone in an atomic or molecular orbital" (Halliwell and Gutteridge 1989). Radicals are formed when a covalent bond is broken, but one electron from each of the pair shared remains with each atom. The presence of one or more unpaired electrons causes the species to be attracted slightly to a magnetic field (i.e., to be paramagnetic). This can alter the chemical reactivity of the species and causes the interaction of free radicals with other macromolecules to damage their structure and function. When two free radicals encounter one another, they can combine their unpaired electrons and form a covalent

bond. If a free radical meets non-radicals, a new free radical is produced, and chain reactions can be induced, resulting in damage to cell function (Halliwell 1992; 1994).

Major free radicals (both ROS and RNS) include superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide radical ($NO^{\bullet}$), and nitrogen dioxide ($NO_2^{\bullet}$). Non-radical species mainly consist of singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), ozone (O_3), and peroxynitrite ($ONOO^-$) (Halliwell 1996; Juurlink and Paterson 1998).

Under normal circumstances, approximately 3% of oxygen consumed by mitochondria is reduced to the superoxide anion rather than being completely reduced to water (Fridovich 1986). However, under hypoxic conditions, such as ischemia, more superoxide anion is produced (Bast et al. 1991). Reperfusion after ischemia further increases the superoxide anion production (Richter and Kass 1991). The accumulation of intracellular Ca^{2+} in ischemia/reperfusion is partially responsible for the remarkable generation of superoxide anion (Richter and Kass 1991), since the rise of Ca^{2+} activates proteases that convert xanthine dehydrogenase to xanthine oxidase (Sussman and Bulkley 1990). Xanthine oxidase can catalyze hypoxanthine, one of the metabolites from ATP, to xanthine and then uric acid using molecular oxygen as the electron acceptor (Lazzarion et al. 1992). Under normal circumstances this reaction is catalyzed by xanthine dehydrogenase using NAD^+ as the electron acceptor (Sussman and Bulkley 1990). During and following ischemia, ATP metabolites such as hypoxanthine accumulate due to continued consumption and decreased synthesis of ATP. When reperfusion occurs, it further provides the substrate, oxygen, to xanthine oxidase. As a result of the increase in hypoxanthine, xanthine oxidase and oxygen, the generation of superoxide anion is significantly elevated. Superoxide anion is also produced from metabolism of arachidonic acid by lipoxygenase and cycloxygenase at

the expense of α-nicotinamide adenine dinucleotide phosphate (NADPH) (Kukreja et al. 1986).

Although superoxide anion is a mild oxidant, it disturbs cell function in several ways: 1) inactivation of thiol-containing proteins by oxidizing thiols directly (Fridovich 1995), 2) de-esterification of membrane lipid to release arachidonic acid, 3) conversion of NADH to the NAD radical that reacts with oxygen to produce more superoxide anion, 4) production of other ROS such as peroxynitrite (reaction 2a), hydroxyl radical (reaction 2a), and hydrogen peroxide (reaction 2b) (Beckman et al. 1990; Crow et al. 1994; Denicola et al. 1995), 5) release of Fe^{2+} from ferritin stores by reducing Fe^{3+} to Fe^{2+} (reaction 2c) (Funk et al. 1985), and 6) reaction with GSH to form the glutathiyl radical and further to form GSSG (reaction 2d, 3i) (Winterbourn and Metodiewa 1994).

(2a) $NO^{\bullet} + O_2^{-\bullet} \longrightarrow ONOO^- + H^+ \longrightarrow ONOOH \longrightarrow OH^{\bullet} + NO_2^{\bullet}$

(2b) $2O_2^{-\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

(2c) $O_2^{-\bullet} + Fe^{3+} \longrightarrow Fe^{2+} + O_2$

(2d) $O_2^{-\bullet} + 2GSH \longrightarrow 2GS^{\bullet} + H_2O_2$

The hydrogen peroxide generated from the SOD reaction (reaction 2b) is a relatively unreactive oxidant that can easily cross cell membranes. Consequently, if hydrogen peroxide is not removed immediately, it dismutates into the most powerful oxidant, hydroxyl radical, in the presence of Fe^{2+} (reaction 2e); this is known as the Fenton reaction (Halliwell and Gutteridge 1989). Fe^{3+} is formed and reduced back to Fe^{2+} by superoxide anion (reaction 2c). Also, it can produce singlet oxygen by reaction with other ROS such as hypochlorite (reaction 2f) (Steinbeck et al. 1992), peroxynitrite (reaction 2g) (Di Mascio et al. 1994), and superoxide anion (reaction 2h), a reaction known as the Haber-Weiss reaction (Khan and Kasha 1994).

(2e) $H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^{\bullet} + OH^-$

(2f) $H_2O_2 + OCl^- \longrightarrow H_2O + Cl^- + {}^1O_2$

(2g) $H_2O_2 + ONOO^- \longrightarrow {}^1O_2 + ONO^- + H_2O$

(2h) $H_2O_2 + O_2^{-\bullet} \longrightarrow OH^\bullet + OH^- + {}^1O_2$

The hydroxyl radical formed from hydrogen peroxide dismutation (reaction 2e) in the presence of Fe^{2+} is the most powerful radical. It interferes with cell function by oxidation of thiol-containing proteins such as glutathione reductase and GPx (Tabatabaie and Floyd 1994), breakage of the deoxysugar phosphate backbone of DNA strands (Breen and Murphy 1995), and interaction with polyunsaturated lipids (Halliwell and Gutteridge 1989). Uniquely, the hydroxyl radical inactivates electron-carrying proteins and ATPase in mitochondria, resulting in damage of their function (Zhang et al. 1990).

Other reactive species are also generated during and following ischemia. Singlet oxygen, for instance, a powerful oxidant, is formed as outlined above (reaction 2f, 2g, 2h). Peroxynitrite ($ONOO^-$) is produced by the reaction between nitric oxide radical and superoxide anion (reaction 2a). It undergoes protonation to form peroxynitrous acid, which can easily split into two powerful oxidants, hydroxyl radical and nitrogen dioxide (reaction 2a). These reactive species also can readily oxidize protein, DNA base, and polyunsaturated fatty acids (Halliwell 1995; Juurlink and Paterson 1998).

2.2.3 Lipid Peroxidation and Protein Oxidation in Stroke

Peroxidation of cell membrane lipids and oxidation of proteins represent two key types of cellular damage that can occur in the presence of increased amounts of reactive species. Lipid peroxidation is initiated by formation of a lipid carbon-centered radical ($L^\bullet$) by interaction between polyunsaturated lipids (LH) in membranes and the

hydroxyl radical (reaction 2i). Lipid radicals, in turn, can react with molecular oxygen to form the lipid peroxyl radical (LOO$^{\bullet}$) (reaction 2j). Once a lipid peroxyl radical is generated, a chain of propagation reactions occurs as a result of the abstraction of a hydrogen atom from the neighboring polyunsaturated fatty acid to produce a new lipid radical (reaction 2k). This leads to damage to the polyunsaturated fatty acids in the cell membrane. The chain reaction can be terminated by vitamin E (reaction 3d) or by the interaction between two lipid radicals (Juurlink and Paterson 1998).

In addition, both Fe^{2+} and Fe^{3+} can react with lipid hydroperoxides (LOOH) to give rise to lipid peroxyl and alkoxyl radicals, which trigger a new chain reaction to destroy cell membranes (reaction 2l and 2m) (Halliwell and Gutteridge 1989). The peroxidation of lipid causes changes in membrane permeability and fluidity (Tretter and Adam-Vizi 1996), and loss of functions such as ATPase activity (Ranchova et al. 1995), thereby exacerbating the damage in brain tissue already caused by the reduction of blood supply.

(2i) $LH + OH^{\bullet} \longrightarrow L^{\bullet} + H_2O$

(2j) $L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$

(2k) $LOO^{\bullet} + LH \longrightarrow L^{\bullet} + LOOH$

(2l) $LOOH + Fe^{2+} \longrightarrow LO^{\bullet} + OH^{-} + Fe^{3+}$

(2m) $LOOH + Fe^{3+} \longrightarrow LOO^{\bullet} + H^{+} + Fe^{2+}$

Enormous evidence indicates that an increase of lipid peroxidation in the central nervous system (CNS) is associated with ischemia. Bromont et al. (1989) reported a significant increase of lipid peroxidation, estimated by a thiobarbituric acid test, in rat brain regions between 8 and 72 hours of ischemia/reperfusion. Similar results were also observed in a study by Mickel et al. (1987) in which lipid peroxidation

was markedly increased in gerbils exposed to a 100% oxygen atmosphere after ischemia.

The ROS/RNS-induced damage to proteins results in the modification of amino acid residues leading to the formation of carbonyl derivatives, such as the oxo acids and aldehydes with the same or one less carbon atom than the original amino acid. For instance, glycine can be oxidized to form glyoxal and glyoxylic acid, or formaldehyde and formic acid (Dean et al. 1997). Previous work suggested that hydroxyl radical and superoxide anion might be responsible for initiating protein oxidation (Fridovich 1983; Davies and Goldberg 1987). This causes conformational and functional alteration in proteins, leading to the fragmentation, aggregation, and increased susceptibility to proteolytic degradation (Yu 1994). For example, the thiol groups on proteins can be oxidized to form disulfide bonds that are not easily reduced back to the free thiol groups, resulting in increased protein cross-linking and altered conformation. The oxidative modification of proteins can modulate biochemical characteristics of proteins. In the case of enzymes, they become catalytically inactive or less active (Stadtman and Oliver 1991).

Increased protein oxidation in the brain, as measured by protein carbonyl content, has been observed during ischemia. Protein carbonyl content was elevated significantly during reperfusion following 10 minutes of ischemia in gerbils (Oliver et al. 1990). However, inconsistent findings were reported by Folbergrová et al. (1993). They failed to demonstrate an increase in oxidative damage to brain protein in rats at 1- or 3- hours after ischemia. This may be due to differences in species and stroke models studied. Protein carbonyl formation is inhibited by GSH in rat liver microsomes in a dose-dependent manner (Palamanda and Kehrer 1992).

2.3 Antioxidant Defense

2.3.1 Enzymatic System

There are several mechanisms that provide protective effects against ROS/RNS, including enzymatic and non-enzymatic systems (Juurlink 1997; Juurlink and Paterson 1998).

Superoxide dismutase, catalase, and GPx are three major components in this enzymatic antioxidant defense system. SOD, consisting of three forms, extracelluar Cu/Zn-dependent SOD, cytosolic Cu/Zn-dependent SOD, and mitochondrial Mn-dependent SOD (Fridovich 1995), acts as the first line of defense against ROS by conversion of superoxide anion into hydrogen peroxide (reaction 2b). The conversion of GSH to GSSG by superoxide anion (reaction 2d) can also be inhibited by SOD (Winterbourn and Metodiewa 1994). Numerous studies demonstrate the important role of SOD in brain ischemia (Chan et al. 1995; Francis et al. 1997). A decrease in SOD activity aggravates brain damage caused by ischemia/reperfusion (Kondon et al. 1997). SOD must cooperate with other enzymes including catalase (reaction 3a) and GPx (reaction 3b) that play a major role in removing hydrogen peroxide as a secondary line of defense (Juurlink 1997). Catalase is mainly located in the peroxisomes of all mammalian cells, but it is also present in mitochondria (Radi et al. 1991b; 1993). The activity of catalase, however, is limited due to its localization and low affinity for hydrogen peroxide in the mM range despite its relatively high capacity (Simmons and Jamall 1988).

GPx, on the other hand, is capable of conversion of any peroxide into water and oxygen. GPx, a family of selenoproteins, is comprised of four identical subunits. Each subunit has one atom of selenium that is involved in the catalytic activity (Flohé 1979). Four types of GPx have been identified so far. They are the classical cytosolic

GPx (GPx1) (Zakowski et al. 1978), gastrointestinal GPx (GPx2) (Chu et al. 1993; Chu and Esworthy 1995), plasma GPx (GPx3) (Takahashi et al. 1987), and phospholipid hydroperoxide GPx (GPx4) (Ursini et al. 1985). GPx1 and GPx4 play a major role in removing hydrogen peroxide and organic peroxides. GPx1, which comprises about 90% of total cellular GPx activity, has the ability to reduce hydrogen peroxide and free fatty acid peroxides (Flohé 1979; Wendel 1980; Maddipati and Marnett 1987). GPx4 acts specifically on all membrane-bound hydroperoxides as well as fatty acid hydroperoxides, cumene hydroperoxide, tert-butyl hydroperoxide, and hydrogen peroxide (Ursini et al. 1985; Maiorino et al. 1990).

GPx1 and GPx4 are widely distributed (Zhang et al. 1989). However, the activities of GPx1 and GPx4 are high in the liver and kidney and low in the brain (De Marchena et al. 1974; Zhang et al. 1989). GPx has a variable affinity for peroxides, and its affinity is positively associated with the concentration of GSH. GPx is, therefore, a GSH-dependent enzyme, i.e., the reduced form of glutathione is required for the reaction (reaction 3b). Oxidized glutathione (GSSG) is produced in the GPx reaction, and it is converted back to the reduced form (GSH) by glutathione reductase (GSSG-R) at the expense of NADPH (reaction 3c). Glutathione reductase containing FAD in its active site catalyzes NADPH to reduce GSSG (Rosemeyer 1987). The affinity of GPx for GSH also depends upon peroxide levels (Flohé 1979; Ursini et al. 1985; Esworthy et al. 1993). In addition, GPx can remove lipid peroxides formed during the chain reaction of lipid peroxidation using GSH as electron donor (reaction 3d), which is important to stop new chain reactions of lipid peroxidation.

The activity of GPx, as well as SOD, is higher in glial cell-enriched regions than in neuronal cell-enriched regions. This is concomitant with lower levels of lipid peroxidation (Geremia et al. 1990), suggesting that GPx is important in ROS-

scavenging in the brain. Neurons from mice with a homozygous null mutation for GPx were found to increase susceptibility to hydrogen peroxide (O'Shea et al. 1998). However, GPx alone appears to provide incomplete protection from oxidative stress due to its low activity in the brain (De Marchena et al. 1974; Sinet et al. 1980). Other members in the family of peroxidases such as thioredoxin-dependent peroxidase, which is present at a high level in the CNS (Chae et al. 1994), may collaborate with GPx to scavenge peroxides in brain tissue (Juurlink 1999). Glutathione-S-transferase, a non-Se-containing GPx, can also remove peroxides, except H_2O_2, in the presence of GSH (Mannervik et al. 1983; Thomson 1985).

(3a) $2\ H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$

(3b) $2GSH + 2\ H_2O_2 \xrightarrow{GPx} 2H_2O + O_2 + GSSG$

(3c) $GSSG + 2NADPH \xrightarrow{GSSG\text{-}R} 2GSH + 2NADP^+$

(3d) $LOOH + 2GSH \xrightarrow{GPx} LOH + GSSG + H_2O$

2.3.2 Non-enzymatic System

Non-enzymatic antioxidants are a group of molecules with low molecular weight, and include vitamin E (α-tocopherol), vitamin C (ascorbate), and GSH. Vitamin E (reduced form, ToH) can terminate the chain reactions of lipid peroxidation by reacting with the lipid peroxyl radical (Burton and Traber 1990). However, during this process, the vitamin E radical (To•) is produced (reaction 3e). The regeneration of reduced vitamin E needs vitamin C (AscH2), which, in turn, forms the ascorbate radical (reaction 3f). Two ascorbate radicals interact and generate dehydroascobate (Asc) and ascorbate (AscH2) (reaction 3g). Ascorbate is regenerated by donation of H^+ from GSH to dehydroascobate in the presence of NADPH (reaction 3h, 3c). In addition to its

ability to recycle vitamin E, vitamin C also scavenges several ROS, such as superoxide anion, hydroxyl radical, and hydrogen peroxide (Halliwell 1994).

(3e) $LOO^{\bullet} + ToH \longrightarrow LOOH + To^{\bullet}$

(3f) $To^{\bullet} + AscH_2 \longrightarrow ToH + AscH^{\bullet}$

(3g) $2AscH^{\bullet} \longrightarrow AscH_2 + Asc$

(3h) $Asc + 2GSH \longrightarrow AscH_2 + GSSG$

2.3.3 The Central Role of GSH in the Antioxidant Defense in the CNS

That GSH is of importance in preventing cells against oxidation is indicated by the findings that relatively small changes in GSH concentration will affect cell function. Thorburne and Juurlink (1996) showed that enhancing GSH concentration in oligodendroglial cells to the level found in astrocytic cells could increase the ability of these cells to scavenge strong oxidants such as the hydroxyl radical. Conversely, the depletion of intracellular GSH causes oxidative damage. Jain et al. (1991) showed that a decrease of GSH in the cerebral cortex by buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase, resulted in damage to mitochondria. Similar findings were demonstrated in other studies using brain cell cultures (Mårtensson et al. 1991; Mizui et al. 1992; Huang and Philber 1996; Wüller et al. 1999). A more recent study showed that a decrease in intracellular GSH resulted in increased traumatic injury of porcine brain cerebral endothelial cells; this injury was reduced when endothelial cell GSH levels were augmented threefold (Gidday et al. 1999). Furthermore, inhibition of GSH synthesis results in death, hind-leg paralysis, impaired spermatogenesis, or cataracts in newborn rats (Calvin et al. 1986; Mårtensson et al. 1989), and also causes CNS dysfunction in humans (Meister and Larsson 1989; Cooper and Meister 1993). These

data support the idea that GSH plays a central role in enabling cells to deal with oxidative stress in the CNS.

GSH exerts its antioxidant effects by several mechanisms. It serves as an electron donator for the GPx reaction (reaction 3b). Also, GSH itself can remove free radicals directly. For instance, it can inactivate peroxynitrite by forming GSSG (Radi et al. 1991a). It removes the hydroxyl radical by generating the glutathiyl radical (reaction 3i), and two glutathiyl radicals then interact to form GSSG (reaction 3j) (Schöneich et al. 1992). The function of GSH to recycle the vitamin E radical in the presence of vitamin C has been indicated above. GSH is also important in maintaining the essential thiol status of proteins by preventing oxidation of thiol groups or by reducing disulfide bonds induced by oxidant stress (reaction 3k).

(3i) $GSH + R^{\bullet} \longrightarrow GS^{\bullet} + RH$

(3j) $2GS^{\bullet} \longrightarrow 2GSSG$

(3k) $Protein\text{-}SSG + GSH \longrightarrow Protein\text{-}SH + GSSG$

Recently, new evidence suggests that GSH is also involved in the activation of NF-κB, which is a redox sensitive process (Flohé et al. 1997). The addition of GSH to the culture medium during quinone-induced oxidative stress inhibits the activation of NF-κB (Pinkus et al. 1996). However, NF-κB is not activated when GSH is depleted in cells (Li et al. 1998). The exact mechanisms are still unclear. GSH also plays a major role in preventing the formation of advanced glycation endproducts (Jain 1998; Ortwerth and Olesen 1998), which are known to be associated with oxidative stress and affect cellular metabolism such as alteration of protein function.

In addition to acting as an antioxidant, GSH participates in the glutathione-S-transferase detoxification reaction by serving as a substrate to form S-substituted derivatives. GSH functions as a storage and transport form of cysteine, and is,

therefore, important in protein and DNA synthesis and cell proliferation. Furthermore, GSH may have effects on neuronal function in the CNS through interfering with signal transduction (Meister 1982; Meister and Anderson 1983; Cooper and Kristal 1997; Bains et al. 1998).

2.3.4 Changes in Brain GSH after Ischemia

Most work indicates that GSH concentration in the brain is changed under conditions of oxidative stress, such as cerebral ischemia. In a study by Rehncrona et al. (1980), it was found that the levels of total glutathione (both GSH and GSSG) in the cortex were decreased to a similar extent in complete and incomplete cerebral ischemia. In this study, 30, 60, and 120 min of ischemia decreased the cortical glutathione content to 87%, 77%, and 45%, respectively, of the control values, indicating that the rate of decrease in GSH levels was closely correlated with the period of ischemia. The cortical glutathione concentrations during reperfusion following 30 min of complete ischemia decreased further to about 80% of control values. These results are consistent with several other studies (Folbergrová et al. 1979; Cooper et al. 1980).

Inconsistent evidence was found by Zaiden and Sim (1996), who reported that GSH content in brain mitochondria remained unchanged during ischemia. However, mitochondrial GSH increased during the first 3 hours of reperfusion; it then declined and showed a significant reduction at 24 hours of reperfusion. Mitochondria are unable to synthesize GSH (Mårtensson et al. 1992), and the levels are maintained by uptake of GSH from the cytosolic pool. The increase in mitochondrial GSH accompanied by the decrease in cellular GSH content (Rehncrona et al. 1980; Cooper et al. 1980)

suggests that GSH moves from the cytoplasm to the mitochondria to provide the protection against oxidative attack during and following ischemia.

The fall in brain GSH during and following ischemia can be explained by several mechanisms. It may be attributed to the imbalance between GSH synthesis and degradation due to the depletion of ATP, which is required in the synthesis of GSH (Rehncrona et al. 1980). The movement of GSH from the cytoplasm to the mitochondria may partially account for the decrease in brain GSH under ischemia conditions. It has also been suggested that GSH has been converted to GSSG by GPx under oxidative stress (Shivakumar et al. 1995). However, several investigators (Folbergrová et al. 1979; Rehncrona et al. 1980; Cooper et al. 1980) failed to find any change in GSSG, while Shivakumar et al. (1995) reported a small increase in GSSG concentration in the brain after ischemia. The discrepancy may in part be because that the amount of GSSG in the brain is small and differences are difficult to quantify. Unchanged net GSSG during and following ischemia may also be the result of translocation of GSSG to extracellular fluids, although there is no evidence that GSSG content is increased in cerebrospinal fluid during ischemia (Rehncrona et al. 1980). On the other hand, glutathione reductase in the brain is highly active (Orlowski and Karkowsky 1976), and may account for an increased regeneration of GSH from GSSG. Finally, the formation of protein-GSH mixed disulfide in the brain after ischemia may contribute to the depletion of GSH. The amount of protein-GSH mixed disulfide is increased in brain regions following cerebral ischemia, which is accompanied by a corresponding loss in protein containing thiol groups (Shivakumar et al. 1995).

The decrease in brain GSH will render the brain cells more vulnerable to the oxidative stress generated during the ischemic insult. Identification of nutritional and pharmacological factors that could maximize GSH concentration may provide a novel

strategy to minimize brain injury induced by ischemia. Also, identifying nutrient deficiencies that may exacerbate the extent of brain damage by depleting brain GSH is of importance.

2.4 Glutathione

2.4.1 Glutathione Metabolism

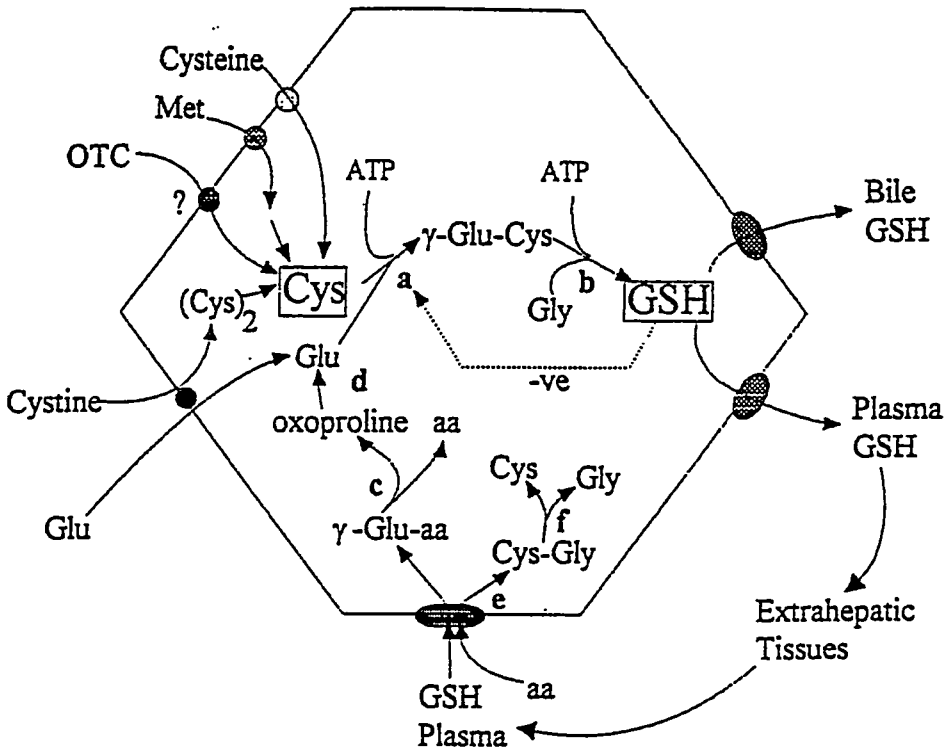
Stable intracellular GSH concentration is achieved by a balance between the rate of utilization by the GPx reaction, the rate of reduction of GSSG to GSH by glutathione reductase, the rate of formation of the conjugate catalyzed by glutathione-S-transferase, and the rate of *de novo* synthesis of GSH (Juurlink 1999).

The metabolism of GSH follows a process called the γ-glutamyl cycle (Figure 2.1), in which GSH is synthesized intracellularly in two ATP-requiring enzymatic steps (Meister 1982; 1988; 1995; Meister and Anderson 1983):

(4a) L-Glutamate + L-cysteine + ATP $\xrightarrow{\text{γ-glutamylcysteine synthetase}}$ γ-glutamylcysteine + ADP +Pi

(4b) γ-Glutamylcysteine + L-glycine + ATP $\xrightarrow{\text{GSH synthetase}}$ L-γ-glutamylcysteinylglycine +ADP + Pi

The first step of GSH synthesis is rate-limiting, and this is catalyzed by γ-glutamylcysteine synthetase (reaction 4a). γ-Glutamylcysteine synthetase is specific for the glutamyl moiety and is regulated physiologically by competitive feedback inhibition of GSH and the availability of its precursor, cysteine (Richman and Meister 1975; Lu 1998). Cysteine is the limiting amino acid for GSH synthesis (Tateishi et al. 1974). In the second step of GSH synthesis, glycine combines with γ-glutamylcysteine, and this



a = γ-glutamylcysteine synthetase
b = glutathione synthetase
c = γ-glutamyl cyclotransferase
d = oxoprolinase
e = γ-glutamyl transpeptidase
f = peptidase

Figure 2.1 γ-Glutamyl cycle of GSH synthesis and degradation.

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is catalyzed by GSH synthetase (reaction 4b). No evidence suggests that the availability of glutamate or glycine influence GSH synthesis, since they can be synthesized via several metabolic pathways in the liver (Bannai and Tateishi 1986).

The breakdown of GSH by the transpeptidase reaction provides substrates for GSH synthesis intracellularly. GSH degradation is initiated once GSH is taken up by extrahepatic cells, and it is catalyzed by γ-glutamyl transpeptidase, which is primarily located at the external surface of cell membranes (Hahn et al. 1978; Griffith and Meister 1979). γ-Glutamyl amino acids and cysteinylglycine are formed during this reaction (Figure 2.1, reaction e). Cysteinylglycine is further cleaved into glycine and cysteine by dipeptidase (Figure 2.1, reaction f). γ-Glutamyl amino acids are then converted into 5-oxoproline and free amino acids by γ-glutamyl cyclotransferase (Figure 2.1, reaction c). γ-Glutamate is formed by the reaction of 5-oxoprolinase, which is an ATP-requiring step (Figure 2.1, reaction d). These free amino acids including cysteine and glutamate provide the sources for intracellular GSH synthesis, as well as for intracellular protein synthesis (Meister and Anderson 1983).

Glutathione is a fairly ubiquitous substance in aerobic life forms. It exists in the tissues in both the reduced form (GSH) and the oxidized form, GSH disulfide (GSSG); the majority (99%) is in the reduced form (Meister and Anderson 1983). The synthesis of GSH mainly occurs in the liver, which has the highest content of GSH among the organs (Kaplowitz et al. 1983). The liver exports GSH predominantly into plasma by sinusoidal efflux at near-maximum rates, thereby providing a constant source of GSH for plasma. Subsequently, plasma GSH supports extrahepatic GSH levels in two ways: a) GSH can be hydrolyzed by brush border γ-glutamyl transpeptidase in extrahepatic tissues such as kidney and intestine to provide cysteine for intracellular GSH synthesis (Bray and Taylor 1993), and b) plasma GSH can be taken up intact by certain tissues

such as at the blood-brain barrier (Kannan et al. 1990). The specificity of uptake is determined by differences in the activity of γ-glutamyl transpeptidase among tissues.

In addition to being transported into plasma, hepatic GSH is also released into the bile by canalicular efflux and enters the small intestine, where it as well as GSH from the diet and desquamated epithelial cells can be cleaved into free amino acids and reabsorbed into the circulation (Taylor et al. 1996).

GSH turns over at a significant rate in many tissues, especially kidney, liver and pancreas (Griffith and Meister 1979). The half-life for turnover of GSH in the rat liver is 1-2 hours (Kaplowitz et al. 1983) whereas it is estimated to be 70 hours in the rat brain (Cooper and Meister 1993).

2.4.2 GSH in the Brain

The GSH content varies among tissues and may also vary among animal strains (Taniguchi et al. 1989). GSH concentration in the brain at 1-3 mM (Cooper and Meister 1993) is relatively low compared with that in the liver at 8-10 mM (Taylor et al. 1996), but is relatively high in comparison with that in the plasma (~15 μM) (Cooper and Kristal 1997) and cerebrospinal fluid (~25 μM) (Cooper and Meister 1993). Cultured astrocytes contain high levels of GSH whereas cultured neurons have low levels (Raps et al. 1989; Yudkoff et al. 1990; Makar et al. 1994; Thorburne and Juurlink 1996). These findings suggest that astrocytes are the major cells that produce GSH, which is supported by other studies in which GSH was primarily localized by mercury orange histochemistry in non-neuronal cells (Slivka et al. 1987; Philbert et al. 1991). Dringen et al. (1999) demonstrated that neurons were less efficient at scavenging radicals than astrocytes due to its low GSH content and GPx acitivity. Consistently, Iwata-Ichikawa et al. (1999) reported that glial cells protect neurons

against oxidative stress by transcriptional up-regulation of γ-glutamylcysteine synthetase for GSH synthesis. The cellular GSH in the brain exists primarily in the cytosol with 10-20% in the mitochondria (Reichelt and Fonnum 1969; Jain et al. 1991).

Although GSH can cross the blood-brain barrier intact (Kannan et al. 1990; Kaplowitz et al. 1996), suggesting the ability of the brain to take up GSH from blood, this is not the major source of brain GSH (Jain et al. 1991). Cysteine is easily taken up by the brain for GSH synthesis (Oldendorf and Szabo 1976). Furthermore, methionine and cystine, the disulfide form of cysteine, are also taken up into the brain (Oldendorf and Szabo 1976; Jain et al. 1991).

Enzymes involved in GSH metabolism including γ-glutamylcysteine synthetase, GSH synthetase, and γ-glutamyl transpepidase are active throughout the brain, but particularly higher in specific areas, such as choroid plexus (Tate et al. 1973; Okonkwo et al. 1974). γ -Glutamyl transpepidase is mainly located in the capillaries and choroid plexus (Albert et al. 1966; Tate et al. 1973; Okonkwo et al. 1974). Thus, GSH is actively metabolized in the choroid plexus (Anderson et al. 1989). These findings indicate that γ-glutamyl transpepidase might be involved in amino acid transport in the CNS, along with other enzymes of the γ-glutamyl cycle (Tate et al. 1973; Orlowski and Wilk 1975; Orlowski and Karkowsky 1976). Apparently, brain cells are capable of maintaining GSH concentration through the γ-glutamyl cycle, although the exact mechanisms have not been well documented.

Brain GSH appears to change with age. It is lowest in newborn rats and increases with age (Nanda et al. 1996). The level of GSH in the brain is decreased notably in aged rats compared with young adults under normal conditions (Chen et al. 1989; Ravindranath et al. 1989; Kudo et al. 1990; Benuck et al. 1995) or under oxidative stress (Benzi et al. 1989).

2.5 Dietary Influences on GSH Concentration

2.5.1 Sulfur Amino Acid Metabolism

While the enzymes of the GSH cycle and their regional specificities provide the basis for intracellular maintenance of tissue GSH concentration, diet and nutritional status can also play a role in optimizing GSH for antioxidant defense.

GSH concentration depends on the availability of cysteine, which is known to respond to dietary protein. Under physiological conditions, cysteine is derived mainly from diet or protein breakdown. In addition to dietary sources, cysteine can also be supplied from the cleavage of cystine. However, this is not a major source of cysteine since cystine is predominantly outside the cell, relatively insoluble in extracellular fluid, and it is poorly taken up into cells under physiological circumstances. Nevertheless, once it enters the cell, cystine is rapidly reduced to cysteine (Lu 1998). Alternatively, cysteine can be supplied by methionine metabolism through the trans-sulfuration pathway, which is a major pathway of methionine degradation (Figure 2.2).

The trans-sulfuration pathway is unique to the liver cell; other GSH-synthesizing systems lack this pathway (Finkelstein 1990). This methionine trans-sulfuration pathway is indeed to maintain methionine concentration in the liver rather than to supply cysteine. That is, when dietary methionine is limited, the activity of cystathionine synthetase (Figure 2.2, d) is reduced, resulting in less amounts of cysteine produced (Tateshi and Sakamoto 1983). Also, when cysteine and GSH levels are normal, less methionine is converted to cysteine due to the inhibition of cystathionine synthetase activity by its end product, cysteine (Tateishi et al. 1982;Finkelstein et al. 1986). Other mechanisms are also involved in maintaining methionine concentration, such as the increase of betaine-homocysteine

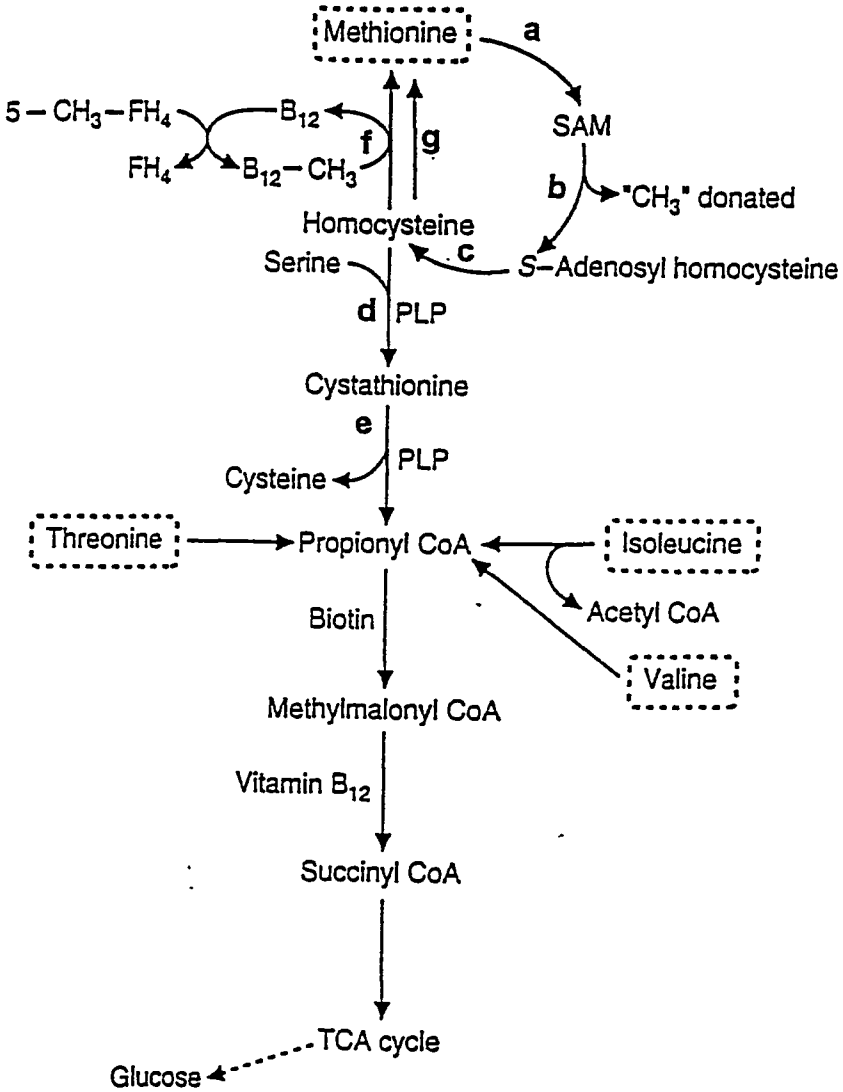

Figure 2.2 Methionine metabolism. Letters represent the following enzymes or reactions: a, methionine adenosyltransferase; b, S-adenosylmethionine-dependent transmethylation; c, adenosylhomocysteinase; d, cystathionine synthase; e, cystathionase; f, methylfolate-homocysteine methyltransferase; and g, betaine-homocysteine methyltransferase.

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methyltransferase (Figure 2.2, g) that catalyzes the conversion of homocysteine to methionine (Finkelstein and Martin 1984). Therefore, in contrast to cysteine, methionine concentration in the liver is relatively insensitive to changes in dietary protein intake or to fasting (Finkelstein et al. 1982).

Cysteine catabolism can occur by both cysteine sulfinate-dependent (transamination) and cysteine sulfinate-independent (direct oxidation) pathways (Figure 2.3). Both pathways lead to the production of pyruvate and sulfate, but only the former pathway can produce taurine (Bella et al. 1999). Cysteine dioxygenase catalyzes the first reaction in the cysteine sulfinate-dependent pathway by which cysteine is oxidized to cysteine sulfate. Two possible catabolic fates are followed, which are transamination to ultimately yeld pyrvate and sulfate by aspartate aminotransferase or decarboxylation to produce taurine by cysteine sulfinate decarboxylase (Bella and Stipanuk 1996).

2.5.2 Regulation of GSH by Diet

Tissue GSH concentration is regulated by diet and nutritional status in three ways: 1) availability of cysteine, the limiting substrate for GSH synthesis, 2) the activities of enzymes that synthesize GSH, such as γ-glutamylcysteine synthetase, and 3) the rate of uptake and efflux of GSH.

Nutritional status influences GSH synthesis in the liver, indicated by several studies in which hepatic GSH levels decrease significantly in rats subjected to starvation (Tateishi et al. 1977; Higashi et al. 1977; Cho et al. 1981). Refeeding fasted animals results in the return of liver GSH concentration to prefasting values (Cho et al. 1981). GSH concentration in the liver undergoes marked diurnal fluctuations, which depends on the food intake rhythm (Beck et al. 1958; Jaeschke and Wendel 1985).

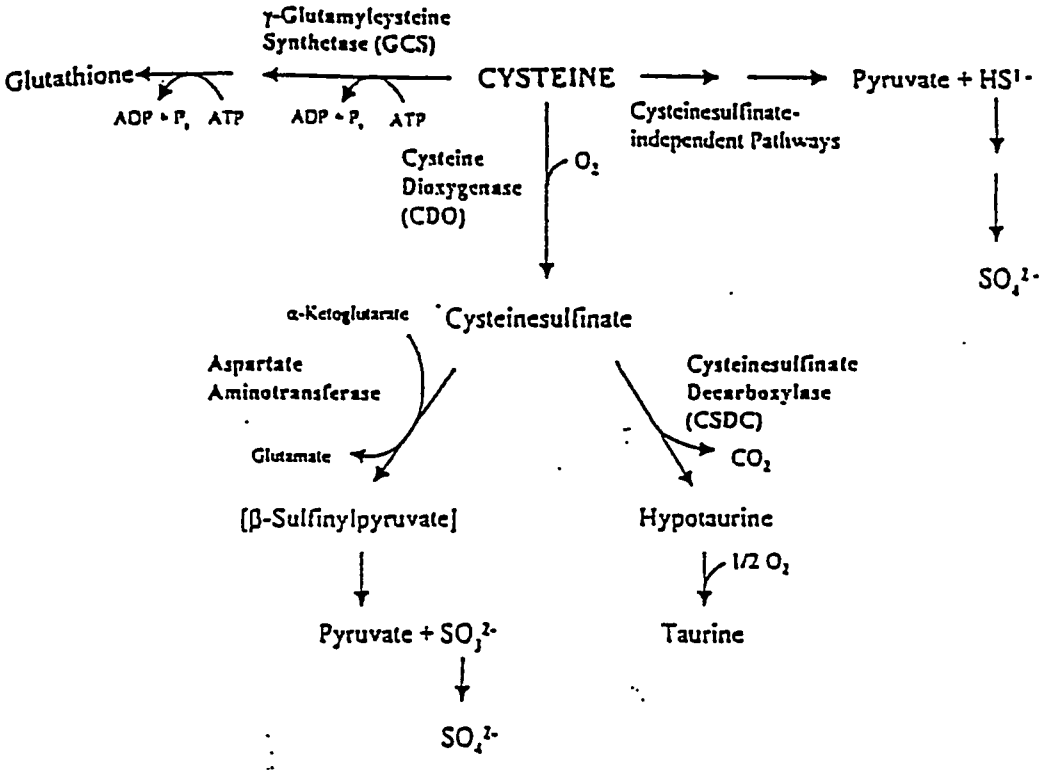

Figure 2.3 Pathways of cysteine metabolism.

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This phenomenon indicates that tissues collected for GSH analysis should be harvested at the same time of day to minimize the diurnal effect.

GSH concentration in the liver is also responsive to low dietary protein content (Bauman et al. 1988a; 1988b; Taylor et al. 1992; 1997; Rana et al. 1996). As dietary protein increases from 0 to 20% casein, hepatic and plasma GSH levels are elevated correspondingly (Hum et al. 1992), suggesting a close correlation between protein content in the diet and hepatic GSH level. Supplementation of sulfur amino acids to a diet low in protein can restore hepatic GSH concentration to the normal level. In a study by Bauman et al. (1988a), rats fed protein-deficient diets (4 and 7.5%) demonstrated a 50 to 60% reduction in hepatic GSH concentration compared with those fed the normal protein diet (15%). Supplementation of the 7.5% protein diet with cysteine, methionine or methionine hydroxy analog to be isosulfurous with the 15% protein diet resulted in an increase in GSH concentration to the level found in the control rats. Similar observations were reported by other investigators (Tateishi et al. 1977; Boebel and Baker 1983). These studies provide evidence that dietary protein regulates tissue GSH concentration by affecting cysteine availability.

GSH concentrations in other tissues are also influenced by protein content and sulfur amino acid level in the diet. Taylor et al. (1992) showed a depletion of GSH in other tissues such as the lung, heart, and spleen, in addition to the liver, of rats fed a 0.5% protein diet. Interestingly, this extremely low protein diet did not deplete GSH concentration in the liver below approximately 30% of that in the control rats (Taylor et al. 1992; 1997). Excess protein in the diet (30%, 40%, or 45%) does not further increase hepatic GSH concentration (Bauman et al. 1988a; b; Hum et al. 1992) beyond the maximum physiological range (8-10 μmol/g), suggesting that hepatic GSH is tightly

regulated. This is probably due to the feedback inhibition of γ-glutamylcysteine synthetase by tissue GSH concentration.

Brain GSH concentration may also be influenced by diet although to a lesser extent than liver GSH. Few studies have examined this question. A 30% depletion of brain GSH concentration was observed in rats fed a cysteine and methionine-free diet for 5 days (Tor-Agbidye et al. 1996). However, liver GSH concentration remained unchanged in this study, which is inconsistent with other studies (Bauman et al. 1988a; Hum et al. 1992; Taylor et al. 1997). In a similarly designed study, GSH concentration showed a 10-14% decline in the neocortex and thalamus of rats fed a sulfur amino acid-deficient diet, while liver GSH concentration was also depressed by 74% (Paterson et al. 1998).

The second regulatory point for tissue GSH concentration is the dietary impact on the activity of γ-gutamylcysteine synthetase. The activity is probably maintained during protein deprivation, since supplementation of the cysteine precusor, OTC, to rats fed a 0.5% protein diet rapidly returns hepatic GSH concentration to the normal level (Taylor et al. 1992). Bella et al. (1996) demonstrated that the activity of γ-gutamylcysteine synthetase was increased with low levels of dietary protein. Furthermore, the modulation of γ-gutamylcysteine synthetase by dietary protein occurs at multiple transcriptional and posttranscriptional levels, which was indicated as a high concentration of γ-gutamylcysteine synthetase mRNA, protein and activity in rats fed protein-deficient diets (Bella et al. 1999).

Finally, GSH concentration is also regulated by diet through an influence on GSH efflux and uptake mechanisms. The efflux rate of hepatic GSH in mice fed a low protein diet was significantly lower than that in the control group (Adashi et al. 1992). This may account for the 30% residual hepatic GSH concentration in rats fed a diet

extremely deficient in protein (Taylor et al.1992). However, conflicting results were observed by Kaplowitz et al. (1983) who reported an increase in efflux rate of hepatic GSH with dietary protein restriction. The exact mechanisms of GSH efflux during protein restriction remain to be discovered.

The activity of the GSH uptake enzyme, γ-glutamyl transpeptidase, is also important in maintenance of extrahepatic GSH concentration (Meister 1989). The study by Taylor et al. (1992) provides indirect evidence that GSH uptake mechanisms are maintained during protein deficiency since the decrease in extrahepatic tissue GSH is much less than that in the liver.

2.6 Glutathione Delivery Agents

2.6.1 Intracellular Cysteine Delivery Systems

Since the maintenance of brain GSH concentration is of key importance in cerebral ischemia, strategies have been devised to attempt to increase brain GSH. Supplementation of the diet with cysteine for GSH synthesis is one approach. However, excess cysteine is toxic when administrated orally or subcutaneously in moderate amounts to animals (Birnbaum et al. 1957; Harper et al. 1970; Olney et al. 1971; Karlsen et al. 1981). Supplementation of 13.6 g cysteine/100 g diet to a diet containing sufficient essential amino acids caused weight loss and death in weanling rats (Birnbaum et al. 1957). A review of older studies suggests that addition of cystine at 4-20% of the diet reduces growth and increases mortality, but the diets were not always nutritionally balanced (Harper et al. 1970). A single subcutaneous injection of cysteine at a dose of approximately 1.5 g/kg body weight to 10 day old mice led to necrosis in retina and hypothalamus (Olney et al. 1971) while administrating cysteine subcutaneously (1.2 g/kg body weight) to rats 4 days after birth was followed by brain

atrophy (Karlsen et al. 1981). The neurotoxic effect of cysteine is probably mediated through cysteine metabolites such as cysteic acid and cysteine sulfinic acid that have neuro-physiological properties similar to the neurotransmitter, glutamate (Karlsen et al. 1981).

GSH concentration may be enhanced after the administration of GSH (Vina et al. 1989; Hagen et al. 1990; Aw et al. 1991). Oral administration of GSH to fasted rats or mice with GSH depletion showed an increase in the GSH concentration in tissues such as small intestine, kidney, lung, and brain but not the liver (Vina et al. 1989; Hagen et al. 1990; Aw et al. 1991). However, this is not always the case (Vina et al. 1989; Mårtensson et al. 1990; Hagen et al. 1990; Aw et al. 1991), since the presence of γ-glutamyl transpeptidase and dipeptidase in the epithelial cell membranes limits the absorption of intact GSH into circulation.

Therefore, several compounds have been developed to provide cysteine for intracellular GSH synthesis. Among these, N-acetyl-L-cysteine (NAC), a compound used for treatment of acetaminophen toxicity, is found to deacetylate in vivo and provide cysteine for GSH synthesis. The location of deacetylation of N-acetyl-L-cysteine in tissues needs to be further investigated. Some side effects have also been observed when N-acetyl-L-cysteine was administered for treatment of overdosage of paracetamol. The major adverse reaction is anaphylaxis including rash, flushing, nausea, vomiting, angioedema, hypotension (Mant et al. 1984). Some have reported N-acetyl-L-cysteine to be less utilized than thiazolidine for hepatic cells increasing GSH levels (Williamson et al. 1982).

L-2-Oxothiazolidine-4-carboxylate (OTC), a novel intracellular L-cysteine delivery system, was developed based on studies of 5-oxoprolinase that converts 5-oxo-L-proline to L-glutamate in the γ-glutamyl cycle (reaction 6a) (Williamson and

Meister 1981; Anderson and Meister 1987). OTC is a 5-oxo-L-proline analog in which the 4-methylene group is replaced by a sulfur group (Figure 2.4). OTC is transported into most cells (Anderson 1997) where it reacts with 5-oxoprolinase to form the unstable intermediate, s-carboxycysteine, which rapidly produces cysteine by decarboxylation (reaction 6b).

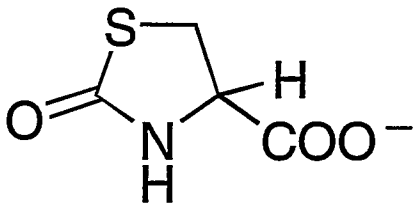


Figure 2.4 The structure of L-2-oxothiazolidine-4-carboxylate

(6a) 5-Oxoproline + ATP $\xrightarrow{\textit{5-oxoprolinase}}$ γ-glutamate + ADP + Pi

(6b) L-2-Oxothiaxolidine-4-carboxylate + $2H_2O$ + ATP $\xrightarrow{\textit{5-oxoprolinase}}$

S-carboxy-L-cysteine + ADP +Pi ⟶ L-cysteine + CO_2

2.6.2 Increase of GSH by OTC

Administration of OTC to animals previously depleted of hepatic GSH by fasting or by feeding a protein-deficient or sulfur amino acid-deficient diet, led to an increase in GSH concentration in the liver and other tissues such as the lung. Intraperitoneal injection of OTC to mice fasted overnight stimulated the formation of GSH in the liver to levels about twice those of fasted control rats (Williamsom and Meister 1981). A similar result was also observed by Moslen et al. (1988). Bauman et al. (1988b) demonstrated that dietary supplementation with OTC resulted in an increase in hepatic GSH concentration in rats fed a diet deficient in protein (7.5%). No change in hepatic

GSH has been found in rats on a normal diet (15% protein) following the administration of OTC.

Depleted GSH concentration in the liver of rats or chicks fed a cysteine-free diet that was adequate in methionine was increased by oral administration of OTC. However, OTC was utilized less efficiently than cysteine for growth and hepatic synthesis of GSH in rats (Chung et al. 1990). OTC also selectively increased GSH levels in other tissues. Oral administration of OTC to protein-energy malnourished rats could increase lung GSH concentration to provide an efficient protective mechanism against oxygen toxicity in lung (Taylor et al. 1992). Similarly, a study by Levy et al. (1998) demonstrated that OTC supplementation enhanced GSH levels in the lung of rats previously exposed to a severely protein-deficient diet. However, no correlation was found between OTC supplementation and the activities of GPx, SOD, and catalase (Levy et al. 1998).

An overshoot phenomenon has been observed in that the elevation in hepatic GSH concentration exceeded the physiological maximum level within 4 hours after administration of OTC to rats with depleted hepatic GSH stores (Taylor et al. 1992). The mechanism responsible for this phenomenon is unknown. It is possible that the feedback inhibition of γ-glutamylcysteine synthetase by GSH is delayed when OTC is administrated to rats with depleted GSH stores.

Total brain GSH concentration is increased after injection of OTC to rats fasted overnight, although the extent of change was much less than in the liver (Anderson and Meister 1989). In this study, various brain regions of rats (cerebellum, hypothalamus, cortex, brain stem, pons, caudate nucleaus) showed a 12 to 25% increase in GSH content; the cortex had the most significant change in GSH content. In another study, total GSH level in the brain was increased at 2 hours and reached a

peak at 4 hours after subcutaneous injection of OTC to the normal rats (Mesina et al. 1989). However, Pileblad and Magnusson (1992) were unable to find the same result. The discrepancy may be due to methodological errors in the analysis of GSH. Recently, Kamencic et al. (1999) reported that injection of OTC shortly after spinal cord trauma resulted in a significant repletion of GSH in spinal cord.

OTC is also being studied in clinical trials in diseases in which the generation of free radicals is believed to play a role in the development of disease, such as adult respiratory distress syndrome (ARDS) (Bernard et al. 1997), coronary artery disease (Vita et al. 1998), and chronic renal failure (Moberly et al. 1998). The therapeutic effect of OTC on HIV replication has been extensively investigated recently in both in vitro and in vivo studies. These studies demonstrate the potential of OTC to inhibit HIV expression and HIV replication in human peripheral blood mononuclear cells (Lederman et al. 1995; Ho et al. 1997). In human trials, whole blood GSH concentrations were increased by administration of OTC in HIV-infected individuals (Kalayjian et al. 1994; Barditch-Crovo et al. 1998).

These studies implicate OTC as a new treatment for stroke patients with low brain GSH levels. The compound may be particularly beneficial in protein-compromised patients if it can be shown that protein status alters brain GSH metabolism.

2.7 Stroke Incidence and Mortality Related to Protein Intake

2.7.1 Epidemiological Evidence

A series of epidemiological studies suggest a protective effect of dietary protein on stroke incidence and mortality. Stroke, which once ranked first among all causes of death in Japan, is now the third leading cause following cancer and heart disease

(Kodama 1993). An investigation of dietary changes from 1955 to 1990 indicated that the consumption of total animal protein had increased accompanied by a decline in stroke mortality (Shimamoto et al. 1989; Mizushima and Yamori 1992; Kodama 1993). An analysis of geographic distribution of cerebrovascular disease mortality and food intakes in Japan found that stroke mortality was negatively related to dietary protein mainly from animal sources (Omura et al. 1987).

A study comparing stroke incidence in Japanese men living in Hawaii and Japan showed that incidence was lower in Hawaii where protein consumption was higher than in those living in Japan (Takeya et al. 1984). An inverse relationship between protein intake and stroke incidence was also found in Japanese men in Hawaii in two other follow-up studies (Kagan et al. 1985; Lee et al. 1988).

Like Japan, stroke incidence in China is among the highest in the world. Recently, there has been a remarkable decline in stroke incidence in this country (Cheng et al. 1995). A 5 year survey undertaken in urban and rural areas of Shijiazhuang, China showed that the incidence of stroke in urban areas was lower than that in rural areas; on the contrary, the animal protein intake is higher in urban areas (Zhang 1993). Such long-term epidemiological studies in western and developed countries have not examined this relationship although stroke incidence and mortality has been declining in countries such as Canada (Petrasovits and Nair 1994). In a 12 year follow-up study of women in Gothenburg, Sweden, no correlation was found between protein intake and stroke mortality and incidence (Lapidus et al. 1986). Also, a 12-year study failed to report this relationship in a southern California cohert (Khaw and Barrett-Connor 1987). This might be due to the different food patterns between western and eastern countries. Over the past 20 years, the proportion of protein in the Asian diet has been increased and the protein sources have been changed from rice

and beans such as soybean to more animal protein (Shimamoto et al. 1989; Mizushima and Yamori 1992). Such changes have not occurred in western countries.

2.7.2 Evidence from the Stroke-Prone Spontaneously Hypertensive Rat

Data from the stroke-prone spontaneously hypertensive rat also provide supportive evidence that dietary protein has a protective effect on stroke incidence and mortality. The stroke-prone spontaneously hypertensive rat was separated from the original genetic strain of spontaneously hypertensive rat (Okamoto and Aoki 1963) by Okamoto et al. (1974). This strain of rat displays a significantly higher blood pressure than the spontaneously hypertensive rat and has an extremely high incidence of stroke during their first year of life. The stroke-prone spontaneously hypertensive rat is now used as one of the most suitable models for studying stroke in humans. It was noted that a high protein diet could ameliorate the adverse effects of excess salt intake on blood pressure and, therefore, decrease the incidence of stroke in stroke-prone spontaneously hypertensive rat (Yamori et al. 1977; 1979). Another study by Yamori et al. (1984) indicated that the stroke-prone spontaneously hypertensive rat had a higher incidence of stroke when fed the diet used in Japan than those fed the diet used at the National Institutes of Health in the U.S. The analysis of these two diets showed that the American diet contained a higher proportion of protein. In a recent study, Sarwar et al. (1999) reported that stroke-prone spontaneously hypertensive rat fed a 10% casein diet had a shorter survival rate (77 days) than that of those fed a 20% casein diet (96 days). Death due to stroke was significantly earlier in animals fed a 10% casein diet compared to those fed a 20% or 40% casein diet.

2.7.3 Protein Status in Stroke Patients

Protein deficiency occurs as a result of insufficient food intake in most cases, and this is frequently associated with a deficiency of energy and other nutrients. Recently, the term protein-energy malnutrition (PEM) is often used instead of protein deficiency to describe these conditions that range from mild to severe. The physical features of mild and moderate PEM include growth retardation, poor musculature, edema, thinness, fragile hair, skin lesions and immune system disorders. Major biochemical changes of PEM are low serum albumin and hormonal imbalance (Latham 1990).

Severe PEM will eventually develop into one of two diseases, kwashiorkor or marasmus, which have very different appearances. Kwashiorkor, characterized by edema, stunting of growth, and wasting of muscles, is caused by severe protein deficiency with moderate energy deficit. Marasmus, which has features including extreme thinness and failure to grow, is the result of both severe protein and energy deficiency (Latham 1990). Severe protein deficiency rarely happens in North America, except under conditions such as malabsorption, infections such as AIDS, renal and hepatic diseases, malignancies, and anorexia nervosa (Latham 1990). PEM has been frequently seen in starving children in poor countries.

Mild to moderate PEM is also not uncommon in both free-living and institutionalized elderly persons in North America. Elderly persons have difficulty in meeting the needs of nutrients that are not decreased with age as is energy requirement (Ausman and Russell 1994). A number of factors may contribute to inadequate intake, such as changes in eating function, depression, chronic diseases, decreased activity, usage of medicine, and inadequate access to food (Lipschitz 1991; Abbasi and Rudman 1994). This situation is more severe in hospitalized or institutionalized elderly persons. It has been reported that 30-40% of elderly patients

admitted to medical units in North America were found to have PEM (Constans et al. 1992). Only 15 out of 49 patients on admission to a rehabilitation unit met protein and energy requirements (Newmark et al. 1981). Similar results were reported by other investigators (Mowé and Bohmer 1991; Mühlethaler et al. 1995; Potter et al. 1995). Furthermore, PEM has a strong independent correlation with 1 year (Sullivan and Walls 1994; Sullivan et al. 1995) and 4.5 year (Mühlethaler et al. 1995) post-discharge morbidity in elderly patients.

Protein-energy status can be assessed by anthropometric and biochemical data. Weight, height, mid-arm circumference, triceps and biceps skinfolds are measured as anthropometric indicators. Biochemical indices of protein status include: 1) somatic protein status such as urinary creatinine excretion and 3-methylhistidine, 2) visceral protein status including total serum protein, and serum levels of albumin, transferrin, retinol-binding protein and prealbumin, 3) metabolic changes such as serum amino acid ratio and nitrogen balance, 4) muscle function tests such as skeletal muscle function after electrical stimulation and hand grip strength, and 5) immunological tests such as lymphocyte count (Gibson 1990). Among these laboratory indices, serum albumin and transferrin are most frequently used to determine protein status in hospital assessment (Gibson 1990).

Poor protein status has been reported among patients at the time of a stroke. In the past, most studies have focused on poor nutritional status in stroke patients as a result of cognitive deficits and hemiparesis occurring secondary to the stroke. These studies demonstrated suboptimal protein-energy status following stroke (Aptaker et al. 1994; Finestone et al. 1995; Finestone et al. 1996). Fewer studies have examined nutritional status at the time of stroke occurrence. It was found that 16 out of 100 patients with acute stroke showed poor protein status, assessed by anthropometric

indicators and three circulating proteins (albumin, transferrin, and prealbumin), on admission to the hospital (Axelsson et al.1988). Patients aged 75 years or older and female patients showed a particularly high prevalence of poor protein status in this study. Dávalos et al. (1996) also reported that 16.3% of patients with an acute stroke had PEM when admitted to the hospital. A more recent survey showed a similar result in which the prevalence of the suboptimal nutritional status was high among stroke patients within 48 hours of admission to the hospital (Gariballa et al. 1998). Serum albumin level also appears to be a predictor of death (Gariballa et al. 1998) and functional outcome (Aptaker et al. 1994) following acute stroke. Older stroke patients with a lower serum albumin level had a higher frequency of medical complications and lower improvement scores.

The nutritional care in the immediate period following stroke is not well documented. A high rate (49%) of malnutrition among stroke patients transferring from the hospital to rehabilitation services was reported (Finestone et al. 1995), suggesting poor nutritional intervention for patients with acute stroke that may aggravate stroke outcome. In addition to other medical therapies, nutritional intervention may also play a role in the outcome of stroke.

Chapter 3 Materials and Methods

3.1 Animals and Diets

Twenty-five male, weanling Long-Evans rats (weighing 50-55 g) were obtained from Charles River (St. Constant, Quebec, Canada). They were placed in a room with temperature controlled at 20-22°C and light from 7:00 h to 19:00 h. Animal care met the requirements of the Canadian Council on Animal Care (CCAC) (1993).

After acclimatization for 2 days on the control diet and tap distilled water *ad libitum*, the rats were randomly assigned to three experimental groups and housed individually in suspended stainless steel cages. The control diet was modified from the AIN-93G diet (Reeves et al. 1993). The ingredients of the control diet are shown in Table 3.1. Vitamin free casein (89.3% protein) was used as the protein source. The amino acid composition of the three diets is shown in Table 3.2. The antioxidant, tert-butylhydroquinone, was left out of the diets to avoid the possibility that this compound could mask the effect of experimental treatment.

The experimental groups were 1) the moderately protein-deficient group (-PRO) (n = 8) fed a diet formulated to contain 8.4% casein (7.5% protein), 2) the OTC-supplemented group (+OTC) (n = 8) fed a protein-deficient diet (8.4% casein) supplemented with 0.453% OTC, which was equivalent in sulfur content to the control diet, and 3) the protein-adequate (control) group (+PRO) (n = 9) fed the diet containing 20% casein (17.9% protein). To confirm protein concentration in the diets, the protein content of the protein-deficient, OTC-supplemented, and protein-adequate diets were

Table 3.1 Composition of three experimental diets[1]

Components (g/kg diet)	Protein-adequate	Protein-deficient	OTC-supplemented
Vitamin free casein[2]	200	84	84
Cornstarch	397.486	397.486	397.486
Dyetrose[3]	132	132	132
Sucrose	100.014	217.754	213.224
Cellulose	50	50	50
Soybean Oil	70	70	70
Mineral Mix[4]	35	35	35
Vitamin Mix[5]	10	10	10
Choline Bitartrate	2.5	2.5	2.5
L-Cystine	3	1.26	1.26
OTC	0	0	4.53

[1]Diets were purchased from Dyets (Bethlehem, PA, USA).
[2]Vitamin free casein contained 89.3% protein.
[3]Dyetrose (Dyets, ethlehem, PA, USA) is dextrinized cornstarch containing 90-94% tetrasaccharides.
[4]Mineral mix prepared in sucrose supplied the following concentration of minerals in g/kg mix: calcium carbonate, 357; potassium dihydrogen phosphate, 196; potassium citrate monohydrate, 70.78; sodium chloride, 74; potassium sulfate, 46.6; magnesium oxide, 24; ferric citrate, U.S.P., 6.06; zinc carbonate, 1.65; manganous carbonate, 0.63; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenate, 0.01025; ammonium paramolybdate tetrahydrate, 0.00795; sodium metasilicate nonahydrate, 1.45; chromium potassium dodecahydrate, 0.275; lithium chloride, 0.0174; boric acid, 0.0815; sodium fluoride, 0.0635; nickel carbonate, 0.0318; ammonium vanadate, 0.0066.
[5]Vitamin mix prepared in sucrose supplied the following concentration of vitamins in g/kg mix: niacin, 3; calcium pantothenate, 1.6; pyridoxine hydrochloride, 0.7; thiamine hydrochloride, 0.6; riboflavin, 0.6; folic acid, 0.2; biotin, 0.02; vitamin E acetate (500 IU/g), 15; vitamin B_{12} (0.1%), 2.5; vitamin A palmitate (500,000 IU/g), 0.8; vitamin D_3 (400,000 IU/g), 0.25; vitamin K_1/dextrose mix (10 mg/g), 7.5.

Table 3.2 Amino acid composition of experimental diets

Components (g/kg diet)	Protein-adequate	Protein-deficient	OTC-supplemented
Alanine	4.64	1.95	1.95
Arginine	6.43	2.70	2.70
Aspartate	11.61	4.88	4.88
Cysteine	0.71	0.30	0.30
Glutamate	37.15	15.60	15.60
Glycine	3.21	1.35	1.35
Histidine	4.64	1.95	1.95
Isoleucine	8.57	3.60	3.60
Leucine	15.72	6.60	6.60
Lysine	13.22	5.55	5.55
Methionine	4.64	1.95	1.95
Phenylalanine	8.93	3.75	3.75
Proline	20.90	8.78	8.78
Serine	9.64	4.05	4.05
Threonine	6.79	2.85	2.85
Tyrosine	9.47	3.98	3.98
Tryptophan	2.14	0.90	0.90
Valine	10.18	4.28	4.28
Cystine	3.00	1.26	1.26

analyzed using the Copper Catalyst Kjeldahl method (Kane 1984) in the Department of Animal and Poultry Science (University of Saskatchewan), and found to be 7.48%, 7.44%, and 17.45%, respectively. The diets were prepared by Dyets (Bethlehem, PA, USA). OTC (L-2-oxothiazolidine-4-carboxylate) powder was supplied by Sigma Chemical Company (St. Louis, MO, USA). The rats in each group had free access to the assigned experimental diet and tap distilled water for 41-43 days. Daily feed intake and weekly body weights were monitored.

3.2 Sample Collection

Tissues were collected on days 41-43 at the same time each day (08:30 AM-12:30 AM) to minimize the effect of the diurnal rhythm on GSH concentration. All three groups were included in random order in each day's collection. Rats were weighed before sacrifice. They were anaesthetized with isoflurane (Isoflo®, Abbott Laboratories) and killed by transcardial perfusion with phosphate buffered saline to eliminate blood contamination of tissues. Livers were removed rapidly, frozen in liquid nitrogen and stored at -70°C until analysis. After decapitation of the animals, brains were immediately sliced at approximately 2mm thickness and dissected on ice to collect striatum, hippocampus, thalamus, neocortex, cerebellum, and brain stem. Brain regions were identified using the rat brain atlas (Paxinos and Watson 1998). The brain sections were frozen in liquid nitrogen and stored at -70°C.

3.3 High Performance Liquid Chromatography Assay of GSH

The method used in this study was first developed by Komuro et al. (1985) and further modified by Katrusiak (1998) and Paterson et al. (1998). The principle of this method is based on the reaction of free thiol groups in proteins with 5, 5'-dithiobis(2-

nitrobenzoic acid) (DTNB), also called Ellman's reagent (Figure 3.1), to give the DTNB-thiol derivatives. Subsequently, DTNB-thiol derivatives are separated by reverse-phase high performance liquid chromatography (HPLC) and detected at 330 nm ultraviolet light. This procedure allows quantitative, sensitive, reliable, and precise measurements of thiol concentration such as GSH (Komuro et al. 1985). Cysteine was also eluted by this method to provide evidence for the protein-deficient status of animals.

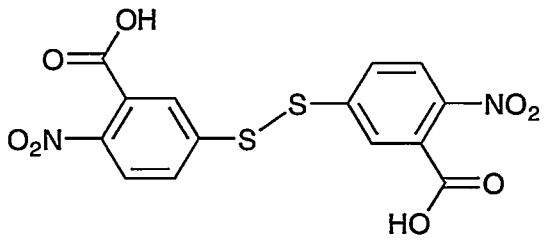


Figure 3.1 The structure of 5, 5'-dithiobis(2-nitrobenzoic acid)

3.3.1 Preparation of Tissue Extracts

Prior to assay, approximately 0.15 g of liver was weighed and homogenized in 10 volumes of cold 5% 5-sulfosalicylic acid containing 0.1mM ethylenediaminetetraacetate, disodium salt (EDTA). The homogenization was performed 5 times at intervals of 6 seconds using a Brinkmann® Polytron Model PT10-35 at speed 6. The brain sections were weighed and homogenized in the same cold solution 5 times at intervals of 6 seconds using a Skil® Pistol Grip Drill Model 6225. The homogenate was kept on ice all the time. The homogenate was then centrifuged in a refrigerated Eppendorf® centrifuge Model 5415C at 13,800 × g for 15 min. The supernate was stored at -70°C until HPLC analysis. The final dilutions for the liver and brain tissues were 1:200 and 1:50, respectively.

3.3.2 Preparation of Reagents

For chromatography of the thiol derivatives, two mobile phases were used. The first mobile phase contained 12.5% methanol (v/v) and 100 mM KH_2PO_4 (pH = 3.85). It was prepared from a stock solution of 0.5 M KH_2PO_4 (adjusted to pH 3.85 with orthophosphoric acid), HPLC grade methanol and deionized, distilled water. The second mobile phase consisted of 40% methanol (v/v) and 100 mM KH_2PO_4 (pH = 3.85). Both mobile phases were filtered before use using a nylon membrane filter with a pore size of 0.45 microns.

A solution of DTNB was prepared by dissolving 396 mg of DTNB in 100 ml of 0.5 M K_2HPO_4 (pH = 7.2). A solution of 5% (w/v) 5-sulfosalicylic acid containing 0.1 mM EDTA was made by dissolving 25 g of 5-sulfosalicylic acid and 0.0186 g of EDTA in 500 ml of deionized, distilled water.

A solution of 7 M orthophosphoric acid was prepared by mixing equal volumes of 85% H_3PO_4 and deionized, distilled water. A solution of 0.5 M Tris-HCl buffer was prepared by dissolving 60.57 g of tris(hydroxymethy)amino-methane in 1 L of deionized, distilled water and adjusting pH to 8.9 with HCl.

3.3.3 Standard Solutions

Internal standard solution (400 µM penicillamine) was made by dissolving D-penicillamine in 5% 5-sulfosalicylic acid containing 0.1 µM EDTA and stored at -20°C.

Standards were prepared from the standard stock solution consisting of 100 µM GSH, 25 µM cysteine, 45 µM dl-homocysteine, and 80 µM cysteinylglycine in 5% 5-sulfosalicylic acid containing 0.1 mM EDTA. A standard curve was generated on a daily basis by 4 standard samples diluted from the stock solution (1:1, 1:2, 1:5, and 1:10 dilution) with 5% 5-sulfosalicylic acid containing 0.1 mM EDTA.

3.3.4 Derivatization

Free reduced thiols (such as GSH, cysteine) were analyzed by reacting with DTNB to form thiol-DTNB derivatives that can be detected by ultraviolet light at 330 nm. The reaction mixture was made up of 0.5 ml of Tris-HCl buffer (pH = 8.9), 0.13 ml of sample or standard, 0.02 ml of 400 μM penicillamine, 0.35 ml of 10 mM DTNB, and 0.05 ml of 7 M H_3PO_4. Orthophosphoric acid was added slowly to the mixture with vortexing to minimize precipitation. The derivatization was performed in duplicate for tissue samples (both the liver and brain sections) to analyze GSH and cysteine concentration.

3.3.5 Chromatography

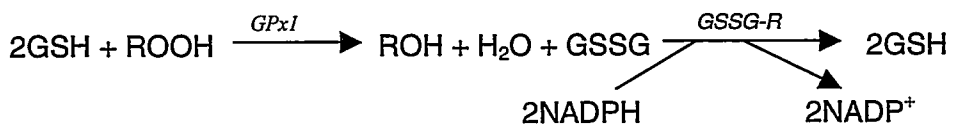
GSH analysis was accomplished with a Shimadzu® (Tokyo, Japan) HPLC system including an X-Act 4-channel degasser, an LC-10AT solvent delivery system, a syringe unit, an SCL-10A system controller, an SIL-10A automatic sample injector, and an SPD-10A variable wavelength spectrophotometric detector. Chromatography of the thiol-DTNB derivatives were performed on a Supelco® (Bellefonte, PA, USA) LC-18-T column (150 x 4.6 mm, 3 μm) at 38°C in a water bath. This was preceded by a Supelguard® LC-18-T precolumn (2 cm). The column was first equilibrated with mobile phase containing 12.5% methanol and 100 mM KH_2PO_4 at a flow rate of 1.1 ml/min. After injection of 50 μL of reaction mixture, isocratic elution with this mobile phase was applied for 9 minutes followed by mobile phase containing 40% methanol and 100 mM KH_2PO_4 for 7 minutes to elute excess DTNB reagent from the column. The column was then regenerated by running with the initial mobile phase for 12 minutes before the next injection of sample. The free thiol-DTNB derivatives absorbed ultraviolet light at 330 nm. Data were collected digitally and analyzed by Shimadzu® Ezchrom version

3.2 chromatography software. Peaks and their retention times were identified automatically based on the standard. Adjustment was made manually to confirm peak identities and baselines. Peak areas were calculated automatically.

A thiol standard curve was obtained by plotting the concentration of thiol standards against peak area ratio of thiol standard to the internal standard. The standard curve was run in duplicate daily. The unknown concentration of GSH or cysteine in the liver and brain regions was determined according to the corresponding thiol standard curve.

3.4 GPx1 Activity Assay

The activity of GPx1 was analyzed in the liver by the coupled enzyme procedure modified from that of Prohaska and Ganther (1976). This assay used cumene hydroperoxide as substrate in the presence of GSH, glutathione reductase (GSSG-R), and α-nicotinamide adenine dinucleotide phosphate (NADPH). The enzymatic reaction was initiated by addition of cumene hydroperoxide. The conversion of NADPH to $NADP^+$ was continuously recorded over 3 minutes as the change in absorbance at 340 nm. This procedure was performed using a microtitre plate technique.



3.4.1 Preparation of Tissue Extracts

The preparation of liver homogenate was the same as that for HPLC analysis as described above. However, the homogenization buffer used contained 0.1 M KCl,

0.02 M KH_2PO_4, and 0.001 M EDTA (pH = 7). The final dilution of liver samples for GPx1 activity assay was 1:1000. The supernate was stored at -70°C before the enzyme assay.

3.4.2 Preparation of Reagents

GSH solution (22 mM) was made by adding 67.6 mg of GSH to 10 ml of incubation buffer, a 0.1M KH_2PO_4 buffer (pH = 7) containing 1 mM EDTA. NADPH stock solution (1.1 mM) was prepared by dissolving 9.2 mg of NADPH powder in 10 ml of 0.1% $NaHCO_3$ buffer (pH = 9). Mercaptosuccinic acid (6.6 mM), an inhibitor of GPx1, was prepared by mixing 24.8 mg of mercaptosuccinic acid with 25 ml of incubation buffer. GSH, NADPH, and mercaptosuccinic acid solution were stored at -70°C. GSSG-R solution was prepared by adding 1.23 µl of a stock solution containing 3,900 Unit/ml GSSG-R to each ml of incubation buffer. Cumene hydroperoxide (1 mM) was made by mixing 1.9 µl of 80% cumene hydroperoxide stock solution with 10 ml incubation buffer. The cumene hydroperoxide and GSSG-R solution were prepared fresh for the assay.

3.4.3 Standard Curve of NADPH

Four NADPH solutions were prepared for the generation of a NADPH standard curve, including one blank and three standards diluted from the 1.1 mM NADPH stock solution (1:100, 1:20, and 1:10) with incubation buffer. NADPH concentration in standards expressed as nmol/ml was calculated by dividing NADPH absorbance by its molar absorption coefficient of 6200 $M^{-1}cm^{-1}$ (Paglia and Valentine 1967). NADPH absorbance was measured by a spectrophotometer (IEC® MicroMax) at 340 nm. To microtitre wells were added 330 µl of each standard in duplicate. The absorbance of

NADPH standard was recorded by a microplate spectrophotometer (SPECTRA max® 340, Sunyvale, CA) at 340 nm using endpoint mode. The data were collected by SOFT max® PRO software. The standard curve of NADPH was then obtained by plotting the concentration of NADPH standards against its absorbance by SOFT max® PRO software.

3.4.4 Enzymatic Assay

A mixture of 100 µl of incubation buffer, 100 µl of liver homogenate, 30 µl of 22 mM GSH, 30 µl of 4.8 units/ml GSSG-R, and 30 µl of 1.1 mM NADPH was added to the microtitre plate in triplicate. The mixture was incubated for 10 minutes at room temperature. The enzymatic reaction was initiated by adding 40 µl of 1 mM cumene hydroperoxide to the mixture. The final concentrations of GSH, NADPH, and cumene hydroperoxide in the assay mixture were 2 mM, 0.1 mM, and 0.12 mM, respectively, and the mixture contained 0.44 Units/ml GSSG-R. The absorbance of each mixture in the well was read at 340 nm every minute for 3 minutes and the change was recorded using kinetic mode in a microplate spectrophotometer (SPECTRA max® 340). The data were collected and analyzed by SOFT max® PRO software.

The difference of NADPH absorbance per minute (Vmax) was converted to NADPH concentration according to the standard curve. This result was divided by protein concentration present per well to obtain GPx1 activity in the absence of the GPx inhibitor, mercaptosuccinic acid.

For measurement of background conversion of NADPH to $NADP^+$, a mixture of 80 µl of incubation buffer, 100 µl of liver homogenate, 30 µl of 22 mM GSH, 30 µl of GSSG-R solution, 30 µl of 1.1 mM NADPH, 40 µl of 1 mM cumene hydroperoxide, and 20 µl of 6.6 mM mercaptosuccinic acid was used for assay. The final concentration of

mercaptosuccinic acid in the assay mixture was 400 μM. Thus, the actual GPx1 activity was calculated by subtracting assay results with GPx inhibitor present from the results without GPx inhibitor.

3.5 Protein Carbonyl Content Assay

Protein carbonyl content in the liver, neocortex, hippocampus, thalamus, striatum, cerebellum, and brain stem was analyzed as a marker of oxdative damage to cell proteins according to a method that was originally established by Levine et al. (1990) and further modified by Folbergrová et al. (1993). This method is based on the reaction of protein carbonyl groups with 2, 4-dinitrophenylhydrazine (DNPH) (Figure 3.2), a classic carbonyl reagent, to form protein-bound hydrazone that can be determined spectrophotometrically by its absorbance at 365 nm. The DNPH technique provides a nonradiochemical and sensitive assay (Levine et al. 1990).

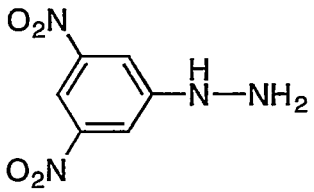


Figure 3.2 The structure of 2, 4-dinitrophenylhydrazine

3.5.1 Preparation of Tissue Homogenate

A small piece of the liver (approximately 0.15 g) was weighed and transferred into a plastic tube containing 10 volumes of 10 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH = 7.4), 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, and 0.6 mM $MgSO_4$. The HEPES buffer also contained protease inhibitors including leupeptin (0.5 μg/ml), pepstatin A (0.7 μg/ml), phenylmethylsulfonyl fluoride

(40 μg/ml), aprotinin (0.5 μg/ml), and 1.1 mM EDTA to prevent proteolysis of oxidized proteins. Preparation of tissue extracts was as follows: the liver tissue was disrupted 5 times at intervals of 6 seconds using a Brinkmann® Polytron Model PT10-35 at a speed of 6. For homogenization of the brain, the brain section was weighed and homogenized in the cold HEPES buffer 5 times for periods of 6 seconds each using a Skil® Pistol Grip Drill Model 6225. The homogenate was kept on ice all the time. The insoluble cellular debris was removed by centrifugation in a refrigerated Eppendorf® centrifuge Model 5415C at a speed of 13,800 × g for 15 min. The supernate was used for determination of protein carbonyl content. The final dilutions for the liver and brain tissues were 1:50 and 1:25, respectively.

3.5.2 Preparation of Reagents

A solution of 10% (w/v) streptomycin sulfate was prepared by dissolving 1 g of streptomycin sulfate in 10 ml of 50 mM HEPES buffer (pH = 7.2). A solution of 0.2% (w/v) 2, 4-dinitrophenylhydrazine (DNPH) was made in 2 M HCl. Trichloroacetic acid solution (20% w/v) was prepared by dissolving 20 g of TCA in 100 ml of deionized, distilled water. A solution of 6 M guanidine-HCl solution was made by mixing 28.659 g of guanidine with 20 mM NaH_2PO_4 buffer (pH = 6.5) to a volume of 50 ml.

3.5.3 Derivatization

Nine volumes of liver or brain homogenate were treated with one volume of 10% streptomycin sulfate (final concentration at 1%) at room temperature for 15 minutes. This step precipitated nucleic acids that also contain carbonyl groups and thus can give falsely high values in the assay. Nucleic acids were removed by centrifugation in a refrigerated Pharmacia® centrifuge at 11,000 × g for 10 minutes.

The clear supernate was divided into three equal aliquots (250 µl) containing approximately 500 µg of protein. One aliquot of clear supernate (blank) was treated with an equal volume of 2 M HCl and the other two (duplicates) with an equal volume of 0.2% DNPH in 2 M HCl. All samples covered with foil were incubated at room temperature for 1 h with vortexing every 10 to 15 minutes. The proteins were precipitated by mixing with an equal volume of 20% trichloroacetic acid, and collected by centrifugation in a refrigerated centrifuge at 11,000 × g for 5 minutes. The supernate was discarded and the pellet was washed three times with ethanol/ethyl acetate (1:1) to remove excess DNPH. The final dry precipitate was redissolved in 250 µl of 6 M guanidine-HCl in 20 mM NaH_2PO_4 buffer (pH = 6.5) in a water bath at 37°C for 20 to 30 minutes. The absorbance of protein-bound DNPH derivatives was recorded by a microplate spectrophotometer (SPECTRA max® 340) at 365 nm using endpoint mode.

The values of DNPD-treated samples were corrected by subtraction of the corresponding blank values. The protein carbonyl content was expressed as nanomoles of carbonyl groups per mg of protein using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$ (Levine et al. 1990). The exact amount of redissolved protein added to the well of the microtitre plate determined the length of the light path used for calculation.

3.6 Protein BCA Assay

The concentration of soluble protein in the liver and various brain regions was determined by the Pierce Bicinchoninic acid (BCA) method (Smith et al. 1985).

3.6.1 Preparation of Tissue Homogenate

The procedure for homogenization of the liver and brain samples was as described in Section 3.6.1. The final dilution of supernate for the BCA assay was 1: 250 for the liver and 1: 50 for brain tissue.

3.6.2 Preparation of Solutions

Protein standards used were 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/ml using bovine serum albumin. Copper sulfate solution (4%) was prepared by dissolving 4 g of copper sulfate in 100 ml deionized, distilled water. Working BCA solution contained one part of 4% copper sulfate solution and 50 parts of bicinchoninic acid (BCA) solution.

3.6.3 BCA Assay

To microtitre wells were added the blank, protein standards, and sample homogenate (20 µl each) in triplicate. To each well, 200 µl of working BCA solution was added. The mixture was incubated at 37°C for 30 minutes. After cooling to room temperature, the absorbance was read spectrometrically (Spectra Max® 340) at 562 nm and the concentration of protein in samples was obtained according to the bovine serum albumin standard curve.

3.7 Statistical Analysis

Statistical software packages Quattro Pro 8 (Corel WordPerfect) were used for compiling and calculating data and for linear regression. Differences among the three experimental groups were determined by one-way analysis of variance followed by protected least significant difference (LSD) using SPSS 8.0.1. A probability of less than 0.05 was considered significant.

3.8 Chemicals

For HPLC analysis, L-cysteine, cysteinylglycine, glutathione, DL-homocysteine, D-penicillamine, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Company. Glutathione reductase was obtained from Fluka Chamie (Switzerland), and cumene hydroperoxide, mercaptosuccinic acid, and NADPH were purchased from Sigma Chemical Company for the GPx1 activity assay. The carbonyl reagent, 2,4-dinitrophenylhydrazine (DNPH), leupeptin, pepstatin A, phenylmethyl-sulfonyl fluoride, and aprotinin, streptomycin sulfate, and guanidine were obtained from Sigma Chemical Company for the protein carbonyl assay. Bovine serum albumin, copper sulfate, and bicinchoninic acid were obtained from Sigma Chemical Company for determination of protein concentration. Several chemicals including 5-sulfosalicylic acid, HPLC grade methanol, potassium dihydrogen phosphate, orthophosphoric acid, tris(hydroxymethy)aminomethane, potassium chloride, sodium hydrogen carbonate, sodium dihydrogen phosphate, sodium chloride, hydrochloric acid, and trichloroacetic acid were obtained from BDH Chemicals. EDTA, HEPES, and magnesium sulfate were obtained from Sigma Chemical Company.

Chapter 4 Results

4.1 Feed Intake and Weight Gain

Feed intake and body weight are presented in Table 4.1. Feed intake during the period of study was the same among the 3 experimental groups. Upon starting the experimental diets, there was no statistical difference in the average weight of the acclimated rats assigned to the 3 experimental groups. During the experimental period, the protein-deficient rats weighed significantly less than the protein-adequate rats at all time points measured ($P < 0.05$) (Appendix A). The average weight of the rats fed the low protein diet was about 10 to 15% less at each time point in comparison with that of the control rats. Overall weight gain at the conclusion of the study was less in the rats with protein deficiency than those fed the protein-sufficient diet. OTC supplementation did not affect the growth of the rats fed the low-protein diet. Throughout the study, the protein-deficient rats administered OTC had similar average weight to that of the rats fed the protein-deficient diet.

4.2 Tissue glutathione concentration

The chromatographic separation is shown in Figure 4.1 and Figure 4.2. The retention time of thiol standards including cysteine, cysteinylglycine, GSH, and homocysteine and the internal standard, penicillamine, are shown in Table 4.2. The first peak at ~1.7 minutes was the solvent front and was followed closely by the large 5-sulfosalicylic acid peak at ~2.1 minutes. The peak at ~4.4 minutes represented the

Table 4.1 Feed intake and body weight in rats[1]

	-PRO[2]	+OTC[2]	+PRO[2]
	Feed Intake (g)		
Week 1	101.3 ± 2.1	95.2 ± 1.7	95.3 ± 2.3
Week 2	252.0 ± 6.8	237.1 ± 3.2	241.0 ± 4.9
Week 3	427.4 ± 12.4	406.2 ± 5.5	414.9 ± 7.2
Week 4	615.1 ± 17.5	589.5 ± 7.7	608.6 ± 9.0
Week 5	805.6 ± 23.0	780.8 ± 10.8	806.6 ± 12.8
Total	1004.3 ± 29.1	971.4 ± 18.4	1017.7 ± 19.0
	Body Weight (g)		
Initial	59.1 ± 1.1	59.4 ± 1.5	59.6 ± 1.2
Week 1	96.8 ± 1.8^{a}	94.6 ± 1.5^{a}	111.8 ± 1.8^{b}
Week 2	146.8 ± 4.4^{a}	143.2 ± 2.1^{a}	175.8 ± 2.3^{b}
Week 3	194.0 ± 6.5^{a}	190. 2 ± 3.3^{a}	229.1 ± 1.0^{b}
Week 4	253.6 ± 9.1^{a}	251.1 ± 5.1^{a}	299.7 ± 4.9^{b}
Week 5	305.5 ± 11.2^{a}	305.1 ± 6.1^{a}	350.8 ± 6.5^{b}
Final	354.2 ± 12.8^{a}	350.1 ± 7.1^{a}	393.7 ± 8.1^{b}
Total Weight Gain (g)	295.1 ± 12.9^{a}	290.6 ± 8.2^{a}	334.1 ± 7.5^{b}

[1]Results are mean ± SEM; n = 8 for the protein-deficient and OTC-supplemented groups, and n = 9 for the protein-sufficient group. Means not sharing a common letter (a,b) in the same row are significantly different ($p < 0.05$). Statistical analysis was by one-way ANOVA followed by least significant difference.
[2]-PRO, +OTC, and +PRO represent the protein-deficient, OTC-supplemented, and protein-sufficient group, respectively.

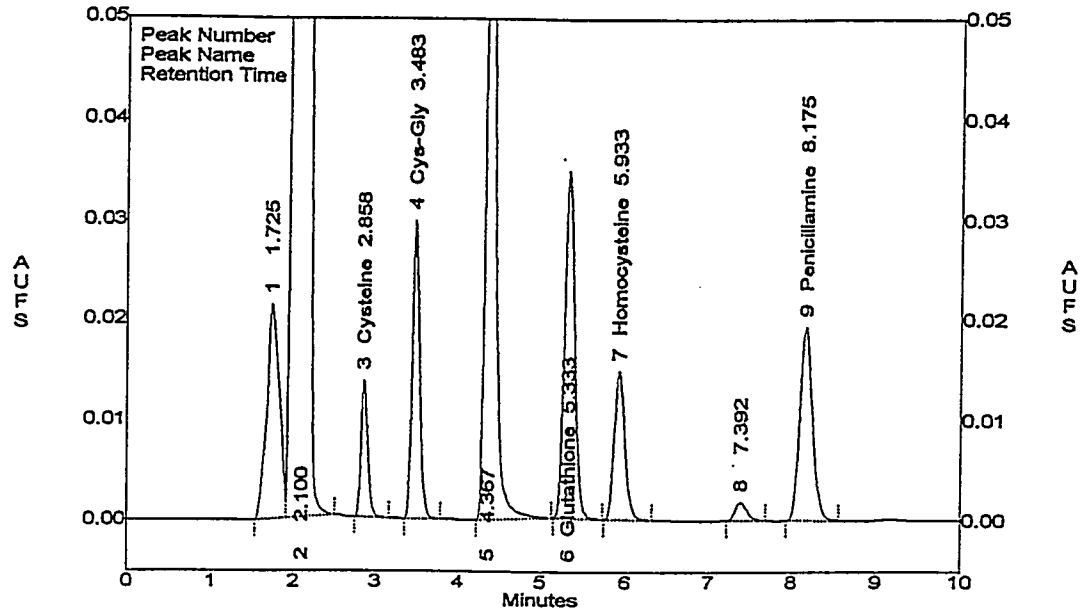


Figure 4.1 Representative chromatogram of a thiol standard containing 25 μM cysteine, 80 μM cysteinylglycine, 100 μM GSH, and 45 μM homocysteine

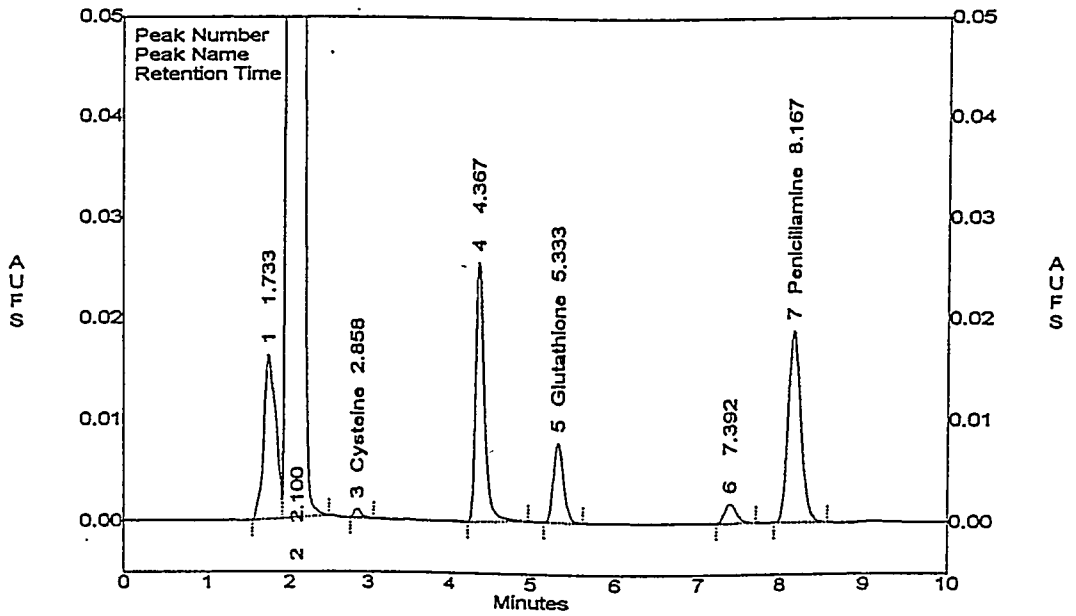


Figure 4.2 Representative chromatogram of thiols in rat neocortex diluted 1:50

DTNB reaction by-product.

Table 4.2 The retention time of thiols

Peak	Retention Time (minutes)
Cysteine	2.9
Cysteinylglycine	3.5
GSH	5.3
Homocysteine	5.9
Penicillamine	8.2

The data on GSH concentration in the liver, neocortex, hippocampus, thalamus, striatum, cerebellum, and brain stem are shown in Table 4.3. GSH was found in high concentration in the liver compared with that in various brain regions. Hepatic GSH concentration was lower in the rats fed the low protein diet than in those fed the adequate protein diet ($P < 0.05$) (Appendix B). Hepatic GSH concentration was decreased ~30% compared with that of the control rats. OTC administered to the rats with protein deficiency significantly increased hepatic GSH concentration to near normal concentration (-PRO vs. +OTC, $P < 0.05$). No significant difference was found between the OTC-supplemented group and the protein-adequate group.

GSH concentration varied among the 6 different brain regions. For the rats fed the normal protein diet, it was highest in the cerebellum (1.88 ± 0.03 μmol/g wet weight), and lowest in the brain stem (1.28 ± 0.01 μmol/g wet weight). The other brain regions had similar GSH concentration ranging from 1.54 ± 0.04 to 1.65 ± 0.03 μmol/g wet weight (Table 4.3) (Appendix C).

GSH concentration was not decreased by protein deficiency in any brain area. Supplementation of the low-protein diet with OTC had no significant effect on GSH concentration in brain regions.

Table 4.3 Glutathione concentration in the liver and brain regions[1]

Experimental Groups[2]	GSH Concentration[3] (μmol/g)						
	Liver	Neocortex	Striatum	Cerebellum	Hippocampus	Thalamus	Brain Stem
-PRO	5.85 ± 0.20[a]	1.50 ± 0.04	1.70 ± 0.06	1.88 ± 0.10	1.70 ± 0.03	1.53 ± 0.04	1.26 ± 0.03
+OTC	7.77 ± 0.37[b]	1.64 ± 0.05	1.75 ± 0.06	1.95 ± 0.02	1.72 ± 0.06	1.47 ± 0.02	1.30 ± 0.02
+PRO	8.50 ± 0.20[b]	1.60 ± 0.03	1.65 ± 0.03	1.88 ± 0.03	1.64 ± 0.03	1.54 ± 0.04	1.28 ± 0.02

[1]Results are mean ± SEM; n=8 for protein-deficient and OTC-supplemented groups, and n=9 for protein-sufficient group. Means not sharing a common letter (a,b) within the same column are significantly different ($P < 0.05$). Statistical analysis was by one-way ANOVA followed by least significant difference.
[2]-Pro, +OTC, and +PRO present the protein-deficient, OTC-supplemented, and protein-sufficient group, respectively.
[3]Expressed on a wet weight basis.

4.3 Tissue Cysteine Concentration

Table 4.4 shows cysteine concentration in the liver and different brain regions. Hepatic cysteine concentration followed the same pattern as GSH concentration (Appendix B). Protein deficiency significantly depressed hepatic cysteine concentration (-PRO vs. +PRO, $P < 0.05$). It caused cysteine concentration to decrease to about 50% of that found in the normal rats. However, the effect of OTC supplementation was less pronounced on cysteine concentration than that on GSH concentration. Cysteine concentration did not significantly increase after administration of OTC to the rats with protein deficiency (-PRO vs. +OTC, $P > 0.05$).

Cysteine concentration also varied among the brain regions. The highest concentration was found in the thalamus (0.100 ± 0.006 μmol/g wet weight), and the lowest was found in the hippocampus (0.041 ± 0.003 μmol/g wet weight). Protein restriction did not affect cysteine concentration in the striatum, hippocampus, thalamus, and brain stem. Cysteine concentration was decreased significantly in the neocortex and cerebellum of the rats with protein deficiency (-PRO vs. +PRO, $p < 0.05$). In these two brain areas, OTC supplementation also elevated cysteine concentration in the protein-deficient rats to the level found in the normal rats (-PRO vs. +OTC, $p < 0.05$).

4.4 Hepatic GPx1 Activity

Table 4.5 presents GPx1 activity in the liver. GPx1 activity in the liver was not statistically different between the protein-deficient group and the protein-adequate group (-PRO vs. +PRO, $p > 0.05$). Administration of OTC to the rats with the protein-deficient diet did not influence GPx1 activity in the liver either (-PRO vs. +OTC, $P > 0.05$; +OTC vs. +PRO, $P > 0.05$).

Table 4.4 Cysteine concentration in the liver and brain regions[1]

Experimental Groups[2]	Cysteine Concentration[3] (μmol/g)						
	Liver	Neocortex	Striatum	Cerebellum	Hippocampus	Thalamus	Brain Stem
-PRO	0.043 ± 0.003[a]	0.064 ± 0.003[a]	0.048 ± 0.005	0.048 ± 0.005[a]	0.040 ± 0.004	0.084 ± 0.007	0.098 ± 0.016
+OTC	0.055 ± 0.002[a]	0.088 ± 0.006[b]	0.053 ± 0.004	0.062 ± 0.004[b]	0.044 ± 0.004	0.103 ± 0.008	0.099 ± 0.004
+PRO	0.086 ± 0.007[b]	0.082 ± 0.004[b]	0.048 ±0.002	0.060 ± 0.001[b]	0.041 ± 0.003	0.100 ± 0.006	0.093 ± 0.004

[1]Results are mean ± SEM; n=8 for protein-deficient and OTC-supplemented groups, and n=9 for protein-sufficient group. Means not sharing a common letter (a,b) within the same column are significantly different (P< 0.05). Statistical analysis was by one-way ANOVA followed by least significant difference.
[2]-Pro, +OTC, and +PRO present the protein-deficient, OTC-supplemented, and protein-sufficient group, respectively.
[3]Expressed on a wet weight basis.

Table 4.5 Glutathione peroxidase activity in the liver[1]

	-PRO[2]	+OTC[2]	+PRO[2]
GPx1 Activity[3]	223.5 ± 24.63[a]	249.6 ± 9.40[a]	214.4 ± 10.56[a]

[1]Results are mean ± SEM; n = 8 for protein-deficient and OTC-supplemented groups, and n = 9 for protein-sufficient group. Means not sharing a common letter (a,b) are significantly different (P< 0.05). Statistical analysis was by one-way ANOVA followed by least significant difference.
[2]-PRO, +OTC, and +PRO denote the protein-deficient, OTC-supplemented, and protein-sufficient group, respectively.
[3]Expressed as nmol NADPH/min/mg protein.

4.5 Protein Concentration of Tissues

Protein concentration in the soluble fraction of the liver, neocortex, hippocampus, thalamus, striatum, cerebellum, and brain stem are demonstrated in Table 4.6. Protein concentration was much higher in the liver than that in brain regions. The decrease in protein concentration in the liver of the rats with the protein-deficient diet was pronounced (-PRO vs. +PRO, P< 0.05). Hepatic protein concentration in the protein-deficient rats was lowered to about 73% of that found in the control rats. However, OTC supplementation did not increase hepatic protein concentration in the rats with protein deficiency (Appendix E).

Mean protein concentration in brain regions ranged from 29.0 ± 0.5 mg/g wet weight to 39.3 ± 1.6 mg/g wet weight. Protein deficiency did not affect protein concentration in brain regions. OTC supplementation did not change protein concentration in 6 different brain regions of the protein-deficient rats (Appendix E).

4.6 Protein Carbonyl Content

Table 4.7 shows the protein carbonyl content in the liver and different brain regions of rats in the 3 experimental groups. Protein carbonyl contents were similar in

Table 4.6 Protein concentration in the liver and brain regions[1]

Experimental Groups[2]	Protein Concentration[3] (mg/g)						
	Liver	Neocortex	Striatum	Cerebellum	Hippocampus	Thalamus	Brain Stem
-PRO	83.8 ± 2.8[a]	35.1 ± 2.1	34.6 ± 1.0	36.4 ± 1.5	35.7 ± 1.5	33.2 ± 1.0	29.0 ± 0.6
+OTC	87.5 ± 4.1[a]	36.6 ± 1.6	33.9 ± 1.2	38.6 ± 0.5	34.0 ± 1.5	30.3 ± 1.3	29.2 ± 0.6
+PRO	114.9 ± 6.3[b]	39.3 ± 1.6	34.0 ± 1.6	38.7 ± 1.0	36.0 ± 1.2	30.0 ± 0.9	29.0 ± 0.5

[1]Results are mean ± SEM; n=8 for protein-deficient and OTC-supplemented groups, and n=9 for protein-sufficient group. Means not sharing a common letter (a,b) within the same column are significantly different ($P < 0.05$). Statistical analysis was by one-way ANOVA followed by least significant difference.
[2]-Pro, +OTC, and +PRO present the protein-deficient, OTC-supplemented, and protein-sufficient group, respectively.
[3]Expressed on a wet weight basis.

the liver and brain regions. A significant increase in the formation of hepatic protein carbonyl groups was found in the rats with protein deficiency compared with that in the control rats ($P < 0.05$). However, OTC administration to the rats with protein deficiency did not decrease the formation of protein carbonyl groups in the liver (Appendix F). No difference in protein carbonyl groups in brain regions was found among the three experimental groups.

Table 4.7 Protein carbonyl content in the liver and brain regions[1]

Experimental Groups[2]	Protein Carbonyl Content (nmol/mg protein)						
	Liver	Neocortex	Striatum	Cerebellum	Hippocampus	Thalamus	Brain Stem
-PRO	5.02 ± 0.16[a]	5.57 ± 0.22	6.63 ± 0.30	5.27 ± 0.24	7.71± 0.59	6.40 ± 0.29	6.13 ± 0.17
+OTC	5.03 ± 0.20[a]	5.63 ± 0.16	6.32 ± 0.15	4.92 ± 0.18	7.59 ± 0.52	5.61 ± 0.19	6.20 ± 0.13
+PRO	4.28 ± 0.14[b]	5.41 ± 0.14	6.34 ± 0.11	5.01 ± 0.17	8.10 ± 0.58	6.32 ± 0.27	6.29 ± 0.26

[1]Results are mean ± SEM; n=8 for protein-deficient and OTC-supplemented groups, and n=9 for protein-sufficient group. Means not sharing a common letter (a,b) within the same column are significantly different ($P < 0.05$). Statistical analysis was by one-way ANOVA followed by least significant difference.
[2]-Pro, +OTC, and +PRO present the protein-deficient, OTC-supplemented, and protein-sufficient group, respectively.

Chapter 5 Discussion

The model used in this study to examine nutritional regulation of brain GSH represents a protein deficiency, and not a protein-energy restriction. Moderate protein deficiency did not affect feed intake of rats. Those rats fed a low protein diet for 6 weeks grew more slowly; weight gain was lowered by 12% in comparison with the control rats, indicating a suboptimal growth. These results are supported by previous studies in which rats fed the same level of protein had retarded growth, despite normal feed intake (Bauman et al. 1988a; b). Other studies in which rats were fed diets containing less than 6% protein demonstrated a significant decrease in feed intake and a remarkable reduction in weight gain (Eisenstein and Harper 1991; Hum et al. 1992; Rana et al. 1996).

Oral supplementation of OTC, a cysteine precursor, to rats fed a protein-deficient diet did not effectively increase body weight. This is consistent with the previous observation by Bauman et al. (1988b) and can be explained by an inadequate supply of essential amino acids for protein synthesis. Different species may differ in their ability to utilize OTC for growth, as rats have been shown to utilize OTC less effectively than chicks (Chung et al. 1990). OTC supplementation can support maximal growth in rats fed diets deficient only in cysteine (Chung et al. 1990; Jain et al. 1995).

Coincident with suboptimal growth, soluble protein content in the liver of rats with protein deficiency was lowered to 73% of that found in the control rats in this study. This finding agrees with a previous study (Eisenstein and Harper 1991) in which

a decrease in hepatic protein content was observed when protein intake was restricted. This is probably due to the reduced rate of protein synthesis in the liver during protein deficiency resulting from the limited amino acid supply, or decreased capacity of protein synthesis, or the combination of both (Eisenstein and Harper 1991). However, brain protein content was not decreased in rats fed the 7.5% protein diet. The results are similar to the findings of Viana et al. (1996) and Agarwal et al. (1981). The latter reported an unchanged protein content in the brain of rats fed a 10% casein diet.

However, protein content in both liver and brain were relatively low compared with previous studies in which it was approximately 170 mg/g in the liver (Eisenstein and Harper 1991) and 80-170 mg/g in the brain (Agarwal et al. 1981; Viana et al. 1996). The inconsistencies may be due to different assays for determining protein concentration and rat strains used in the different studies. The Lowry method was used to measure protein content in three previous studies (Agarwal et al. 1981; Eisenstein and Harper 1991; Viana et al. 1996). However, whether the total protein content or the soluble protein fraction was analyzed in previous studies was not clear.

The exact mechanisms involved in the maintenance of protein content in the brain during moderate protein deficiency are unknown. Little change has been observed in amino acid levels in the brain in response to moderate protein deficiency. Rats fed a 8% protein diet did not show a decrease in most free amino acid concentrations in the cerebellum (Velazoquez et al. 1993). Similarly, no changes in aspartate, glutamate, glutamine, alanine, and γ-amino-butyric acid (GABA) were found in the rat brain during mild to moderate protein deficiency (10% casein); this was accompanied by normal activities of glutamate-related enzymes (Agarwal et al. 1981). Therefore, it can be speculated that the supply of free amino acids was adequate for

protein synthesis in the brain during moderate protein deficiency in the present study; the activities of enzymes for protein synthesis were probably not changed either. However, severe protein deficiency (5% casein diet) has been shown to inhibit the activities of enzymes for glutamate metabolism and to reduce the concentration of certain free amino acids in rat brain (Agarwal et al. 1981). Consistent with the finding that OTC supplementation did not effectively optimize animal growth, depleted protein content in the liver of protein-deficient rats was not restored to a normal level by OTC treatment.

In the current study, we examined whether moderate restriction of dietary protein had an impact on tissue GSH concentrations. Hepatic GSH concentration of rats on the protein-deficient diet was about 70% of that found in rats fed the control diet, indicating that hepatic GSH is responsive to protein intake. This result is strongly supported by numerous previous observations (Beck et al. 1958; Tateishi et al. 1977; Cho et al. 1981; Jaeschke and Wendel 1985; Bauman et al. 1988a; 1988b; Hum et al. 1992). The response is, in fact, due to the sulfur amino acid content in the diet, since Bauman et al. (1988a) found that a 7.5% protein diet supplemented with either cysteine or methionine to be equivalent in sulfur amino acid content to the 15% protein (control) diet restored hepatic GSH concentration to the normal level. It has also been reported that supplementation of a protein-free or cysteine-free diet with varied levels of cysteine led to a gradual increase of GSH concentration in rat liver (Tateishi et al. 1977).

Supplementation of OTC normalized hepatic GSH concentration in rats with protein deficiency in our study. This result is in agreement with other observations that administration of OTC can elevate depleted GSH concentration in the liver by providing the substrate, cysteine, for GSH synthesis (Bauman et al. 1988b; Chung et al. 1990;

Jain et al. 1995). Bauman et al. (1988b) found that the supplementation of OTC to rats fed a diet deficient in protein increased hepatic GSH concentration to normal levels found in rats with a diet sufficient in protein. All of these studies indicate that the availability of cysteine is one of the major determinants of the regulation of hepatic GSH.

In addition to cysteine availability, the second predominant determinant of the rate of GSH synthesis is the activity of γ-glutamylcysteine synthetase, which is increased with low levels of dietary protein (Bella and Stipanuk 1996; Bella et al. 1996; 1999). Unfortunately, the activity of γ-glutamylcysteine synthetase has not been determined in the present study. However, it can be postulated that the activity of γ-glutamylcysteine synthetase was maintained during protein deficiency, indicated by the elevation of hepatic GSH concentration by supplementation of OTC in rats with protein deficiency.

Along with the decrease in hepatic GSH was an approximately 50% reduction in cysteine concentration in the liver of rats fed the 7.5% protein diet. This result indicates that moderate protein-deficient status was achieved in rats fed a 7.5% protein diet, and supports the conclusion that hepatic cysteine concentration is sensitive to changes in dietary protein intake (Tateishi et al. 1977; Finkelstein et al. 1982). The depression of cysteine concentration in the liver of rats with protein deficiency was also observed by Bauman et al. (1988a; b).

When protein is deficient in the diet, cysteine concentration in the liver is decreased remarkably. Although cysteine can be supplied by synthesis from methionine via the trans-sulfuration pathway, this process is limited since methionine supplied by dietary protein is also decreased in rats fed a low protein diet. Alternatively, the activity of system Xc^- that transports cystine was found to be induced when

intracellular cysteine concentration was depleted in an in vitro study, resulting in the transportation of extracellular cystine into cells to form cysteine (Takada and Bannai 1984). However, it is not known if it occurs in the intact liver at present. Dietary protein, therefore, is the major determinant of cysteine concentration and accounts for the depression in cysteine concentration in rat liver.

The magnitude of the reduction in cysteine concentration did not parallel that of GSH concentration in the liver of rats during protein deficiency. Other studies have shown that cysteine concentration itself could regulate cysteine metabolism to GSH, sulfate, and taurine. Stipanuk et al. (1992) demonstrated that the percentage of total metabolism resulting in production of each of three major cysteine metabolites, GSH, sulfate, and taurine, was remarkably affected by cysteine availability. Low cysteine availability favored its utilization for the synthesis of GSH; high cysteine availability favored its catabolism to produce sulfate and taurine. Further studies indicated that the different effects of dietary protein on cysteine metabolism were due to the reciprocal regulation of activities of cysteine dioxygenase for cysteine catabolism and γ-glutamylcysteine synthetase for GSH synthesis (Bella and Stipanuk 1996; Bella et al. 1996). The activity of cysteine dioxygenase was observed to be lower in rats fed a protein-deficient diet (10%) compared with those fed a normal protein diet (20%). On the contrary, the activity of hepatic γ-gutamylcysteine synthetase was two times higher in the protein-deficient group (Bella et al. 1996; 1999). Thus, when the dietary protein level was low, less cysteine was catabolized to sulfate and taurine; the percentage of cysteine for GSH synthesis was increased. It is, therefore, not surprising to see that the extent of the decrease in hepatic GSH concentration was less than that of cysteine concentration during moderate protein deficiency.

In contrast to hepatic GSH concentration, cysteine concentration in the liver did not show a statistically significant increase with OTC supplementation, although there was a small degree of elevation. This result is not supported by the study of Bauman et al. (1988b) in which cysteine concentration in the liver of rats with protein deficiency was returned to a normal level after OTC administration. The reason for the discrepancy is unclear. One previous study indicated that the capacity of rat hepatocytes to take up and to metabolize OTC was lower than that for uptake and metabolism of cysteine and methionine (Coloso et al. 1991). A further study demonstrated that the limited utilization of OTC by rat hepatocytes was due to its slow rate of uptake and intracellular conversion to cysteine (Banks and Stipanuk 1994). These findings may explain the relatively low hepatic cysteine concentration found in rats with OTC supplementation. In addition, a limitation of the current study is that the concentration of cysteine in tissues of the control rats is low compared with that reported by some other investigators (Bauman et al. 1988a; 1988b; Anderson and Meister 1989; Paterson et al. 1998). This is probably due to the fact that the method used in this study is better suited for GSH analysis than for cysteine analysis. Limitations in the latter relate to the limit of detection (Katrusiak 1998) and the high susceptibility of cysteine to oxidation (Anderson and Luo 1998).

OTC appears to be partitioned preferentially for GSH synthesis. GSH production accounts for 78% of total cysteine metabolism products from OTC, whereas it accounts for 33% of total metabolites from cysteine (Banks and Stipanuk 1994). Other observations also suggest that low concentration or availability of cysteine results in a high percentage of utilization of cysteine for GSH synthesis versus sulfate and taurine formation (Stipanuk et al. 1992). Our findings that relatively low hepatic

cysteine concentration is accompanied by normal GSH concentration in the liver after OTC administration also suggest that OTC is preferentially utilized for GSH synthesis.

Dietary protein intake was found to have a much less pronounced effect on brain GSH concentration in the current study. Compared with hepatic GSH, brain GSH concentrations ranging from a mean of 1.28 to 1.88 μmol/g wet weight in the control rats were relatively low. GSH was highest in the cerebellum and lowest in the brain stem. These findings are not quite consistent with other observations in which the highest level was in the forebrain and neocortex, and the lowest level was in the brain stem (Chen et al. 1989; Kudo et al. 1990; Benuck et al. 1995). However, GSH concentration in all brain regions measured in the study was in the expected physiological range (1-2 μmol/g wet weight) (Cooper and Kristal 1997). The discrepancy may be due to various methods used to measure GSH concentration, different rat strains, or different ages of the animals. In aging, levels of GSH in the brain and other tissues are decreased (Chen et al. 1989; Ravindranath et al. 1989; Benuck et al. 1995). Observations similar to ours that cerebellum GSH concentration is as high as or higher than that in the neocortex have also been reported (Folbergrová et al. 1979; Ravindranath et al. 1989). Paterson et al. (1998) also observed similar results for GSH concentration in the neocortex, striatum, hippocampus, and thalamus.

The unique finding in the present study is that GSH concentration was not decreased by moderate protein deficiency in the neocortex, striatum, hippocampus, thalamus, cerebellum, and brain stem. Cysteine concentration in most brain regions of rats was also not reduced by protein deficiency. Neocortex and cerebellum were exceptions.

Although the brain has the capacity to synthesize GSH, the overall rate of GSH synthesis is relatively slow in the brain compared to that in the liver (Sekura and

Meister 1974; Griffith and Meister 1979), resulting in the accumulation of cysteine in the brain. This partly explains why the cysteine concentration in some brain regions such as the neocortex, thalamus, and brain stem is similar to that found in the liver of the control rats, whereas GSH concentration is much lower.

The unchanged brain GSH concentration with protein deficiency is in agreement with two previous studies using either male Albino young rats or Wistar pregnant rats. These studies demonstrated no change in GSH concentration in the whole brain of rats fed a 8% casein diet (Srivastava et al. 1989; Khanna et al. 1992). However, Srivastava et al. (1989) also observed unaltered GSH concentration in the liver, kidney, and testis of rats fed the protein-deficient diet, which is inconsistent with the majority of reports (Bauman et al. 1988a; b; Hum et al. 1992) and calls into question the validity of these findings.

The differences in these results and previous reports that sulfur amino acid deficiency depresses brain GSH (Tor-Agibidye et al. 1996; Paterson et al. 1998) are likely related to the difference in models used. In these studies, sulfur amino acids were taken out of the diet completely. The alternative pathway to synthesize cysteine, the trans-sulfuration pathway, was also limited as methionine was lacking in the diet. This leads to an extremely low availability of cysteine for GSH synthesis. In the present study, sulfur amino acids were present in the diet although in suboptimal amount. Apparently, the availability of cysteine was not decreased significantly in the brain. It has also been shown in the liver that the ability to synthesize GSH is compromised to a much greater degree than the ability to synthesize protein during dietary sulfur amino acid insufficiency (Hunter and Grimble 1997). Perhaps this phenomenon also occurs in the brain. In addition, the adult sulfur amino acid deficient animals studied by Paterson et al. (1998) experienced severe weight loss, suggesting a much greater rate of protein

degradation than that of protein synthesis. Protein-dependent functions, such as the activities of enzymes involved in GSH synthesis and uptake, may be impaired as well. In the present study, adaptations to the low protein diet may take place to spare body proteins and preserve their functions (Torun and Chew 1999). The capacity of GSH synthesis in the rats with the moderately low protein diet was likely sustained resulting in the stability of brain GSH.

The mechanisms for maintaining brain GSH during moderate protein deficiency are not well documented. Although the brain can take up intact GSH from blood, the major source of brain GSH is from intracellular synthesis of GSH in brain tissue. Cysteine, as well as methionine and cystine, is readily taken up by the brain from blood for GSH synthesis (Cooper and Kristal 1997). In the present study, unaltered cysteine concentrations in most brain regions, except neocortex and cerebellum, suggest that the uptake systems for sulfur amino acids from blood are well preserved or even induced by moderate protein deficiency. However, direct evidence is absent. Taylor et al. (1992) have provided indirect evidence for the high priority to maintain the enzymes for GSH synthesis in the liver during severe protein deficiency. The activity of hepatic γ-glutamylcysteine synthetase is also unaffected by dietary sulfur amino acid intake (Hunter and Grimble 1997), providing direct evidence that the capacity of GSH synthesis is maintained in some tissues during malnutrition. The same may be true in the brain. Although the activities of enzymes involved in the γ-glutamyl cycle, such as γ-glutamylcysteine synthetase, GSH synthetase, γ-glutamyl transpeptidase, and γ-glutamylcyclotransferase, are present throughout the brain, and are especially high in the choroid plexus (Okonkwo et al. 1974), little work has been done to examine their response to dietary protein deprivation.

Hepatic efflux of GSH to blood may also increase in protein deficiency to maintain brain GSH pools. The rate of GSH efflux into plasma and the clearance rate of plasma GSH increases in rats during fasting (Kaplowitz et al. 1983), suggesting that export of GSH to extrahepatic tissues might be increased. However, a conflicting result was reported by Adashi et al. (1992) who found that the rate of efflux of hepatic GSH was actually lower in protein-deficient rats. The exact role of GSH efflux mechanisms in the regulation of extrahepatic GSH concentration during dietary protein restriction is still under investigation.

Brain GSH concentration seems also to be highly regulated, as has been reported in tissues such as liver. This is supported by the findings of normal GSH concentrations in the neocortex and cerebellum of protein-deficient rats in spite of decreased cysteine concentrations. Furthermore, OTC supplementation did not further increase GSH concentration in brain regions in which GSH concentration was maintained during protein deficiency.

Oral administration of OTC selectively increased cysteine concentration in the neocortex and cerebellum, which represent the only brain regions in which cysteine decreased with protein deficiency. This result is in accordance with a previous study (Anderson and Meister 1989), which demonstrated an overall increase in cysteine concentration in the hypothalamus, neocortex, cerebellum, brain stem, caudate nucleus, and pons after injection of OTC to fasted rats. Mesina et al. (1989) also reported a small increase in brain GSH concentration after subcutaneous injection of OTC. These results suggest that OTC is an effective vehicle for the transport of cysteine into the brain for GSH synthesis when GSH is depleted.

Because of the close relationship between GSH concentration and GPx1 activity, the effect of protein deficiency on this enzyme in the liver has been determined

in our study. The activity of GPx1 in the brain was not determined because it is very low compared with that in the liver (De Marchena et al. 1974; Paterson et al. 1998), which makes it difficult to measure. Also, other enzymes present in the brain that consume the same substrate interfere with interpretation of the result in the brain. In our study, hepatic GPx1 activity showed no significant change within the 3 experimental groups, suggesting that GPx1 activity in the brain may not be affected by moderate protein deficiency.

Most previous studies have demonstrated a decrease in hepatic GPx activity by protein deficiency (Bauman et al. 1988a; Pelissier et al. 1990; 1993; Zhu et al. 1992; Huang and Fwu 1992; 1993; Rana et al. 1996). However, one study reported an increase in hepatic GPx activity of rats fed either a 5% or 10% protein diet in comparison with that of rats fed a 20% protein diet (Sambuichi et al. 1992). The reasons for this discrepancy are complicated, and may be due to differences in assays used to measure GPx activity, rat strains, dietary protein contents, or experimental time periods.

Most important, the balance between stimulation and inhibition of GPx activity caused by a protein-deficient diet may account for these inconsistent results. On the one hand, a low protein diet is known to cause a decrease in GSH synthesis, and GPx activity is highly dependent on GSH level (Flohé 1977). On the other hand, a low protein diet increases the relative energy intake from carbohydrate in comparison with a normal protein diet. In the present study, protein deficiency did not affect total feed intake of the animals, but the energy intake from carbohydrate was relatively increased in rats fed the low protein diet. Both increase in carbohydrate and decrease in protein have been suggested to increase the generation of radicals by several mechanisms. High carbohydrate and low protein diets stimulate the secretion of thyroid hormone

(Tulp et al. 1979), which could promote the uptake of oxygen, consequently leading to increased formation of radicals. Secondly, a low protein diet diminishes plasma proteins and decreases the level of transferrin (Golden and Ramdath 1987), an inhibitor of the formation of the hydroxyl radical and lipid peroxidation (Halliwell 1987). At the same time, it increases the concentration of ferritin and free iron, which is involved in hydroxyl radical reaction (Halliwell 1987). Thirdly, the depletion of GSH resulting from low dietary protein also contributes to an increase in the rate of the generation of radicals. The formation of radicals upregulates GPx activity by inducing the new synthesis of GPx as well as other radical-scavenging enzymes such as SOD (Rister and Baehner 1976; Stevens and Autor 1977). In the present study, the balance of these factors resulted in unchanged hepatic GPx activity in the face of protein deficiency. OTC supplementation did not affect the activity of GPx1 in the liver of rats with protein deficiency.

Protein carbonyl content was also measured in the liver and brain as a marker of oxidative damage in the present study. Brain protein carbonyl content was unchanged by moderate protein restriction and OTC supplementation. To our knowledge, there has not been much investigation of the effect of nutritional status on protein oxidation. Aksenova et al. (1998) have previously reported that the age-associated increase of protein oxidative damage in the cortex, cerebellum, and hippocampus was diminished by dietary restriction. The unchanged protein oxidation in the brain regions under protein deficiency conditions corresponds with the maintenance of GSH in these tissues.

Hepatic protein carbonyl content significantly increased in the liver of rats on the low protein diet, paralleling the decrease in hepatic GSH concentration in the present study. However, hepatic protein carbonyl content in the OTC-supplemented

group was increased in the presence of a normal GSH concentration so low rates of oxidative damage to proteins cannot be solely attributed to the maintenance of brain GSH.

In summary, protein deficiency in the rat was achieved in the present study, indicated by the following results: 1) reduced weight gain accompanied by normal energy intake, 2) decreased protein content in the liver, and 3) depressed hepatic cysteine and GSH concentration. Hepatic GSH and cysteine concentration was responsive to dietary protein intake. GSH concentration in the liver was decreased by protein restriction primarily as a result of the low availability of cysteine. The moderate degree of protein deficiency achieved in this study did not influence brain GSH concentration. Cysteine concentration was maintained in most brain regions, except neocortex and cerebellum, under the condition of moderate protein restriction, and oxidative damage to proteins was not evident as measured by protein carbonyl content. OTC supplementation did not further increase GSH concentration in brain regions in which GSH levels were preserved in protein deficiency. However, it significantly reversed the decline in cysteine concentration in neocortex and cerebellum produced by protein deficiency, suggesting OTC is an effective system for delivering cysteine to brain tissue.

We cannot exclude the possibility that the brain GSH results might have been different in a more severe protein deficiency model. Furthermore, the age of animals used in the study may also be a factor. Brain GSH concentration decreases significantly in aged rats (Ravindranath et al. 1989) probably as a result of an increase in the formation and release of free radicals as the brain ages (Benzi and Moretti 1995).

The important question to address in future studies is whether brain GSH pools can be maintained when protein deficiency is present in an animal model of stroke. Evidence exists that brain GSH concentration is depleted as a result of increased oxidative stress during and following ischemia/reperfusion. This may be exacerbated by suboptimal protein-energy status since brain GSH under conditions of cerebral oxidative insult will be determined by the balance among its utilization, *de novo* synthesis, and reduction of oxidized glutathione. This type of study would also allow an evaluation of the extent of brain damage that occurs when protein deficiency is present during cerebral ischemia. A model of acute sulfur amino acid deficiency that depletes GSH in some brain regions by 10-14% has been shown to exacerbate neuronal damage in a rat model of stroke (Paterson et al. 1999).

At present, there is little effective treatment for stroke once it occurs. Our findings as well as others that administration of OTC repletes cysteine and GSH in some brain regions imply that OTC can be used therapeutically in stroke patients with depleted brain GSH by providing adequate cysteine for GSH synthesis. Future study should investigate whether OTC supplementation to animals with poor protein status can restore brain GSH and diminish the oxidative stress and brain damage seen in stroke. This should provide direct evidence as to whether nutritional status influences ischemia-induced brain damage.

It may also be important that another product of cysteine metabolism, taurine, is also a potent antioxidant that participates in the remove of radicals such as HOCl (McLoughlin et al. 1991; Stapleton and Bloomfield 1993). Taurine has been shown to protect cells from radical-induced damage in an ischemia/reperfusion model of the heart (Barry et al. 1997; Raschke et al. 1995). However, the influence of dietary protein, especially sulfur amino acids, on taurine production has received less attention

than effects on GSH synthesis. Whether protein deficiency limits taurine production during ischemia/reperfusion and exacerbates oxidative damage in the brain is a question worthy of further study.

Other mechanisms that may explain the inverse relationship between protein intake and stoke mortality and incidence should also not be ignored. For example, impaired blood coagulation, which is associated with the low protein diet, may promote the incidence of stroke (Chang et al. 1997). A protein-rich diet increases urinary volume and Na excretion to ameliorate hypertension (Yamori et al. 1984). Also, an increase in high quality protein intake can alter the physical characteristics of vascular walls and thereby improve regional cerebral blood flow in spite of severe hypertension (Yamori et al. 1979).

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