Role of hydrogen sulfide in modulating the life and death of cells

by:

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Dedications

This thesis is dedicated to :

My Mother and Father

My Wife Tahereh

My Son Atabak

My Brothers and Sisters

Abstract
Role of hydrogen sulfide in modulating the life and death of cells

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Hydrogen sulfide was found to be more toxic to hippocampal slice cultures than isolated hepatocytes. Chloride ion channel blockers decreased H_2S cytotoxicity suggesting that H_2S enters the cells via these channels. Intracellular H_2S was formed from cysteine metabolism as the cystathionine γ-lyase inhibitor propargylglycine prevented cystinedimethylester (CDME) toxicity towards GSH-depleted hepatocytes but not neurons. However the cystathionine β-synthase inhibitor aminooxyacetate decreased CDME toxicity in neurons but not hepatocytes. H_2S toxicity was decreased by $ZnCl_2$ by complexing H_2S or accelerating H_2S oxidation.

H_2S also prevented Zn^{2+}:8-hydroxyquinoline toxicity in hepatocytes. ROS scavengers DMSO and catalase, metal chelators neocuproine, TPEN and desferoxamine and endocytosis inhibitors chloroquine and methylamine also decreased Zn^{2+} hepatoxicity suggesting that zinc displaces Fe/Cu from storage proteins that have been endocytosed in lysosomes. Hydrogen sulfide was also protective against NO and hypoxia induced toxicity in hepatocytes.

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Summary of abbreviations

AdoHcy	S-adenosyl-L-homocysteine
AOA	Aminooxyacetate
CBS	Cystathionine β-synthase
CDME	Cystine dimethylester
CSE	Cystathionine γ-lyase
Cys	Cysteine
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
GABA	Gamma Amino Butyric Acid
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)
HBSS	Hank's Balanced Salt Solution
Hcy	Homocysteine
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
IREs	Iron Responsive Elements
MAO	Monoamine Oxidase
Met	Methionine
MNNG	1-Methyl-3-nitro-1-nitrosoguanidine
NMDA	N-Methyl -D- Aspartate

NO	Nitric Oxide
PPG	Propargylglycine
RDA	Recommended Dietary Allowance
ROS	Rective Oxygen Species
SE	Standard Error
TfR	Transferrin Receptor
TPEN	N,N,N'N'-tetrakis(2-Pyridyl-Methyl)Ethylenediamine

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Chapter 1

INTRODUCTION

1.1 General Introduction

Hydrogen sulfide (H_2S) is the noxious odor of rotten eggs. Few realize that it is as lethal as hydrogen cyanide but is far more insidious and much more common (Burnett et al., 1977). H_2S is used or encountered in diverse industries ranging from farming and brewing to tanning, glue making, rubber vulcanizing, heavy water production and most prominently in oil and gas exploration and refining (Beck et al., 1981). It is a major occupational health hazard (Beauchamp et al., 1984). For example, fatalities associated with "sour" gas wells are caused by H_2S (Beauchamp et ak., 1984). In addition, excessive hydrogen sulfide formation from colonic bacteria has been suggested as a cause of ulcerative colitis (Roediger et al., 1997). The toxicology of H_2S should be therefore of interest to molecular toxicologists.

Hydrogen sulfide is produced endogenously and has a physiological role. It may modulate enzymes of the glycolytic and gluconeogenesis pathways (Wood 1982) and regulate iron/sulfur clusters found in intracellular electron transport systems. Fe-S clusters in iron-sulfur proteins (also called non-heme iron proteins) play a critical role in a wide range of reduction reactions in biological systems (Stryer 1995). Physiological concentrations of hydrogen sulfide have recently been suggested to have a signalling role analogous to that of NO. Physiological concentrations of hydrogen sulfide selectively enhanced NMDA receptor-mediated responses and facilitated the induction of hippocampal long term potentiation (Abe and Kimura, 1996). The physiological role of

H_2S in modulating cell function is therefore of interest to cell physiologists.

In this thesis, two aspects of hydrogen sulfide action have been studied: cytotoxic and cytoprotective effects. A comparative approach was taken using two cell types, neurons of the brain hippocampal region and hepatocytes, in order to examine the prevalence and mechanisms of the different sulfide dependent processes that influence cell death and cell survival. Although our ultimate aim is to study the role of hydrogen sulfide in brain function and dysfunction, hepatocytes proved to be convenient in terms of working out protocols and proving principles. This work complemented other projects in Dr. O'Briens lab exploring hydrogen sulfide toxicity and looking for antidotes.

Hypothesis

Our hypothesis was that hydrogen sulfide acts intracellularly to have helpful or harmful actions depending on conditions. These action could be initiated as the result of known intracellular reactions of hydrogen sulfide including modulation of iron sulfur complexes, formation of persulfides, precipitation of zinc, interaction with reactive oxygen species. Because little is known about the specific reactions of hydrogen sulfide at this stage our goal was to survey the actions of H_2S and to develop protocols to study those actions. In order to test the hypothesis that hydrogen sulfide acts intracellularly we used the membrane permeable cysteine precursor cystine dimethylester. Cysteine in turn can be metabolized to hydrogen sulfide although the enzymes producing H_2S are different in brain from hepatocytes with distinct and specificinhibitors, thereby allowing a direct test of our hypothesis. We also tested the hypothesis by using anion channel blockers to

reduce entry of HS^-. Again different chloride channel blockers are effective in hepatocytes and brain cells allowing a direct test of the hypothesis.

We have also carried out some experiments to test direct and indirect interactions between hydrogen sulfide and Zn^{2+}or other metals. Because certain metals like Zn^{2+} can combine directly with hydrogen sulfide we examined the ability of metals to reduce the toxicity of hydrogen sulfide. Many of these metals can themselves be toxic at higher concentrations or when delivered intracellularly using the membrane permeable metal chelator 8-hydroxyquinoline. Therefore we predicted that under certain conditions hydrogen sulfide could be cytoprotective. Alternatively hydrogen sulfide might produce some other effects unrelated to direct sequestration of metals that is nevertheless cytoprotective. To study this we determined and stablished the time course of hydrogen sulfide in the cell suspension and examined the influence of preincubation with hydrogen sulfide on subsequent toxic actions of metals. Finally, we tested the ability of hydrogen sulfide to reduce reactive oxygen species generated by hepatocytes whaen incubated with zinc, hypoxia or nitric oxide.

1.2 Endogenous hydrogen sulfide reactions.

1.2A. Interactions with metal ions

Hydrogen sulfide can combine with a number of metal ions.

Iron/sulfur clusters. Intracellular electron transport systems that are required for oxidative phosphorylation, photosynthesis and nitrogen fixation involve proteins containing Fe/S clusters e.g. ferredoxin (Stryer 1995). Several types of Fe-S clusters are known. In the simplest kind, a single iron atom is tetrahedrally coordinated to the

sulfhydryl groups of four cysteine residues of the protein. A second kind, denoted by [2Fe-2S], contains two iron atoms and two inorganic sulfides, in addition to four cysteine residues. A third type designated as [4Fe-4S], contains four iron atoms, four inorganic sulfides, and four cysteine residues. NADH-Q reductase contains both [2Fe-2S] and [4Fe-4S] clusters. Iron atoms in these Fe-S complexes cycle between Fe^{2+} (reduced) or Fe^{3+} (oxidized) states (Stryer 1995). The Fe/S clusters are assembled from sulfide, ferrous ions and apoprotein thiols. The bifunctional role of aconitase as a citric acid cycle enzyme catalyzing the conversion of citrate to isocitrate and as an iron regulatory protein is controlled by its Fe-S cluster. In this way cellular iron levels can be modulated by cellular signals e.g. oxidative stress (Paraskeva and Hentze 1996). Sulfide reductively activates aconitase by reforming the Fe-S clusters (Kennedy et al., 1994). Recently it has been suggested that this enzyme may act as an iron or oxidant sensor in the cell (Rouault and Klausner, 1996).

Iron is indispensable to the function of the cells, and the uptake and sequestration of iron is regulated by a set of genes that are highly conserved in mammalian cells. Synthesis of the transferrin receptor (TfR), which is responsible for iron uptake, is regulated to respond to metabolic needs. When intracellular iron supplies are adequate, the mRNA for the TfR is rapidly degraded, resulting in a decrease in TfR biosynthesis. When intracellular iron levels are low, mRNA levels and biosynthesis of the TfR increase, while the rate of translation and biosynthesis of ferritin, an iron-sequestration protein, decreases. These regulatory changes are mediated by a post-transcriptional regulatory system that involves binding of iron-regulatory proteins to stem-loop structures in the

mRNA known as iron responsive elements (IREs). When cellular iron levels are low, iron regulatory proteins bind to IREs, where they inhibit translation (ferritin) or mRNA degradation (TfR) (Klausner et al., 1993). Tight regulation of iron uptake and distribution is necessary because excess iron can be toxic, particularly because iron species and oxygen can interact to form reactive oxygen species, including superoxide and hydroxyl radical.

The initial purification of the IRE-binding protein, now called IRP1, has led to insights into the mechanism by which iron levels are sensed (Klausner et al., 1993). Human IRP1 was shown to have significant (approximately 30%) sequence homology to mitochondrial aconitase, an enzyme that has been crystallized and extensively characterized (Rouault et al. 1991). Mitochondrial aconitase activity depends on an iron-sulfur cluster, a finding that was initially revealed by Mössbauer spectroscopy (Kent et al., 1980) and was later confirmed biochemically and crystallographically (Kennedy and Beinert 1988, Lauble et al., 1992). A [4Fe-4S] cluster like that of mitochondrial aconitase was found in IRP1 (Kennedy et al., 1992), and analysis of recombinant IRP1 led to the observation that the protein functioned as a cytosolic aconitase in iron-replete cells, whereas it functioned as a high affinity IRE-binding protein in iron depleted cells (Rouault and Klausner, 1996). Use of previously established methods for the in vitro assembly or disassembly of the iron - sulfur cluster of mitochondrial aconitase revealed that the two forms of this bifunctional protein could be interconverted in vitro (Haile et al., 1992) and that mutations of cysteine residues involved in cluster ligation produced an apoprotein that bound RNA constitutively, regardless of the iron status of the cell (Philpott et al., 1994).

Therefore it is reasonable to assume that hydrogen sulfide is essential for the assembly of iron-sulfur clusters and that this action may control the expression of proteins involved in the adaptation of cells to changes in redox states.

There are other examples where iron-sulfur clusters regulate cellular responsiveness to redox states. The aerobic-anaerobic transcription switch action of the FNR (fumarate nitrate reduction) regulatory protein of Escherichia coli occurs via its [4Fe-4S] cluster (Green et al. 1996). The FNR protein is a redox-responsive transcription regulator that activates and represses a family of genes required for anaerobic and aerobic metabolism. Its $[4Fe\text{-}4S]^{2+}$ clusters remain intact under anaerobic conditions but are degraded to $[3Fe\text{-}4S]^{+}$ by limited oxidation with air, or are completely lost on prolonged air exposure. Incorporation of $[4Fe\text{-}4S]^{2+}$ clusters increases site-specific DNA binding about 7-fold compared with apo-FNR (Green et al., 1996).

Heme Metals. H_2S complexes heme Fe^{3+} and Cu^{2+} of cytochrome oxidase which results in oxidase inactivation (Beauchamp et al., 1984). Recently it was concluded that the inhibition of respiratory enzymes may be responsible for the H_2S-induced alteration of neuronal function in structures such as the hippocampus and other neurological tissues (Roth et al., 1997).

Nitrite has been extensively used in the treatment of cyanide poisoning. The action of nitrite in cyanide poisoning is due to the production of methemoglobin and the binding of free cyanide in blood as the biologically inactive, but reversible complex,

cyanomethemoglobin. Methemoglobin also binds sulfide to form sulfmethemoglobin (Smith, 1997). Pretreatment of animals with sodium nitrite or human methemoglobin has decreased mortality after parenteral sodium sulfide or inhalation of H_2S (Smith and Gosselin 1976). However, in serious human poisoning, nitrites (300 mg or 10 ml of 3% sodium nitrite intravenously over 4 to 5 minutes) were ineffective because (1) the lifetime of sulfide in oxygenated blood is short (15 to 20 minutes) (Beck et al., 1981), (2) only doses of nitrite that caused 60% to 80% methemoglobinemia prevented mortality (in animals) when the nitrite was given 2 minutes after sulfide injection, (Beck et al., 1981), (3) sulfide was not bound as strongly to methemoglobin as cyanide (Smith and Gosselin 1966).

Zn^{2+} and Cd^{2+} Sulfide salts of metals such as Zn^{2+} and Cd^{2+} are not soluble and sulfide has been extensively used in the Timm's method for the localization of Zn in the brain tissues (Frederickson, 1989). Thus sulfide will combine with a variety of metals and theoretically could be used to modulate their actions. Conversly, metal ions may be able to modulate the actions of hydrogen sulfide.

1.2B. Persulfide modulation

The development of a specific gas dialysis and ion chromatography technique coupled with an electrochemical detection method for sulfide determination has enabled brain sulfide level to be measured (Goodwin et al., 1989). Initial measurements raised the possibility that some proportion of an administered hydrogen sulfide dose is subsequently complexed in a chemically latent or cryptic molecular form within brain tissue that does not readily hydrolyze so as to liberate hydrogen sulfide but which decomposes in weak

acid and readily undergoes air oxidation. These are properties of sulfane-sulfur compounds (R-S-SH) known as persulfides (Wood, 1987). Persulfide formation theoretically may disrupt or modulate the function of a wide variety of enzymes and physiologically important proteins.

Persulfides are present in the active sites of the enzymes xanthine oxidase, aldehyde oxidase and short-chain acyl-CoA dehydrogenase (Tiffany et al. 1997). Physiological levels of hydrogen sulphide also bind to enzymes of the glycolytic and glyconeogenetic pathways and modulate their activity resulting in a possible regulatory mechanism for these pathways (Ogasawa et al. 1997).

Monoamine oxidase, the principle catabolic enzyme of monoamines, can be inactivated by H_2S (Warenycia et al., 1989b) leading to increases in the brain levels of catecholamines and 5-hydroxytryptamine. MAO inhibition and the consequent increases in brain stem monoamines may be important in central respiratory drive failure after hydrogen sulfide poisoning. Dithiothreitol (DTT) is reported to prevent the inactivation of MAO by hydrogen sulfide (Mangani and Haakanson, 1992), thus suggesting a molecular mechanism consistent with known persulfide chemistry.

In addition to a possible modulation of GABA receptors in the hippocampus, it has been demonstrated that NaHS has a direct inhibitory effect on hippocampal neurons (Reiffenstein et al., 1992). These effects were suggested to be due to the opening of a K^+ channel and activation of Na^+/K^+ ATPase. These actions may be related to persulfide formation on those proteins. Persulfide formation may also explain the reversible

inhibition of an inward current in neuroblastoma cells caused by the coapplication of taurine and H_2S (Warenycia et al., 1989c).

1.2C Endogenous Sources of Sulfide and other forms of reactive sulfur species

Hydrogen sulfide, depending on the pH of the medium , exists in three forms:

$H_2S \leftrightarrow H^+ + HS^- \leftrightarrow 2\,H^+ + S^{2-}$

At a physiological pH of 7.4, approximately one-third of H_2S exists as the undissociated form and two-thirds as the hydrosulfide anion (Reiffenstein et al., 1992). Although it has not been possible to determine which form of H_2S is active, the general term "hydrogen sulfide" is used here to describe both H_2S and HS^-.

H_2S can also be formed as an endogenous metabolic product with a physiological and pathological role. Endogenous H_2S can be formed from cysteine by pyridoxal-5'-phosphate-dependent enzymes, including cystathionine β-synthetase and cystathionine γ-lyase (Stipanuk and Beck, 1982, Grffith, 1987, Erickson et al., 1990). The hydrogen sulfide-producing enzyme cystathionine β-synthetase is highly expressed in the hippocampus and cerebellum. Furthermore brain homogenates readily produce H_2S (Abe and Kimura, 1996). Physiological concentrations of hydrogen sulfide facilitate the induction of long term potentiation in the hippocampus, suggesting that endogenous H_2S functions as a neuromodulator in the brain (Abe and Kimura, 1996).

Smooth muscle containing tissues such as the ileum, portal vein and thoracic aorta express another H_2S-producing enzyme, cystathionine γ-lyase. H_2S relaxes these smooth muscles, and low concentrations of H_2S synergize smooth muscle relaxation induced by

NO in the thoracic aorta (Hosoki et al., 1997) again suggesting a modulatory physiological role for H_2S.

Like smooth muscle, the liver contains high levels of cystathionase as well as mercaptopyruvate sulfurtransferase. Cystine which is produced by oxidation of cysteine is metabolized by rat hepatocyte γ-cystathionase to thiocysteine and HS^- (Stipanuk and Beck, 1982). Cysteine is also metabolized by hepatocyte cysteine aminotransferase to β-mercaptopyruvate which is converted to S° and H_2S by a sulfurtransferase (Meister et al., 1954). The atomic sulfur can be reduced to sulfide by cysteine which can then promote thiol oxidation (Wood 1982) via the following reaction:

$$2RS^- + S^\bullet \leftrightarrow RSSR + S^{2-}$$

Atomic sulfur, which can also exist as polysulfide, reacts with mercaptans to form persulfide, which can generate sulfide by the following reaction (Wood 1982):

$$RS^- + S^\bullet \leftrightarrow RSS^-$$

$$RSS^- + RS^- \leftrightarrow RSSR + S^{2-}$$

1.3. Metabolism of sulfur-containing amino acids

In order to understand the significance of the endogenous production of hydrogen sulfide it is useful to review the metabolism of sulfur containing amino acids. Although cysteine is the major amino acid which produces hydrogen sulfide, methionine is also important because its metabolism can lead to the production of cysteine. Therefore, the pathways of methionine metabolism relevant to cysteine metabolism are discussed.

1.3A Methionine metabolism.

The first step in methionine (Met) metabolism is the formation of the high-energy sulfonium compound, S-adenosyl-L-methionine (AdoMet). AdoMet serves as the methyl donor for essentially most known biological methylation reactions with the notable exception of those involved in the methylation of L-homocysteine (Hcy). The methyl group of AdoMet is transferred to a nitrogen, oxygen, or sulfur atom of a wide range of compounds in reactions catalyzed by numerous methyltransferases e.g. for the formation of creatine from guanidinoacetate. The co-product of transmethylation, S-adenosyl-L-homocysteine (AdoHcy), is hydrolyzed to yield adenosine (Ado) and Hcy by AdoHcy hydrolase (EC 3.3.1.1), Hcy can then be remethylated to Met or condensed with serine to form cystathionine. Hcy may be remethylated to Met in reactions catalyzed by Met synthase or in reactions catalyzed by betaine-Hcy methyltransferase. It may also be used for resynthesis of AdoHcy by reversal of hydrolysis catalyzed by AdoHcy hydrolase or irreversibly converted to cystathionine via the reaction catalyzed by cystathionine ß-synthase. The formation of cystathionine commits the Met molecule to catabolism by the transsulfuration pathway (Stipanuk, 1986). These transmethylation reactions and the transsulfuration pathway are depicted in Figure 1.1.

1.3B Cysteine Metabolism.

Cysteine is metabolized by animal cells to yield either taurine (2-aminoethanesulfonate) and CO_2 or sulfate, urea and CO_2 (Krijgsheld et al., 1981). Several pathways of Cys metabolism have been demonstrated, but the physiological roles of these are not well understood.

Figure 1.1 Mammalian methionine metabolism (adapted from Griffith 1987). The numbers marked with parentheses correspond to the following enzymes or metabolic processes: 1. Methionine adenosyltransferase, an ATP-dependent reaction; 2. Various S-adenosylmethionine methyltransferases; 3. Adenosylhomocysteinase; 4. Betaine-homocysteine methyltransferase; 5. 5-Methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase); 6. Cystathionine β-synthase; 7. Cystathionine γ-lyase

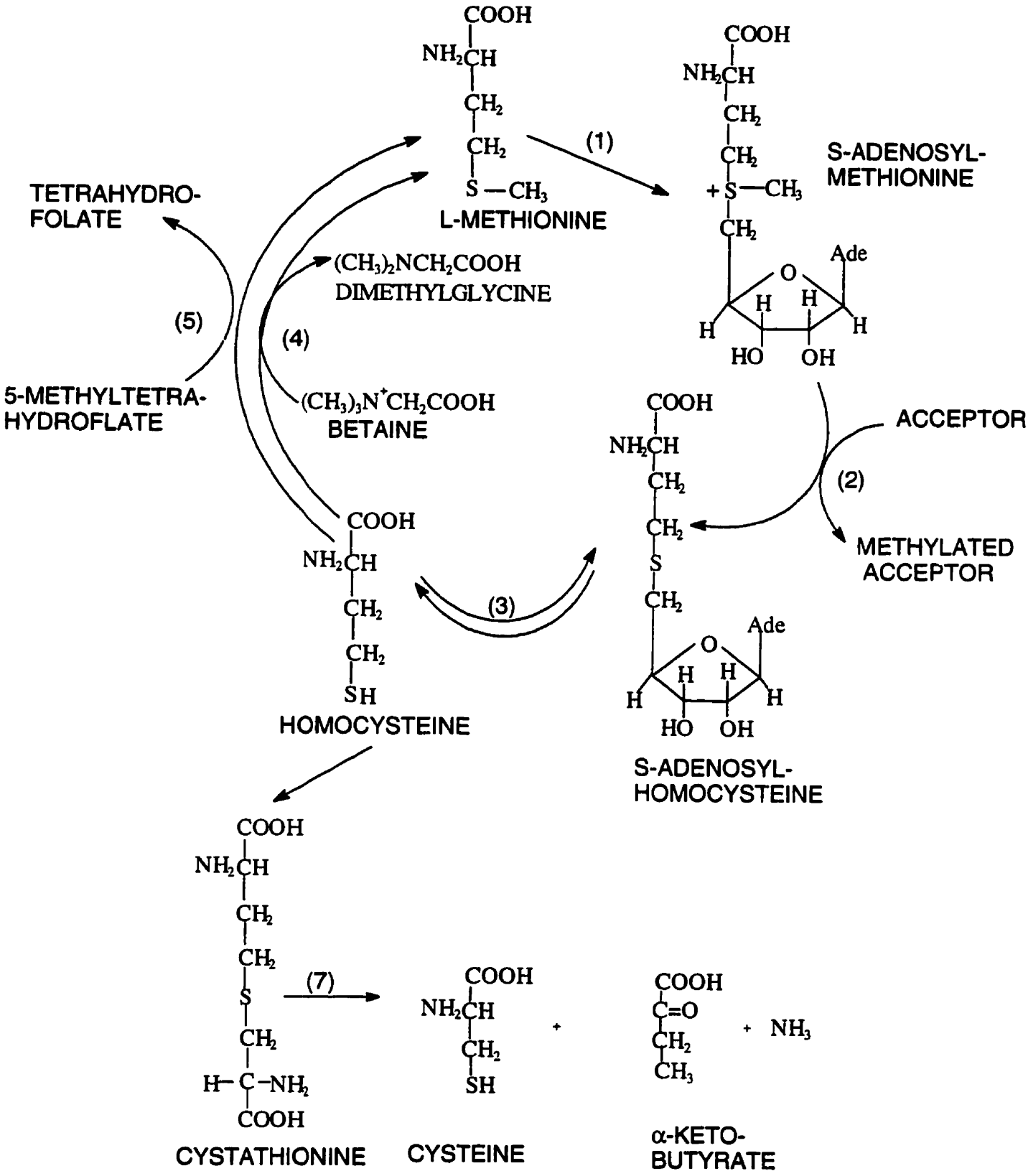


Figure 1.1- Metabolism of methionine

The cysteinesulfinate pathway of Cys metabolism is depicted in Figure 1.2. The oxidation of Cys to cysteinesulfinate is catalyzed by Cys dioxygenase (EC 1.13.11.20), an iron-containing enzyme that seems to be specific for Cys, with an apparent K_m of 0.45 mM (Yamaguchi et al., 1978). Cysteinesulfinate may then be decarboxylated to hypotaurine (2-aminoethanesulfinate) by the action of cysteinesulfinate decarboxylase (EC 4.1.1.29), a pyridoxal 5'-phosphate-dependent enzyme that has an apparent K_m for cysteinesulfinate of 0.045-0.17 mM. Brain glutamate decarboxylase (EC 4.1.1.15) can also catalyze the decarboxylation of cysteinesulfinate (Oertel et al., 1981). Alternatively, cysteinesulfinate may undergo transamination or oxidative deamination to form the putative intermediate, ß-sulfinylpyruvate, which spontaneously decomposes to yield pyruvate and sulfite (Singer and Kearny, 1956). In mammals, hypotaurine is apparently oxidized to taurine via a poorly characterized enzymatic reaction that requires NAD^+, Cu^{2+}, and O_2 (Oja and Kontro, 1981) with an apparent K_m for hypotaurine of 0.20 mM (Oja and Kontro, 1981).

The cysteinesulfinate pathway seems to be the major route of taurine formation in mammals. This conclusion is supported by 1) the detection of labeled cysteinesulfinate and taurine in tissues of animals given labeled Cys (Pasantes-Morales et al., 1980), 2) the presence of the enzymes involved in the conversion of Cys to cysteinesulfinate and hypotaurine in most mammalian tissues (De La Rosa and Stipanuk, 1985), and 3) Hepatic cysteinesulfinate decarboxylase levels correlates with the capacity of animals to synthesize taurine (De La Rosa and Stipanuk, 1985, Hardison et al., 1977).

Figure 1.2 Mammalian cysteine metabolism. The numbers marked with parentheses correspond to the following enzymes or metabolic processes: 1. Ribosomal protein synthesis ; 2. Formation of protein disulfide bonds; 3. Cysteine aminotransferase and probably other aminotransferases; 4. 3-Mercaptopyruvate sulfurtransferase; 5. Cystathionine γ-lyase; 6. Poorly characterized mitochondrial activity; 7. Cysteine dioxygenase; 8. Aspartate aminotransferase; 9. Spontaneous reaction; 10. Cysteinesulfinate decarboxylase (sulfinoalanine decarboxylase); 11. Hypotaurine oxidase 12. Cysteinesulfinate decarboxylase (sulfinoalanine decarboxylase); 13. γ-Glutamylcysteine synthetase (glutamate-cysteine ligase); 14. Glutathione synthase; 15. Combined activities of γ-glutamyltransferase and various dipeptidases acting on cysteinylglycine; 16. Enzymatic and nonenzymatic oxidation of glutathione to glutathione disulfide followed by the cleavage reactions listed as reaction 15; 17.Various thiol oxidases; 18. Enzymatic and nonenzymatic transhydrogenations (disulfide interchange reactions) with glutathione coupled to glutathione reductase; 19. See figure 1.1.

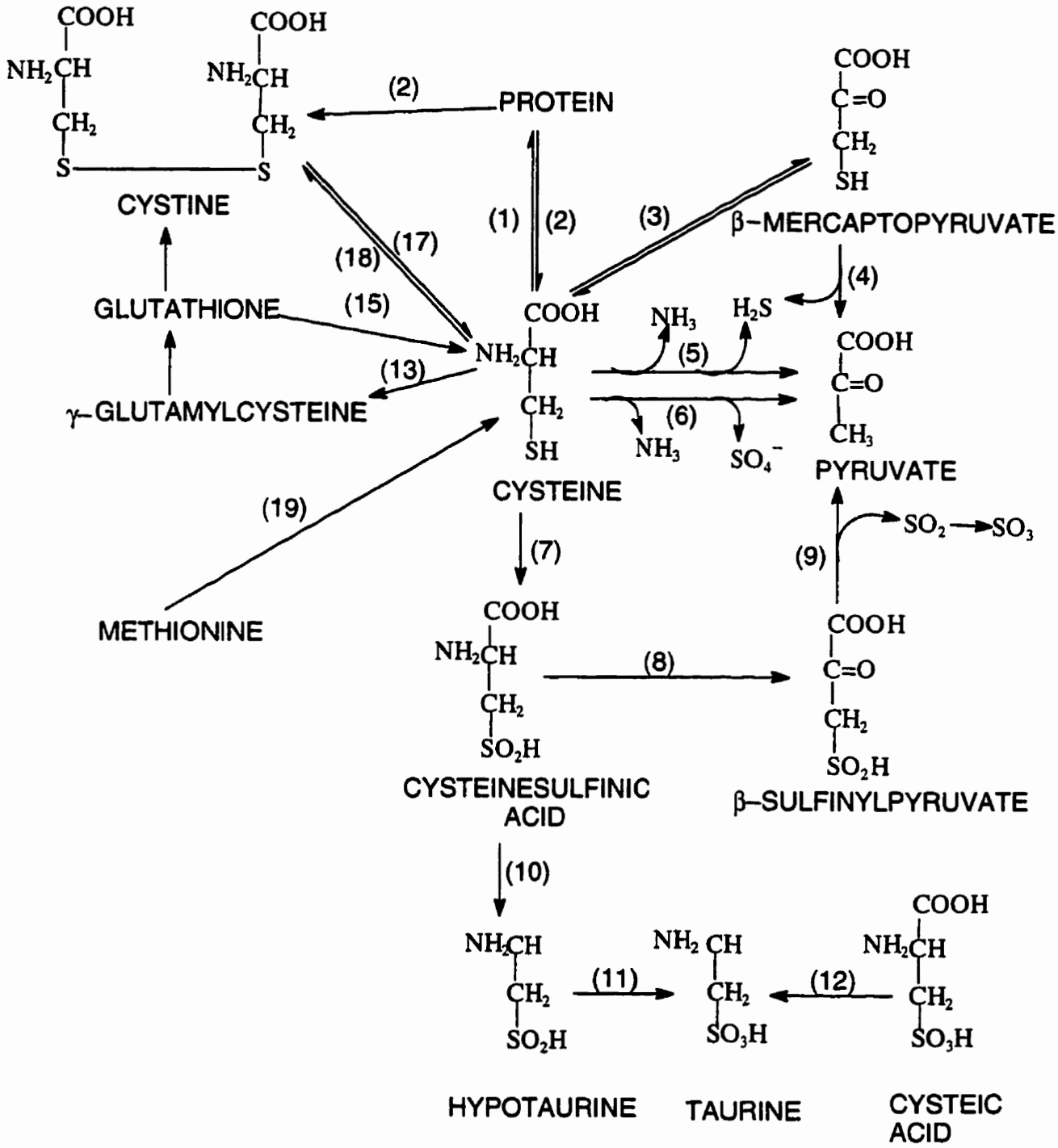


FIGURE 1.2- METABOLISM OF CYSTEINE

About 70-90% of the cysteinesulfinate oxidized by rat hepatocytes in vitro or by rats or mice in vivo is converted to hypotaurine plus taurine (Stipanuk and Rotter, 1984); this indicates that, under normal conditions, the rat or mouse metabolizes cysteinesulfinate primarily to taurine rather than to pyruvate and sulfite.

Taurine is used in conjugation reactions (i.e. bile acids) but does not otherwise appear to be further metabolized by mammalian tissues (Fellman et al., 1978).

Cysteine desulfuration pathways Two pathways for hepatic H_2S formation by cysteine desulfuration have been proposed: 1) a **sulfurtransferase** catalyzed desulfuration of β-mercaptopyruvate (Meister, 1959) which is formed from cysteine transamination (Agaki, 1982). 2) a **cystathionase** catalyzed cleavage of cystine to pyruvate, ammonia, and thiocysteine, followed by further enzymatic or nonenzymatic reaction of thiocysteine with the enzyme or another thiol to reform a disulfide accompanied by sulfide release (Stipanuk and Beck, 1982). The apparent K_m of rat liver cystathionase for L-cystine has been estimated to be about 0.03-0.07 mM compared with 0.8-3.5 mM for L-cystathionine and 15-20 mM for L-homoserine (Yamanishi and Tuboi, 1981). Thus, cystine should compete favorably with other substrates for the cystathionase.

Cyst(e)ine is metabolized by rat enterocytes to pyruvate and inorganic sulfur but not to taurine. About one half of cyst(e)ine metabolism appears to involve its oxidation to cysteinesulfinate and the transamination of cysteinesulfinate to the putative intermediate sulfinylpyruvate, which decomposes to yield sulfite and pyruvate. The remainder of Cys catabolism in enterocytes appears to involve the **γ-cystathionase** catalyzed release of

sulfide from cyst(e)ine prior to its oxidation. Sulfide released from cyst(e)ine in this reaction appeared to be oxidized to thiosulfate in enterocytes before being reduced by GSH to sulfite and oxidized to sulfate (Coloso and Stipanuk 1989). γ-Cystathionase, thus, catalyses both the cleavage of cystathionine to cysteine plus α-ketobutyrate and ammonium ions as well as the cleavage of cyst(e)ine to form pyruvate, sulfide and ammonium ions.

Cysteine metabolism by brain cells involves the hydrogen sulfide producing enzyme **cystathionine β-synthase** (CBS) which has been found to be highly expressed in the hippocampus and cerebellum. Brain homogenates produce hydrogen sulfide in the presence of cysteine and pyridoxal 5′-phosphate. The production of hydrogen sulfide is inhibited by the CBS inhibitors hydroxylamine and aminooxyacetate and is activated by S-adenosyl-L-methionine (AdoMet), indicating that CBS contributes to the production of endogenous H_2S. The cystathionine γ-lyase inhibitors D,L-propargylglycine and β-cyano-L-alanine do not suppress the production of hydrogen sulfide in the brain (Abe and Kimura, 1996), although these inhibitors suppress H_2S production effectively in the liver and kidney (Stipanuk and Beck 1982). It is unlikely that the other pyridoxal 5′-phosphate-dependent enzyme, cysteine aminotransferase, contributes to the production of endogenous H_2S, because it requires a pH well above physiological levels (Stipanuk and Beck 1982).

These observations indicate that CBS is the major enzyme that produces endogenous brain hydrogen sulfide and strongly suggest that hydrogen sulfide can be produced in the

brain under physiological conditions. There is also evidence that cysteine levels are increased during hypoxia/ischemia (Slivka and Cohen, 1993, Puka-Sundvall 1997) which can lead to increased levels of hydrogen sulfide. Sulfide may therefore have a role in modulating brain cell resistance to hypoxia.

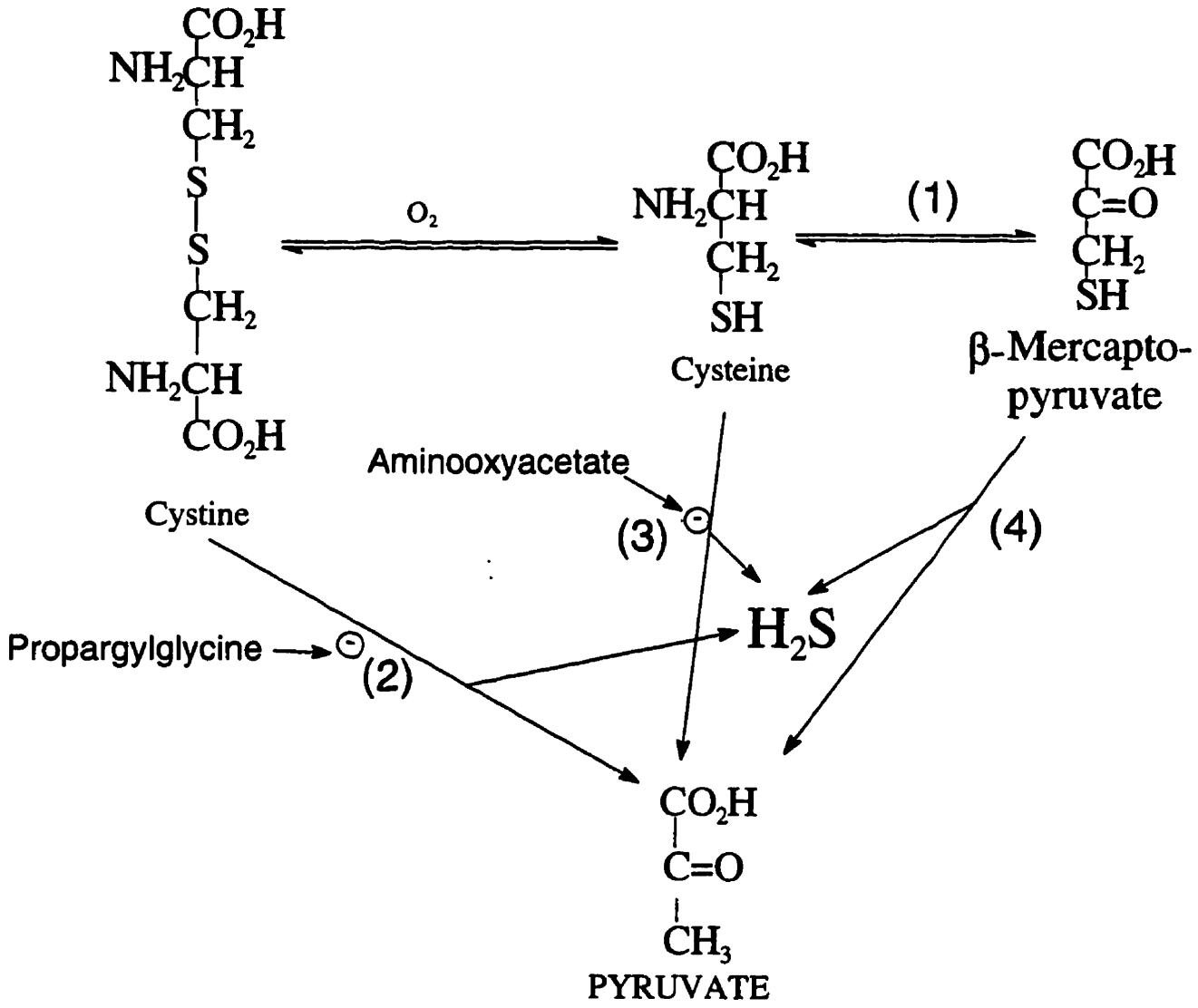
1.4 Summary of endogenous production of hydrogen sulfide

In figure 1.3 a summary of endogenous sources for hydrogen sulfide has been shown. Hydrogen sulfide can be formed from cysteine by the action of cystathionine β-synthase, an enzyme which is highly expressed in the brain. This process is inhibited by aminooxyacetate. Cysteine can be oxidized to cystine and cystathionine γ-lyase, which is highly expressed in the liver, can produce hydrogen sulfide from cystine. This reaction is inhibited by propargylglycine. Cysteine can also be converted to β-mercaptopyruvate by the action of cysteine aminotransferase, an enzyme which is expressed in the liver. Finally, 3-mercaptopyruvate sulfurtransferase (which is expressed in the liver) forms hydrogen sulfide from β-mercaptopyruvate (Figure 1.3).

1.5 Summary of the hypothesis and plan of the thesis

Hydrogen sulfide can have both toxic and physiological roles. Traditionally it has been viewed as a cytochrome oxidase poison, which blocks the mitochondrial respiration chain, in much the same way as cyanide and carbon monoxide (Beck et al., 1981). Hydrogen sulfide has also been suggested to have a signaling role analogous to that of NO (Abe and Kimura, 1996). Hydrogen sulfide levels may be increased (especially in the

Figure 1.3 Endogenous production of Hydrogen sulfide



(1) Cysteine aminotransferase (Liver)

(2) Cystathionine γ-lyase (Liver)

(3) Cystathionine β-synthetase (Brain)

(4) 3-Mercaptopyruvate sulfurtransferase (Liver)

brain) during hypoxia/ischemia. Therefore, modulation of hydrogen sulfide levels may be protective under such conditions. Finally, H_2S can interact with metals which themselves can be protective or toxic depending on conditions. We therefore set out to explore the protective and toxic actions of hydrogen sulfide and the role of metal ions in modulating these cytoprotective and cytotoxic actions. We examined the effects of exogenous hydrogen sulfide and the consequences of the induction of endogenous sulfide production in the cells using cysteine precursors. The specific hypotheses have already been stated on page 2.

Outline of the thesis

In chapter 2, the materials and methods used in these experiments are described.

In chapter 3, the toxicity of hydrogen sulfide towards hepatocytes and organotypic hippocampal slices have been compared and the possibility of endogenous production of hydrogen sulfide has been discussed.

In chapter 4, the protective actions of zinc and other metals against hydrogen sulfide and their possible mechanisms of action have been discussed.

In chapter 5, the physiological role of hydrogen sulfide and its protective action against oxidative stress and ischemia/reperfusion injury has been discussed.

Finally, chapter 6 includs a summary and general conclusions. I carried out all experiments in this thesis. However, GSH levels measurement in hepatocytes was partly carried out by Mr. Bin Wu.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Collagenase (from Clostridium histoliticum) was purchased from Worthington Biochemicals corporation (Freehold, NJ). HEPES and bovine serum albumin were obtained from Boehringer-Menheim (Montreal, Canada). AOA, dihydroxyacetone, cysteine, cystine, CDME, hydroxycobalamine, catalase, TPEN, deferoxamine, chloroquine, methylamine, MNNG, and L-alanine were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest purity that was commercially available.

2.2 Preparation of hepatocytes

Hepatocytes were isolated from adult male Sprague-Dawley rats (220-250g), by collagenase perfusion of the liver as described by Moldeus et al (1978). Cell viability was measured by Trypan blue exclusion method, and the hepatocytes used in this study were at least 85-90% viable. Cells were preincubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 12.5 mM Hepes for 30 min under an atmosphere of 10% O_2 /5% CO_2/N_2 at 37°C in continuously rotating 50 ml round bottom flasks before the addition of chemicals. Stock solutions of chemicals were made either in incubation buffer or in DMSO and added to the hepatocyte suspensions at the indicated time points. Hepatocytes were incubated in an atmosphere of 10% O_2 /5% CO_2/N_2 or 95% O_2 /5% CO_2 at 37°C.

Glutathione depleted hepatocytes were prepared by incubating the hepatocytes with 200 μM 1-bromoheptane for 30 minutes as previously described (Khan and O'Brien 1991). Glutathione-S-transferase transfers the heptyl group of 1-bromoheptane to GSH to form heptyl-S-glutathione and release bromide. Bromoheptane unlike the other GSH depleting agents is not itself toxic to the cells even at ten times the concentration required to deplete hepatocyte GSH. Bromoheptane also depletes hepatocyte GSH much more rapidly and more completely than other GSH depleting agents (Khan and O'Brien 1991).

2.3 Intracellular GSH in isolated hepatocytes was measured in deproteinized samples (5% metaphosphoric acid) after derivatization with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene, by HPLC, using a μBondapak NH_2 column (Water associates, Milford, MA)(Reed et al., 1980). GSH and GSSG were used as external standards. A Waters 6000A solvent delivery system, equipped with a model 660 solvent programmer, a Wisp 710A automatic injector, and a data module were used for analysis. The procedure is based upon the initial formation of S-carboxymethyl derivatives of free thiols with iodoacetice acid followed by conversion of free amino groups to 2,4-dinitrophenyl derivatives by reaction with 1-fluoro-2,4-dinitribenzene (FDNB). Determination of nanomole levels of GSH and GSSG is possible with this method. Briefly, 0.8 ml of cell suspension was spun at half maximal rate for 40 seconds and cell pellet was resuspended in 0.8 ml of fresh medium. 0.2 ml of 25% metaphosphoric acid was added to the sample and after 10-60 minutes the sample was centrifuged at maximal rate for 5 minutes. 0.5 ml of supernatant and 0.05 ml of iodoacetic acid were mixed in 200-300 mg of sodium

bicarbonate. The mixture was sealed and left in the dark and room temperature for 1 hour. Then 0.5 ml of FDNB solution (1.5% v/v in ethanol) was added to the sample and the sample was sealed. The sample was left in the dark for 4 hours at room temperature and then analyzed by HPLC.

2.4 Determination of hydrogen sulfide concentration in the medium containing hepatocytes was carried out as described by Nashef et al. (1977). Briefly 3 ml samples of medium containing hepatocytes were centrifuged for 3 min at 1000 rpm. 20 µl of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) solution (10 mM) was added to 2 ml of supernatant and tha absorbance at 412 nm was read at 2 min in spectrophotometer. In this method one mole of hydrogen sulfide reacts with one mole of DTNB and produces two moles of the thiol anion, 5-thio-2-nitrobenzoate, and one mole of free sulfur. Concentrations of hydrogen sulfide down to 3.7×10^{-6}M could be easily determined with DTNB (Nashef et al., 1977).

2.5 Preparation of Hippocampal Slice Cultures

Organotypic hippocampal slice cultures were prepared according to the method of Stoppini et al. (1991) with minor modifications (Abdel-hamid and Tymiansky, 1997) . Briefly, seven-day old postnatal Wistar rat pups were anesthetized with ketamine (50 mg/Kg, intraperitoneal) and sacrificed by decapitation. The hippocampi were dissected out under sterile conditions and incubated for 5-10 min in ice-cold Hank's balanced salt solution (HBSS) supplemented with d-glucose (to a total of 6.5 mg/ml) and 20 mM HEPES acid / HEPES sodium salt (pH 7.2). Using a tissue slicer, 400 µm tissue transverse hippocampal slices were obtained and replaced in ice-cold HBSS for a further 15-20 min. A fire polished

Pasteur pipette was used to transfer the individual slices to 30 mm sterile Millicel-CM 0.4 μm tissue culture plate inserts . Slices lacking complete cell layers (CA1-CA3 and DG) were discarded. The inserts were placed in 6-well tissue culture plates along with 1 ml of culture medium (CM) consisting of 50% MEM, 25% Horse serum, 25% Earl's balanced salt solution. This mixture were supplemented with d-glucose and HEPES similar to the HBSS. Both HBSS and CM contained penicillin G and streptomycin sulfate (5.0 units/ml and 5 μg/ml respectively). The slices were then incubated at 36.5°C in 100% humidity and 5% CO_2 in air until they were used. Feeding of the slices was carried out by total replacement of the CM with 0.9 ml of fresh CM twice per week.

2.6 Assessment of neuronal cell death by image analysis using fluorescence microscopy. The intensity of propidium iodide fluorescence in the CA1 subfields of the cultures was used as an index of cell death. Propidium iodide (PI) is not fluorescent by itself but becomes fluorescent when bound to DNA. PI cannot cross the cell membrane when the integrity of cell membrane is intact but can enter the dead cells and binds to the nuclei. The slice culture medium was exchanged with the medium containing PI (1 μg/ml) 24 hours before doing the experiments. Chemicals were dissolved in HBSS and were diluted to desired concentrations in HBSS. At this time of the experiment, the slice cultures were washed once with HBSS and then incubated with HBSS containing chemicals for 60 minutes. Then the slices were washed with CM once and incubated in fresh CM containing PI (1 μg/ml) for 24 hours. The first measurement of fluorescence intensity was performed 2 hours before exposing the slices to the experimental treatment (background fluorescence) and the second one was made 24 hours after the experimental

treatment. The cultures were examined using a Zeiss Axiovert 100 TV inverted fluorescent microscope and a 10X Achrostigmat lens. Fluorescent images were obtained using a Cohu CCD camera (model number 4912) and were digitized, using Axon Imaging Workbench 2.1 software running under Microsoft Windows. After the second measurement of propidium iodide fluorescence intensity, the remaining cells were killed by exposing the cultures to 0.1% digitonin. The fluorescence intensity 2 hours after digitonin was set equal to 100% damage to CA1. The fluorescence intensity measured 2 hours before adding toxins (Figure 2.1A) was subtracted from those measured 24 hours after the experimental treatment (Figure 2.1B) and after digitonin (Figure 2.1C). Then the values (fluorescence intensity) obtained from the CA1 region 24 hours after treatment were expressed as a percentage of the values obtained following digitonin (Figure 2.1).

2.7 Data analysis.

A propidium iodide (PI) fluorescence intensity measurement for any given slice culture consisted of the averages of the fluorescence intensity values of pixels in selected areas of the image. This value was proportional to the number of injured cells in the present preparation ((Newell et al., 1995; Abdel-hamid and Tymiansky 1997; (Figure 2.1)). Four non-overlapping fields each encompassing almost ¼ of the CA1 region visible in the slice were selected and total fluorescence in these fields were measured. These values were averaged to obtain a mean value. Because the measurement was not confocal there was considerable scattered light in white matter of the slice. Nevertheless this scattered light would be proportional to the number of stained dead cells. Each PI fluorescence measurement (Figure 2.1B) was background subtracted (Figure 2.1A) and normalized to

the maximal fluorescence (Figure 2.1C) obtained from the same slice after a 2 hour exposure to 0.1% digitonin. Thus, the percentage of cell death in each experiment was expressed as:

$$\%\text{Cell death} = ((F_t - F_0) / (F_{total} - F_0)) \times 100$$

Where F_0 = background fluorescence at the start of experiments, F_t = slice fluorescence 24 hours after treatment with drugs, and F_{total} = maximal fluorescence after digitonin.

2.8 Statistical analysis

The statistical significance of differences between control and treated groups was evaluated using unpaired students 't' test done by PSI-Plot version 5.02 for Windows (Poly Software International) and significantly different groups were chosen when $p < 0.01$.

Figure 2.1. Images taken from CA1 region of hippocampal slice cultures. Propidium iodide was added to CM 24 hrs before exposing the slices to the experimental treatment. The first measurement of fluorescence intensity was performed 2 hours before exposing the slices to the treatment (background fluorescence)(A) and the second one 24 hours after treating the culture (B). After the second measurement of propidium iodide fluorescence intensity, the remaining cells were killed by exposing the cultures to 0.1% digitonin. The fluorescence intensity 2 hours after digitonin was set equal to 100% damage to CA1 (C). Because the measurement was not confocal there was considerable scattered light in white matter of the slice. Images were displayed using a 256-pseudocolor spectrum from violet to red with black indicating no fluorescence and white indicating saturation.

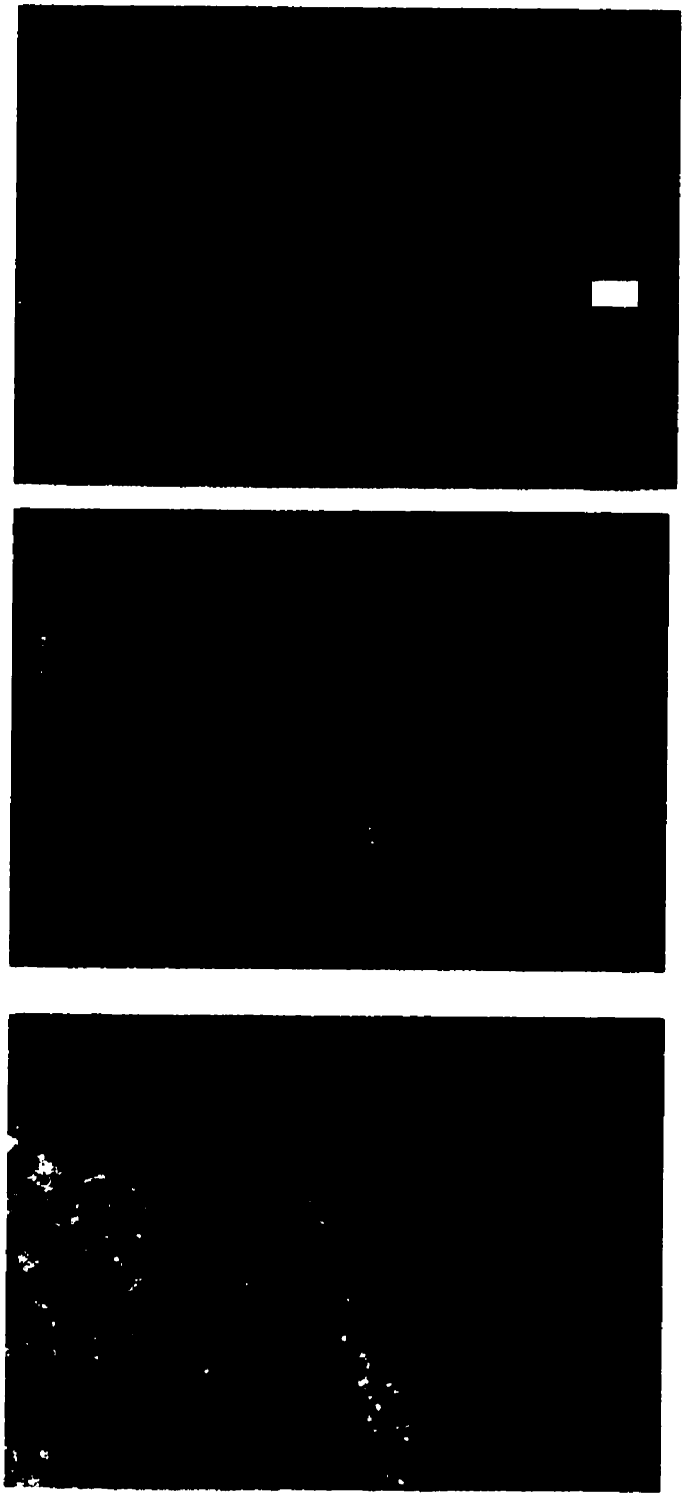


Fig 2.1

Chapter 3

The cell death induced by hydrogen sulfide and sulfide precursors in hippocampal slice cultures and in isolated hepatocytes

3.1 Introduction

As discussed in the introduction the industrial sources of H_2S are major occupational health hazards and yet H_2S produced endogenously may have a physiological role (Abe and Kimura, 1996). This is particularly true in the brain where the sulfur containing amino acid cysteine is found in high concentrations. We have studied H_{2S} toxicity in both brain slices and isolated hepatocytes. Isolated hepatocytes were prepared as described by Moldeus et al., 1978; Niknahad and O'Brien 1995 and organotypic hippocampal slice cultures were prepared as described by Stoppini et al., 1991; Abdel-hamid and Tymiansky, 1997. We were particularly interested in the action of H_2S in the brain but preliminary experiments were conducted with hepatocytes.

Endogenous H_2S can be produced from L-cysteine mainly by two pyridoxal-5′-phosphate-dependent enzymes, cystathionine β-synthetase and cystathionine γ-lyase (Abe and Kimura, 1996, Stipanuk and Beck, 1982). The H_2S-producing enzyme cystathionine β-synthetase is highly expressed in the hippocampus and cerebellum, and brain homogenates produce H_2S (Abe and Kimura, 1996). It has been reported that the cleavage of cyst(e)ine by cystathionine γ-lyase is an important physiological pathway for cysteine catabolism in rat liver (Drake et al. 1987). Other peripheral tissues such as ileum, portal

vein and thoracic aorta also express the H_2S-producing enzyme cystathionine γ-lyase (Hosoki et al., 1997). Both enzymes have distinct and specific blockers. Thus, we could test the hypothesis that lipid soluble cysteine precursor cystine dimethylester (CDME) was toxic as the result of the production of intracellular cyst(e)ine. We predicted that propargylglycine a cystathionine γ-lyase inhibitor (Stipanuk and Beck 1982, Drake et al., 1987) would decrease the toxicity of cystine dimethylester in the liver cells while aminooxyacetate an inhibitor of cystathionine β-synthase (Abe and Kimura, 1996) would decrease its toxicity in brain slices. We also predicted that cyst(e)ine is toxic as a result of H_2S production and thus these blockers would not be protective against exogenous hydrogen sulfide. Another test of the hypothesis H_2S acts intracellularly is to explore the action of anion channel blockers in reducing HS^- flux across the membrane. Again different chloride channel blockers were protective against hydrogen sulfide in hepatocytes and brain slices.

3.2 Results

Cytotoxicty in hepatocytes was assessed by trypan blue exclusion and in hippocampal slices by the increase in propidium iodide fluorescence (see chapter 2). NaHS induced a dose dependent cell death in both hepatocytes and hippocampal slice cultures. Hippocampal slice cultures were more susceptible to NaHS as NaHS at 100 μM was not toxic to hepatocytes but caused almost 60% cytotoxicity in hippocampal slices. Because H_2S was escaping from the solutions in which the cells were suspended we had to seal the flasks or 6-well plates in order to get reproducible toxicity results with hydrogen sulfide.

3.2.1 Detoxification of hydrogen sulfide by GSH

NaHS at 0.3 mM caused almost 100% cell death in GSH depleted hepatocytes. Since NaHS at 0.5 mM caused only 65% toxicity in normal cells those results suggests that GSH is involved in detoxification of H_2S (tables 3.1 and 3.2).

3.2.2 Metabolism of hydrogen sulfide by hepatocytes

NaHS was added to the hepatocyte suspension in a sealed vial and its concentration determined at different time intervals (as described in methods (chapter 2)). At 100 μM concentration, more than 90% of the H_2S disappeared within 30 minutes whereas in the absence of hepatocytes only 30% of the H_2S had disappeared in this time (figure 3.1). These results suggest that any protective action of H_2S is due to the products of the reaction between HS^- and cell components. One major product would be persulfide aducts.

In another set of experiments hepatocytes were incubated with NaHS. After different times the cell suspensions were centrifuged with half maximal speed for 1 min and the cells resuspended in fresh medium. Resuspension of hepatocytes was only protective if it was done within 20 minutes of incubation. These results suggest that cells are committed to death within 20 minutes of exposure to H_2S even though they may take two hours to die (table 3.2). This problem with stability suggests that H_2S may be even more potent than our results indicate.

3.2.3 Blocking the chloride ion channels protects the cells against H_2S

As discussed in the introduction, at a physiological pH, two thirds of the H_2S exists as HS^-. Thus we hypothesised that H_2S has to cross the membrane via anion channels in order to act intracellularly.

Table 3. 1 Propargylglycine is not protective against NaHS toxicity in GSH depleted hepatocytes

Treatment	% Cytotoxicity (% trypan blue uptake)		
	60 min	120 min	180 min
GSH depleted hepatocytes	18 ± 3	23 ± 3	27 ± 3
NaHS 0.3 mM	65 ± 3[a]	95 ± 4[a]	98 ± 2[a]
+ propargylglycine 200 μM	70 ± 3	98 ± 2	100
propargylglycine 200 μM	20 ± 3	25 ± 3	28 ± 4

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of $10\%O_2/5\%CO_2/N_2$. The chemicals were present in cell suspensions during the experiment. Propargylglycine was added immediately before NaHS and the flasks containing cells with NaHS were sealed for 30 minutes to get reproducible toxicity with NaHS. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes ($P<0.01$)

Table 3.2 Prevention of NaHS hepatotoxicity by hepatocyte resuspension in fresh buffer

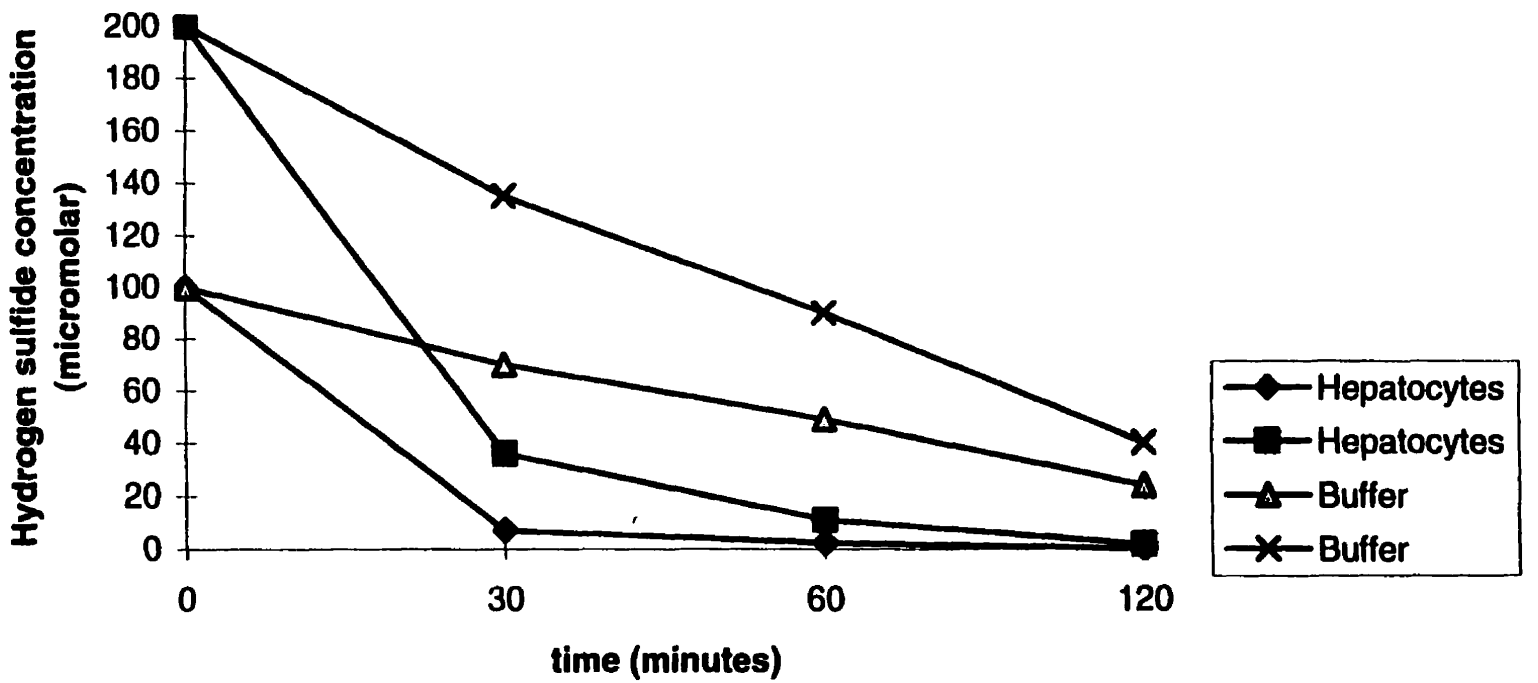
Treatment	% Cytotoxicity at		
	60 min	120 min	180 min
Control	18 ± 3	20 ± 3	22 ± 3
NaHS 0.5 mM	45 ± 3[a]	58 ± 4[a]	65 ± 4[a]
NaHS 0.5 mM (resuspended at 20′)	22 ± 2[b]	26 ± 4[b]	28 ± 4[b]
NaHS 0.5 mM(resuspended at 40′)	46 ± 3	58 ± 4	66 ± 4
NaHS 0.5 mM(resuspended at 60′)	48 ± 3	62 ± 4	70 ± 4

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of $10\%O_2/5\%CO_2/N_2$. NaHS was added to the cell suspensions and the flasks containing cells were sealed for 30 minutes to get reproducible toxicity with NaHS. At different times after adding NaHS the cell suspensions were centrifuged with half maximal speed for 1 min and the cells resuspended in fresh medium. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes ($P<0.01$)

b: Significant difference in comparison with metal NaHS treated hepatocytes ($P<0.01$)

Figure 3.1 Time course of hydrogen sulfide concentration in solutions. NaHS was added to the solutions (200 or 100 micromolar) and its concentration was measured at indicated times using DTNB.(Results are from one experiment).



Blocking the chloride ion channels by picrotoxin in hippocampal slices (Labrakakis et al., 1997) was significantly protective against H_2S indicating that chloride ion channels were involved in the neurotoxicity of H_2S (table3.3). Glycine and alanine which are believed to block Cl^- channels in hepatocytes (Carini et al., 1997) prevented H_2S induced cytotoxicity indicating the involvement of these channels in the hepatotoxicity of H_2S (table 3.4). It seems likely therefore that HS^- anions cross the cell membrane via chloride ion channels and act intracellularly.

3.2.4 Hepatotoxicity of Cystine dimethylester

As discussed in the introduction, intracellular sulfide formation should be induced by loading the cells with sulfur containing amino acids such as cysteine and cystine or their derivatives. Cystine dimethylester was not toxic to the normal hepatocytes at 0.5 mM. However, cystine dimethylester induced 95% cytotoxicity in GSH depleted hepatocytes within 3 hours. Cytotoxicity was prevented by propargylglycine, a cystathionine γ-lyase inhibitor (table 3.5). Dihydroxyacetone, a H_2S trap, also prevented cystine dimethylester toxicity (table 3.4).

The toxicity of H_2S in GSH depleted cells was not prevented by propargylglycine (table 3.1), suggesting that this agent is not having a direct action against H_2S.

3.2.5 Endogenous production of high concentrations of H_2S causes neurotoxicity

In order to test our hypothesis that it is intracellular H_2S that is neurotoxic, the susceptibility of hippocampal slices to the H_2S precursors cysteine,

Table 3.3. Protective effect of picrotoxin against H_2S neurotoxicity

Treatment	% Cytotoxicity at 24 hours
Control	0
NaHS 0.5 mM	58 ± 10
+ Picrotoxin 100 μM	14 ± 5[a]
Picrotoxin 100 μM	10 ± 4

Hippocampal slice cultures were exposed to the indicated chemicals for 60 minutes in HBSS and the plates treated with NaHS were sealed for 30 minutes. The proportion of the increase in PI fluorescence 24 hrs after adding chemicals to that 2 hrs after digitonin was set as the percentage of cytotoxicity. The results are from three animals and four slices from each animal and are presented as mean ± SE.
a: Significant difference in comparison with NaHS treated slices ($P<0.01$)

Table 3.4 Protective effect of glycine, alanine and dihydroxyacetone against NaHS hepatototoxicity

Treatment	% Cytotoxicity at		
	60 min	**120 min**	**180 min**
Control	18 ± 3	20 ± 3	22 ± 3
NaHS 0.5 mM	40 ± 3[a]	58 ± 4[a]	65 ± 4[a]
+ Glycine 2 mM	28 ± 3[b]	32 ± 4[b]	35 ± 4[b]
+ Dihydroxyacetone 10 mM	27 ± 2[b]	30 ± 2[b]	32 ± 2[b]
+ Alanine 3 mM	21 ± 2[b]	26 ± 2[b]	28 ± 3[b]

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of $10\%O_2/5\%CO_2/N_2$. Antidotes were added to the cell suspensions immediately before NaHS and the flasks were sealed for 30 minutes. The chemicals were present in the suspensions during the experiment. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes ($P<0.01$)

b: Significant difference in comparison with metal NaHS treated hepatocytes ($P<0.01$)

Table 3.5 Prevention of the toxicity of Cystine dimethylester ester in GSH depleted hepatocytes by propargylglycine

Treatment	% Cytotoxicity at		
	60 min	120 min	180 min
Control	18 ± 3	20 ± 3	22 ± 3
+Cystine dimethylester ester 0.5 mM	19 ± 2	21 ± 3	24 ± 3
GSH depleted hepatocytes	18 ± 3	23 ± 3	27 ± 3
+Cystine dimethylester ester 0.5 mM	91 ± 4[a]	94 ± 4[a]	96 ± 4[a]
+ Cystine dimethylester ester 0.5 mM + propargylglycine 200 μM	22 ± 3[b]	27 ± 3[b]	28 ± 4[b]
+ propargylglycine 200 μM	20 ± 3	25 ± 3	28 ± 4

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of $10\%O_2/5\%CO_2/N_2$. Hepatocyte GSH was depleted by incubating the cells with 200 μM 1-Bromoheptane for 30 minutes and then the chemicals were added to the medium. The chemicals were present in the suspensions during the experiment. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.
a: Significant difference in comparison with control hepatocytes ($P<0.01$)
b: Significant difference in comparison with metal CDME treated hepatocytes ($P<0.01$)

cystine and cystine dimethylester ester were investigated. At equimolar concentrations the more hydrophobic cystine dimethylester was more toxic than cysteine or cystine as expected if it entered cells more readily (table 3.6). In contrast to results with hepatocytes (see above) the cystathionine γ-lyase inhibitor, D,L-propargylglycine did not prevent the toxicity of each of these sulfur containing amino acids (table 3.6). The neurotoxicity of cystine dimethylester, however, was prevented by amino-oxyacetate, an inhibitor of cystathionine β-synthase indicating that the observed toxicity could be attributed to hydrogen sulfide production by this enzyme (table 3.7). These results are consistent with the lack of expression of cystathionine-γ-lyase in hippocampal cells and the prominent expression of cystathionine-β-synthetase in that tissue.

3.3 Discussion

Endogenous H_2S is formed from cysteine in the brain primarily by CBS (Abe and Kimura, 1996). The production of H_2S in the brain is suppressed efficiently by the CBS inhibitors hydroxylamine and amino-oxyacetate and is strongly potentiated by the CBS activator, S-adenosylmethionine (AdoMet) (Abe and Kimura, 1996). In contrast, the expression in the brain of the other H_2S-producing enzyme, CSE, is undetectable by Northern blot analysis (Abe and Kimura, 1996). CSE inhibitors D,L-propargylglycine and β-cyano-L-alanine did not suppress H_2S production in the brain (Abe and Kimura, 1996), although they suppressed the production of H_2S efficiently in the liver and kidney (Griffith, 1987). It is unlikely that the other pyridoxal 5′-phosphate-dependent enzyme, cysteine aminotransferase, contributes to the production of H_2S, because it requires a pH well above physiological levels (Griffith, 1987). The cystathionine γ-lyase inhibitor, D,L-

Table 3.6. Propargylglycine does not prevent the toxicity of sulfur containing amino acids in hippocampal slice cultures

Treatment	% Toxicity at 24 hrs
Cysteine 1mM	23 ± 3
// + PPG 200 μM	24 ± 2
Cystine 1mM	12 ± 2
// + PPG 200 μM	10 ± 3
Cystine dimethylester 1 mM	48 ± 4
// // + PPG 200 μM	50 ± 3
PPG 200 μM	0
Control	0

Hippocampal slice cultures were exposed to the indicated chemicals in HBSS for 60 minutes and the proportion of the increase in PI fluorescence 24 hrs after adding chemicals to that 2 hrs after digitonin was set as the percentage of cytotoxicity. The results are from three animals and four slices from each animal and are presented as mean ± SE.

Table 3.7. Aminooxyacetate prevents Cystinedimethylester induced cytotoxicity in hippocampal slices

Treatment	% Toxicity at 24 hrs
Cystine dimethylester 1 mM	49 ± 5
Cystine dimethylester 1 mM + AOA 100 μM	30 ± 3[a]
Cystine dimethylester 1 mM + AOA 50 μM	28 ± 3[a]
AOA 100μM	0
Control	0

Hippocampal slice cultures were exposed to the indicated chemicals in HBSS for 60 minutes and the proportion of the increase in PI fluorescence 24 hrs after adding chemicals to that 2 hrs after digitonin was set as the percentage of cytotoxicity. The results are from three animals and four slices from each animal and are presented as mean ± SE.

a: Significant difference in comparison with Cystine dimethylester ester treated slices ($P<0.01$)

propargylglycine did not prevent the toxicity of sulfur containing amino acids (table 3.6). The toxicity of cystine dimethylester, however, was prevented partly by aminooxyacetate, suggesting that cystathionine β-synthase mediated cystine toxicity (table 3.7). All of these data suggest that H_2S can be produced in the brain and have a physiological or pathophysiological role in normal and pathological conditions, respectively.

Picrotoxin, a chloride ion channel blocker, was significantly protective against H_2S indicating that chloride ion channels were also involved in the toxicity of H_2S (table 3.3). It remains to be determined whether picrotoxin will also prevent toxicity induced by a H_2S precursor like cystine dimethylester. Our prediction is that picrotoxin will not be protective in this case.

Difference in glutathione levels between brain cells and hepatocytes may explain much of the difference in sensitivity in the absence of glutathione depletion but this needs to be determined. On the other hand, in neurons, cystine dimethylester is first hydrolyzed to cystine by intracellular esterases and then reduced to cysteine by GSH and finally, H_2S is produced from cysteine by the action of cystathionine β-synthase. Therefore, intracellular GSH is necessary for the neurotoxicity of H_2S precursors such as cystine dimethylester. This can be tested by measuring the toxicity of cystine dimethylester in GSH depleted neurons (in this case CDME should not be toxic) or by loading neurons with reduced GSH before exposing them to CDME (in this case CDME should be more toxic than in the absence of GSH).

Chapter 4

Cytoprotective actions of Zn^{2+} and other metals against hydrogen sulfide toxicity

4.1 Introduction

The hippocampus contains relatively high concentrations of zinc. Primarily located within vesicles in granule neurons of fascia dentata, zinc has been postulated to act as an enzyme cofactor and serve as an intracellular messenger substance (Frederickson and Danscher, 1990). The histochemical localization of zinc with Timm's stain reaction uses sulfide to precipitate zinc so that it is well known that zinc complexes sulfide (Frederickson 1989).

We have explored intracellular interactions of H_2S and zinc levels. Our hypothesis was that zinc might be protective against hydrogen sulfide toxicity. We also predicted that other metals would also be protective against hydrogen sulfide by increasing its oxidation. 8-Hydroxyquinoline is a lipophilic metal chelator which has been presumed to be able to transfer metals such as iron and nickel into the cells (Borg-Neczak and Tjälve 1994, Oubidar et al., 1996). Here we show that hippocampal slices can be loaded with zinc using the lipid soluble/membrane permeable zinc:8-hydroxyquinoline complex and that this intracellular zinc reduces hydrogen sulfide toxicity in this type of cells. Zinc:8-hydroxyquinoline complex was toxic to hepatocytes and Zn^{2+} alone was protective against hydrogen sulfide toxicity.

4.2 Results

4.2.1 Zinc is protective against hydrogen sulfide neurotoxicity

Endogenous hydrogen sulfide concentrations in the brain can reach levels as high as 50-

160 μM (Abe and Kimura, 1996). Results in table 4.1, however, show that 100 μM exogenous hydrogen sulfide was toxic in the CA1 region of organotypic hippocampal slice cultures. Hydrogen sulfide induced cytotoxicity was decreased by 50% by the lipid soluble and cell permeable zinc:8-hydroxyquinoline complex (50 μM). $ZnCl_2$ at 100 μM also prevented 0.5 mM NaHS induced cytotoxicity (table 4.2).

4.2.2 Protective effect of Zn^{2+} against hydrogen sulfide hepatotoxicity

As shown in table 4.3 NaHS at 0.5 mM induced 65% cytotoxicity in normal hepatocytes in 3 hours at 37°C under an atmosphere of 10% O_2/85% N_2/5% CO_2. Hepatocyte cytotoxicity did not occur if $ZnCl_2$ was present at the time of exposure to NaHS (table 4.3). We did not use 8-hydroxyquinoline complex of zinc because it was toxic to hepatocytes.

4.2.3 Protective effect of other metals against hydrogen sulfide hepatotoxicity

We examined various mechanisms by which zinc decreases the toxicity of NaHS. Zinc could act by forming an insoluble zinc precipitate or by catalyzing NaHS oxidation. Both of these actions could be induced by other metals such as Cu^{2+}, Ni^{2+} and Pb^{2+}. Therefore, we tested the effect of these metals on the toxicity of NaHS and found that Ni^{2+} and Pb^{2+} were protective against NaHS in hepatocytes (table 4.4).

4.3 Discussion

Zinc and other metals such as Cu^{2+} and Ni^{2+} were protective against NaHS toxicity (tables 4.1-4). The protection of hepatocytes against NaHS by metals in our experiments can be explained by the catalytic role of metals in sulfide oxidation. Hydrogen sulfide can

Table 4.1. Protective effect of Zinc –8-hydroxyquinoline against H_2S neurotoxicity

Treatment	% cytotoxicity
$ZnCl_2$ 50μM	6 ± 3
$ZnCl_2$ 50μM + 8-Hydroxyquinoline 100 μM	10 ± 5
8-Hydroxyquinoline 100 μM	10 ± 5
NaHS 100 μM	32 ± 3
$ZnCl_2$ 50μM+ 8-Hydroxyquinoline 100 μM + NaHS 100 μM	16 ± 4[a]

Hippocampal slice cultures were exposed to the indicated chemicals for 60 minutes in HBSS and the plates treated with NaHS were sealed for 30 minutes. The proportion of the increase in PI fluorescence 24 hrs after adding chemicals to that 2 hrs after digitonin was set as the percentage of cytotoxicity. The results are from three animals and four slices from each animal and are presented as mean ± SE.
a: Significant difference in comparison with NaHS treated slices ($P<0.01$)

Table 4.2. Prevention of NaHS toxicity by Zinc in hippocampal slices

Treatment	% toxicity
NaHS 0.5mM	58 ± 6
NaHS 0.5mM + $ZnCl_2$ 100μM	22 ± 4[a]
$ZnCl_2$ 100 μM	18 ± 3

Hippocampal slice cultures were exposed to the indicated chemicals for 60 minutes in HBSS and the plates treated with NaHS were sealed for 30 minutes. The proportion of the increase in PI fluorescence 24 hrs after adding chemicals to that 2 hrs after digitonin was set as the percentage of cytotoxicity. The results are from three animals and four slices from each animal and are presented as mean ± SE.
a: Significant difference in comparison with NaHS treated slices ($P<0.01$)

Table 4.3 Prevention of NaHS toxicity by zinc in isolated hepatocytes

Treatment	% Cytotoxicity at		
	60 min	**120 min**	**180 min**
Control	18 ± 3	20 ± 3	22 ± 3
NaHS 0.5 mM	45 ± 3[a]	58 ± 4[a]	65 ± 4[a]
+ $ZnCl_2$ 0.1 mM	20 ± 3[b]	35 ± 4[b]	38 ± 4[b]

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 10%O_2/5%CO_2/N_2. $ZnCl_2$ was added before NaHS and the flasks treated with NaHS were sealed for 3o minutes. The chemicals were present in the suspensions during the experiment. Samples were taken at mentioned time intervals and cell death was assessed by trypan blue uptake. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes (P<0.01)

b: Significant difference in comparison with NaHS treated hepatocytes (P<0.01)

Table 4.4 Prevention of NaHS hepatotoxicity by other metals

Treatment	% Cytotoxicity at		
	60 min	120 min	180 min
Control	18 ± 3	20 ± 3	22 ± 3
NaHS 0.5 mM	45 ± 3[a]	58 ± 4[a]	65 ± 4[a]
+ $NiCl_2$ 0.1 mM	32 ± 2[b]	35 ± 3[b]	40 ± 3[b]
+ $NiCl_2$ 0.25 mM	23± 2[b]	25 ± 2[b]	27 ± 3[b]
+ Pb acetate 0.1 mM at 30′	32 ± 2[b]	40 ± 3[b]	45 ± 3[b]
+ Pb acetate 0.1 mM at 60′	40 ± 2	57 ± 2	60 ± 3
+ $MnCl_2$ 0.1 mM	40 ± 3	50 ± 3	52 ± 3

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 10%O_2/5%CO_2/N_2. Antidotes were added immediately before NaHS and the flasks were sealed for 30 minutes. The chemicals were present in the suspensions during the experiment. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue uptake. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes ($P<0.01$)

b: Significant difference in comparison with NaHS treated hepatocytes ($P<0.01$)

be oxidized to atomic sulfur which can also react with thiols and disulfides and is toxic. However, further oxidation of atomic sulfur to non-reactive metabolites such as sulfite (SO_3^{2-}) and sulfate (SO_4^{2-}) results in a detoxification. In order to increase intracellular concentrations of metals we used 8-hydroxyquinoline which forms a lipid soluble complex with metals and permeates the cells (Borg-Neczak and Tjälve 1994, Oubidar et al., 1996).

The hippocampus contains high concentrations of zinc located within vesicles in granule neurons of fascia dentata It has been postulated that zinc may act as an enzyme cofactor and serves as an intracellular messenger substance (Frederickson and Danscher, 1990).

It has also been shown that acute intoxication with hydrogen sulfide can increase aspartate, glutamate, glutamine, GABA, glycine, taurine and alanine in the brainstem but has no effect on the level of these amino acids in cerebral cortex, striatum or hippocampus. The reduced effect on the levels of hippocampal amino acids may be due to the presence of large amounts of intracellular zinc. In this region Zn^{2+} prevented the toxicity of exogenous hydrogen sulfide (tables 4.1 & 4.2).Thus, it is possible that in the presence of zinc, hydrogen sulfide may be precipitated as zinc sulfide salt. Alternatively, Zn^{2+} is also known to be a potent non-competitive antagonist of NMDA responses in cultured hippocampal neurons (Krigsheld et al., 1981), and cortical neurons (Yamaguchi et al., 1978). Thus the protective action of Zn^{2+} may be secondary to its NMDA blocking activity. In preliminary results, we found that APV an NMDA receptor antagonist decreased H_2S toxicity. The interactions between hydrogen sulfide and NMDA receptors

needs to be clarified.

Chapter 5

Cytoprotection by hydrogen sulfide against oxidative stress induced toxicity in hepatocytes

5.1 Introduction

Zn^{2+} is an essential element for the normal growth and development of plants and animals. It functions as an integral part of a number of enzymes and therefore is important in many types of synthesis in the body, including RNA and DNA (Calesnick and Dinan, 1998). In vitro studies have shown that Zn^{2+} deficiency causes decreased cell proliferation and cytotoxicity in cultures (Thomas and Caffrey, 1991), whereas Zn^{2+} supplementation has the effect of preventing programmed cell death (Cohen and Duke, 1984). On the other hand, it has been shown that 10-50 μM Zn^{2+} selectively suppressed DNA synthesis and growth factor expression in primary fetal hepatocytes (Townsend et al., 1994). Zn^{2+} at higher concentrations (100-150 μM) also accentuated the toxicity in cultured skin fibroblasts from reactive oxygen species generated by hydrogen peroxide and hypoxanthine/xanthine oxidase reactions (Richard et al, 1993). It has also been reported that Zn^{2+} causes cell condensation with DNA fragmentation in human Chang liver cells in a dose dependent manner (Paramanatham et al., 1997). Similarly in neuronal cultures Zn^{2+} induces apoptotic cell death (Koh et al., 1996).

Here we show that exogenous hydrogen sulfide protects hepatocytes against Zn^{2+} toxicity. We have also shown that intracellular generation of hydrogen sulfide using the cystine precursor (cystine dimethylester) protects hepatocytes against Zn^{2+} induced toxicity. Thus

endogenously produced hydrogen sulfide may paradoxically have a protective role under certain conditions. Possible mechanisms responsible for this protective role are discussed.

5.2 Results

5.2.1 Protective effect of hydrogen sulfide towards Zn^{2+} induced cytotoxicity

Loading hepatocytes with Zn^{2+} (complexed with 8-hydroxyquinoline) under the atmosphere of 95% O_2/5% CO_2 led to increasing cell death as measured by trypan blue exclusion. Sodium hydrosulfide protected hepatocytes against Zn^{2+} toxicity in a dose dependent manner being most effective at 100 μM NaHS (table 5.1). Sodium hydrosulfide at 100 μM was more protective than the Cu^{2+} chelator neocuproine 100 μM (tables 5.1 and 5.4). Sodium hydrosulfide at higher concentrations (500 μM or more), caused hepatocyte cytotoxicity.

Preincubating hepatocytes with sodium hydrosulfide (100 μM) for 30-120 minutes before the addition of Zn^{2+} also markedly increased their resistance to Zn^{2+} (table 5.2). It is notable that the enhanced resistance occurred at a time when most of hydrogen sulfide had been metabolized (see figure 3.1) so that direct interaction between Zn^{2+} and H_2S is unlikely.

5.2.2 Protective effect of ROS scavengers towards Zn^{2+} toxicity

Reactive oxygen species (ROS) scavengers DMSO or catalase protected hepatocytes against Zn^{2+} toxicity indicating that ROS formation contributed to the toxicity of zinc (table 5.3).

Table 5.1. Protective effect of hydrogen sulfide towards Zn^{2+} hepatocyte toxicity

Treatment	Cytotoxicity at 60 min	120 min	180 min
Control (95% O_2/5% CO_2)	18 ± 2	19 ± 2	19 ± 2
8-Hydroxyquinoline 60 μM	21 ± 2	28 ± 3	28 ± 3
$ZnCl_2$ 20 μM + 8-Hydroxyquinoline 60 μM	43 ± 3[a]	95 ± 5[a]	100[a]
+ NaHS 100 μM at 5′ (sealed for 30′)	33 ± 2[b]	35 ± 3[b]	41 ± 3[b]

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 95%O_2/5%CO_2. NaHS was added 5 minutes after Zn:8-hydroxyquinoline complex and the flasks were sealed for 30 minutes. The chemicals were present in the cell suspensions during the experiment. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes ($P<0.01$)

b: Significant difference in comparison with $ZnCl_2$ treated hepatocytes ($P<0.01$)

Table 5.2. Protective effect of preincubating NaHS towards Zn^{2+} hepatocyte toxicity

Treatment	Cytotoxicity at 60 min	120 min	180 min
Control (10% O_2/5% CO_2/N_2)	18 ± 2	19 ± 2	19 ± 2
8-Hydroxyquinoline 60 μM	21 ± 2	26 ± 3	28 ± 3
$ZnCl_2$ 20 μM + 8-Hydroxyquinoline 60 μM	40 ± 3[a]	50 ± 3[a]	64 ± 3[a]
+ NaHS 100 μM (preincubated for 30 min)	23 ± 2[b]	30 ± 3[b]	38 ± 3[b]
+ NaHS 100 μM (preincubated for 90 min)	27 ± 3[b]	34 ± 3[b]	41 ± 3[b]

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 10%O_2/5%CO_2/N_2. NaHS treated hepatocyte were preincubated with it for 30 or 60 minutes (as indicated in the table) and then $ZnCl_2$ + 8-hydroxyquinoline was added. The chemicals were present in the suspensions during the experiment. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.
a: Significant difference in comparison with control hepatocytes (P<0.01)
b: Significant difference in comparison with zinc treated hepatocytes (P<0.01)

Table 5.3. Protective effect of ROS scavengers towards Zn^{2+} hepatocyte toxicity

Treatment	Cytotoxicity at 60 min	120 min	180 min
Control (10% O_2/5% CO_2/N_2)	18 ± 2	19 ± 2	19 ± 2
8-Hydroxyquinoline 60 µM	21 ± 2	26 ± 3	30 ± 3
$ZnCl_2$ 20 µM + 8-Hydroxyquinoline 60 µM	40 ± 3[a]	50 ± 3[a]	64 ± 3[a]
+ DMSO 15 µl/ml	26 ± 2[b]	34 ± 3[b]	39 ± 3[b]
+ Catalase 200 units/ml	24 ± 2[b]	31 ± 3[b]	36 ± 3[b]

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 10%O_2/5%CO_2/N_2. The chemicals were present in the suspensions during the experiment. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes ($P<0.01$)

b: Significant difference in comparison with zinc treated hepatocytes ($P<0.01$)

Table 5.4. Protective effect of metal chelators towards Zn^{2+} induced hepatocyte toxicity

Treatment	Cytotoxicity at		
	60 min	**120 min**	**180 min**
Control (10% O_2/5% CO_2/N_2)	18 ± 2	19 ± 2	19 ± 2
8-Hydroxyquinoline 60 μM	21 ± 2	26 ± 3	30 ± 3
$ZnCl_2$ 20 μM + 8-Hydroxyquinoline 60 μM	40 ± 3[a]	50 ± 3[a]	64 ± 3[a]
+ TPEN 20 μM	22 ± 2[b]	23 ± 3[b]	24 ± 3[b]
+ Neocuproine 100 μM at 5'	25 ± 2[b]	40 ± 3[b]	49 ± 3[b]
+ Desferoxamine 100 μM	26 ± 3[b]	28 ± 3[b]	29 ± 3[b]

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 10%O_2/5%CO_2/N_2. The chemicals were present in the suspensions during the experiment. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes ($P<0.01$)

b: Significant difference in comparison with zinc treated hepatocytes ($P<0.01$)

5.2.3 Effect of metal chelators and endocytosis inhibitors on the cytoxicity of Zn^{2+}

The Cu^{2+} chelator neocuproine, the Cu^{2+}, Zn^{2+} chelator TPEN and the Fe^{3+} chelator desferoxamine protected hepatocytes against the toxicity of Zn^{2+} (table 5.4). The endocytosis inhibitors chloroquine and methylamine also prevented zinc toxicity suggesting that zinc displaces Fe/Cu from storage proteins that have been endocytosed in lysosomes (table 5.5).

5.2.4 Effect of preincubating hepatocytes with hydrogen sulfide on hypoxia induced cell death

Another way of generating ROS is paradoxically the exposure of cells to hypoxic conditions (Khan and O'Brien 1995). It has been proposed that the following sequence of events results in hypoxic injury. The lack of oxygen and thus mitochondrial dysfunction inhibits NADH oxidation and results in a more reduced cellular redox state ($NADH/NAD^+$). Mitochondrial ATP formation is inhibited and cellular ATP levels are decreased by ATP utilizing cellular functions (Khan and O'Brien 1995). However they concluded that prolonged reductive stress resulting in oxygen activation and not ATP depletion is responsible for hypoxic injury. Hypoxia may also convert xanthine dehydrogenase to xanthine oxidase (Wu et al., 1991) which could be responsible for reactive oxygen species formation and cause a loss of membrane integrity.

Hepatocytes were incubated under an atmosphere of 10% O_2/5% CO_2/85% N_2 and sodium hydrosulfide 100μM was added to the medium. The flasks containing hepatocytes were then sealed for 30 minutes so as to prevent the escape of hydrogen sulfide formed by

Table 5.5. Protective effect of endocytosis inhibitors towards Zn^{2+} hepatocyte toxicity

Treatment	Cytotoxicity at 60 min	120 min	180 min
Control (10% O_2/5% CO_2/N_2)	18 ± 2	19 ± 2	19 ± 2
8-Hydroxyquinoline 60 μM	21 ± 2	26 ± 3	30 ± 3
$ZnCl_2$ 20 μM + 8-Hydroxyquinoline 60 μM	40 ± 3[a]	50 ± 3[a]	64 ± 3[a]
+ Chloroquine 100 μM	31 ± 3	36 ± 3[b]	39 ± 3[b]
+ Methylamine 30 mM	34 ± 3	38 ± 3[b]	40 ± 3[b]

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 10%O_2/5%CO_2/N_2. The chemicals were present in the suspensions during the experiment. The samples were taken at mentioned time intervals after adding chemicals and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes (P<0.01)

b: Significant difference in comparison with zinc treated hepatocytes (P<0.01)

the hydrolysis of sodium hydrosulfide. The cells were then incubated in 10% oxygen/5% CO_2/85% N_2 for 90 minutes and after that they were exposed to hypoxia. Hepatocytes were more resistant to hypoxia if pretreated with hydrogen sulfide (table 5.6).

5.2.5 Effect of preincubating hepatocytes with hydrogen sulfide on GSH levels

Glutathione is the most abundant antioxidant in the cell, where it is found predominantly in two redox forms: reduced (GSH) and oxidized (GSSG). Its protective action is based on oxidation of the thiol group of its cysteine residue with the formation of GSSG, which in turn is catalytically reduced back to the thiol form (GSH) by glutathione reductase (Meister and Anderson 1983). GSH has been known as a substrate for GSH S-transferases and GSH peroxidase, enzymes that catalyze the reactions for the detoxification of xenobiotic compounds or reactive oxygen species and free radicals (Meister 1991). GSH can also inactivate ROS as demonstrated here for hydroxyl radical (Juurlink 1997):

$$GSH + OH^{\bullet} \rightarrow GS^{\bullet} + H_2O$$

$$2\,GS^{\bullet} \rightarrow GSSG$$

We were therefore interested in measuring GSH levels following incubation with NaHS. In a preliminary experiment involving a single suspension of cells we measured changes in GSH levels following NaHS exposure. Hepatocytes were incubated under an atmosphere of 10% O_2/5% CO_2/N_2. Sodium hydrosulfide 100μM was added and the flask was sealed for 30 minutes. Control cells from the same animal were treated identically only those were not exposed to NaHS. Then the cells were incubated under 95% oxygen/5% CO_2 for 90 minutes, cell samples were taken at regular intervals and GSH

Table 5.6. Cytoprotective effect of hydrogen sulfide towards hypoxia induced toxicity in hepatocytes

Treatment	% Cytotoxicity at 60 min	% Cytotoxicity at 120 min
Control (10% O_2/5% CO_2/N_2)	16 ± 2	17 ± 2
Hypoxia	38 ± 3[a]	60 ± 4[a]
+ Preincubation with NaHS 100 μM (for 120 min)	25 ± 2[b]	35 ± 3[b]

The hepatocytes exposed to hypoxia were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 100%N_2. The flasks containing hepatocytes preincubated with hydrogen sulfide were sealed for 30 minuts and then incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 10%O_2/5%CO_2/N_2 befor exposure to hypoxia. The samples were taken at mentioned time intervals after hypoxia and cell death was assessed by trypan blue exclusion. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes (P<0.01)

b: Significant difference in comparison with hypoxia treated hepatocytes (P<0.01)

levels was measured by HPLC as described in methods. In this single experiment NaHS had only a minor effect on GSH levels that is unlikely to explain the substantial protective action of NaHS (figure 5.1).

5.2.6 Protective effect of hydrogen sulfide against NO

An alternative possibility is that H_2S itself generates radicals that can bind with and neutralize ROS. A precedent for this is the reaction between $O_2^{\cdot}$ and NO (Squadrito and Pryor 1995, Pryor and Squadrito 1995). $O_2^{-\bullet}$ and NO readily react yielding the peroxynitrite anion ($ONOO^-$) (Beckman et al., 1990). The latter decays rapidly once protonated to form the hydroxyl radical ($OH^\bullet$) and nitrogen dioxide ($NO_2^\bullet$):

$$O_2^{-\bullet} + NO^\bullet \rightarrow ONOO^-$$

$$ONOO^- + H^+ \leftrightarrow ONOOH$$

$$ONOOH \rightarrow OH^\bullet + NO_2^\bullet$$

Such a reaction has been demonstrated to occur in vitro (Beckman et al., 1990, Hogg et al., 1992), and oxidation of protein-SH or lipids by $ONOO^-$ has been demonstrated (Radi et al., 1991, Radi et al., 1991, Niknahad and O'Brien, 1995). We therefore examined whether H_2S would be protective against NO induced toxicity.

MNNG (300μM) induced 100% cell death in hepatocytes incubated under an atmosphere of 95% oxygen/5% CO_2 within 3 hours. Sodium hydrosulfide prevented this cytotoxicity in a concentration dependent manner, being the most effective at 200 μM (Table 5.7).

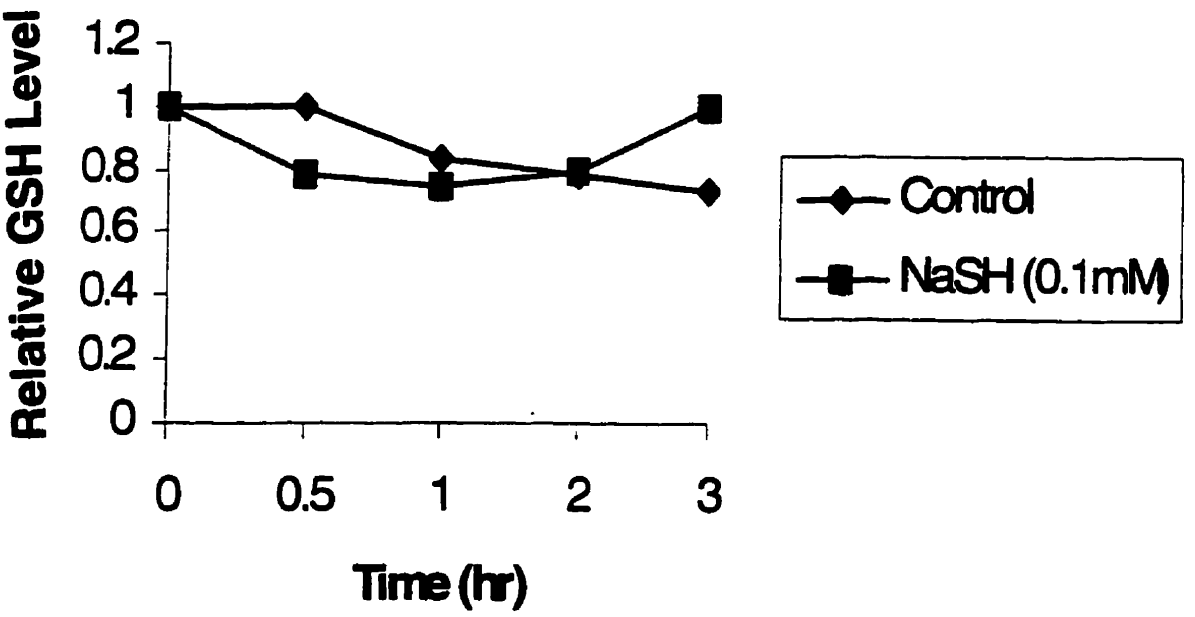


Figure 5.1 Variations in GSH content of hepatocytes in the presence of hydrogen sulfide. GSH levels in isolated hepatocytes in the absence and presence of hydrogen sulfide was measured in deproteinized samples (5% metaphosphoric acid) after derivatization with iodoacetic acid and FDNB by HPLC.(results are from one experiment)

Table 5.7. Cytoprotective effect of hydrogen sulfide towards nitric oxide

	% Cytotoxicity at		
Treatment	**60 min**	**120 min**	**180 min**
Control (95% O_2/5%CO_2)	18 ± 2	20 ± 2	23 ± 3
MNNG 300 μM	85 ± 4[a]	96 ± 4[a]	100[a]
+ NaHS 50 μM	31 ± 3[b]	80 ± 4[b]	89 ± 4
+ NaHS 100 μM	23 ± 2[b]	74 ± 3[b]	88 ± 4
+ NaHS 200 μM	23 ± 3[b]	25 ± 3[b]	34± 3[b]

Hepatocytes were incubated in Krebs-Hensleit solution pH 7.4 at 37°C under the atmosphere of 95%O_2/5%CO_2. NaHS was added immediately after MNNG and the flasks treted with NaHS were sealed for 30 minutes. The chemicals were present in the suspensions during the experiment. The samples were taken at mentioned time intervals and cell death was assessed by trypan blue uptake. The results are from three experiments and are presented as mean ± SE.

a: Significant difference in comparison with control hepatocytes ($P<0.01$)

b: Significant difference in comparison with MNNG treated hepatocytes ($P<0.01$)

5.3 Discussion

Metal fume fever is an acute inflammatory airway reaction that may develop after welding in zinc containing materials and exposure to zinc fumes. The condition is characterized by an increased number of polymorphonuclear neutrophils in the bronchoalveolar lavage fluid and is associated with influenza-like symptoms, such as high fever, chills, myalgia and malaise, that develop 2-24 h after exposure (Nemery 1990) It has been reported that $Zn^{2+}$and ZnO both stimulate oxygen radical formation in human neutrophils and this might contribute to zinc fume fever (Lindhal et al., 1998). Zn^{2+} (10-50 μM) selectively suppressed DNA synthesis and growth factor expression in primary fetal hepatocytes (Townsend et al., 1994). At higher concentrations (100-150 μM) Zn^{2+} also accentuated the toxicity in cultured skin fibroblasts from reactive oxygen species generated by hydrogen peroxide and hypoxanthine/xanthine oxidase reactions (Richards et al., 1993). In our study, ROS scavengers DMSO and catalase protected hepatocytes against Zn^{2+} toxicity indicating that ROS are involved in zinc toxicity (table 5.3).

Clinical evidence of induced copper deficiency with attendant symptoms of anemia and neutropenia, as well as impaired immune function and adverse effects on the ratio of low-density-lipoprotein to high-density-lipoprotein (LDL/HDL) cholesterol have been reported following the consumption of zinc well in excess of the recommended dietary allowance (RDA) (100-300mg Zn/d versus an RDA of 15 mg Zn/d) (Fosmire 1990). In our study, endocytosis inhibitors and Fe/Cu chelators protected hepatocytes against Zn^{2+} toxicity. These results suggest that zinc induced production of ROS may be secondary to

release of iron or copper due to displacement of Fe/Cu from storage proteins that have been endocytosed in lysosomes.

The protective effect of NaHS (table5.1) against Zn^{2+} cytotoxicity may partly results from the precipitation of Zn^{2+} by sulfide. An alternative explanation suggested by the observation that hydrogen sulfide was still protective even when added 30 min before Zn^{2+}, is that NaHS increased GSH biosynthesis (figure 6.1). However, only small changes in GSH levels were observed. Another possibility is that protein persulfides induced by hydrogen sulfide act as a reservoir which complex free Zn^{2+} in the cytoplasm. However, the protective effect of hydrogen sulfide against other oxidative stress inducing agents suggests that hydrogen sulfide or its metabolite(s) exert ROS scavenging activity.

It has been suggested that hypoxia induced hepatocyte injury results from sustained reductive stress and oxygen activation (Khan and O'Brien, 1995). It is possible that pretreatment with hydrogen sulfide activates cellular systems that deal with excessive levels of oxidants, therefore, making the cell resistant to the reductive stress associated with hypoxia. This may be controlled by iron-sulfur clusters (see Introduction).

The effects of nontoxic NaHS concentrations on GSH levels, resulted in an initial GSH depletion which recovered to higher level than the GSH levels of control hepatocytes. For example cysteine levels could be increased as a result of cysteine formation from serine and H_2S by the action of serine dehydratase. This enzyme has been reported to be identical to cystathionine β-synthase (Braunstein et al., 1971, Porter et al., 1974). This is also the principal pathway for cysteine synthesis in plants.

Alternatively the initial GSH depletion as a result of GSH persulfide and GSSG formation by hydrogen sulfide could activate or upregulate γ-glutamylcysteine synthetase, the rate limiting step in GSH biosynthesis. Recently hepatocyte γ-glutamylcysteine synthetase activity and GSH biosynthesis was shown to be increased following GSH depletion by menadione (Ochi 1996) or mercury (Lash and Zalups 1996) which resulted in increased GSH levels above normal and increased resistance to these toxins. Although GSH levels did not change dramatically the possibility of an increased capacity for GSH production needs to be determined.

Sodium hydrosulfide also prevented MNNG induced cytotoxicity. MNNG induced cytotoxicity has been attributed to cytotoxic nitric oxide and peroxynitrite formation (Niknahad and O'Brien, 1995). This likely arises from the reduction of peroxynitrite by hydrogen sulfide as described by the following equation:

$$2^{\cdot}OONO + 2^{\cdot}HS \rightarrow H_2O + S_2O_3^{2-} + 2NO^{\cdot}$$

Alternatively sulfide caused the inactivation of CYP 2E1, the source of superoxide radicals required for peroxynitrite formation (Niknahad and O'Brien, 1995); and CYP 2E1 is known to be readily inactivated by CS_2 (Lauriault et al., 1992).

Thus there are many possible explanations for the protective action of hydrogen sulfide and these will be explored in further experiments.

Chapter 6

Summary and General conclusions

We have investigated the cytotoxic action of exogenously applied H_2S and endogenously produced H_2S (from cystine dimethylester) towards freshly isolated hepatocytes (Moldeus et al., 1978; Niknahad and O'Brien 1995) and organotypic hippocampal slice cultures (Stoppini et al., 1991; Abdel-hamid and Tymiansky, 1997).

NaHS was more toxic to hippocampal slice cultures than isolated hepatocytes. Hydrogen sulfide was much more toxic to GSH depleted hepatocytes compared to the normal cells suggesting that GSH is involved in detoxification of H_2S. The difference in GSH levels in two types of cells (hepatocytes and neurons) may also explain the increased susceptibility of hippocampal slices to hydrogen sulfide. Another explanation for the different susceptibilities could be the difference in the number of cells in 1 ml of the medium, although, in our method it was not possible to determine the number of cells in hippocampal slice cultures.

H_2S toxicity was decreased by chloride ion channel blockers in both hepatocytes (glycine and alanine) and hippocampal slice cultures (picrotoxin). This suggests that hydrosulfide anion possibly enters the cells through chloride ion channels and exerts its effects intracellularly (at a physiological pH of 7.4, one-third of H_2S exists as the undissociated form and two-thirds as the hydrosulfide anion (Reiffenstein 1992)).

The toxicity of hydrogen sulfide was decreased by Zn^{2+} in both hepatocytes and hippocampal slices. Other metals such as Cu^{2+} and Ni^{2+} were protective against NaHS

toxicity (tables 4.1-4). The protection of hepatocytes against NaHS by metals in our experiments can be explained by the catalytic role of metals in sulfide oxidation. H_2S can be oxidized to atomic sulfur which can also react with thiols and disulfides and be toxic. However, further oxidation of atomic sulfur to non-toxic metabolites such as sulfite (SO_3^{2-}) and sulfate (SO_4^{2-}) would detoxify the atomic sulfur.

Cystathionine γ-lyase is highly expressed in the liver and can produce H_2S from cystine (Stipanuk and Beck, 1982). Cystine dimethylester (CDME) a lipid soluble precursor of cystine at 0.5 mM was toxic to the GSH-depleted hepatocytes and this toxicity was prevented by the cystathionine γ-lyase inhibitor propargylglycine. This result suggests that H_2S is formed from cystine by the action of cystathionine γ-lyase and also supports the idea that GSH is involved in the detoxification of H_2S as cystine dimethylester was not toxic to the normal hepatocytes at this concentration.

Cystathionine β-synthase is expressed in the brain and can produce hydrogen sulfide from cysteine (Abe and Kimura, 1996). Cystine dimethylester at 1 mM was toxic to hippocampal slice cultures and its toxicity was prevented by the cystathionine β-synthase inhibitor aminooxyacetate suggesting that the toxicity of cystine dimethylester was as a result of the production of hydrogen sulfide from CDME by the action of cystathionine β-synthase. This also suggests that CDME is hydrolysed to cystine by intracellular esterases and cystine is reduced to cysteine by GSH. Hydrogen sulfide is then formed from cysteine by the the action of cystathionine β-synthase. Thus, intracellular GSH is necessary for the toxicity of CDME. Therefore, CDME at non-toxic concentrations can be used as a model

to study the physiological functions of hydrogen sulfide generated intracellularly.

We also investigated the cytoprotective role of hydrogen sulfide against oxidants in isolated hepatocytes. Zn^{2+} has been reported to stimulate oxygen radical formation in human neutrophils (Lindhal et al., 1998). In our experiments Zn^{2+} when complexed with 8-hydroxyquinoline caused increasing cytotoxicity in hepatocytes that was prevented by non-toxic concentrations of hydrogen sulfide. The ROS scavengers DMSO and catalase protected hepatocytes against Zn^{2+} toxicity suggesting that ROS are involved in Zn^{2+} toxicity. Furthermore, endocytosis inhibitors and Fe/Cu chelators prevented Zn^{2+} toxicity in hepatocytes. These results suggest that zinc induces "ROS" production as a result of displacement of iron or copper from storage proteins that have been endocytosed in lysosomes.

It has been suggested that hypoxia induced hepatocyte injury results from sustained reductive stress and oxygen activation (Khan and O'Brien, 1995). Hydrogen sulfide was protective against hypoxia induced cell death in isolated hepatocytes.

Hydrogen sulfide also prevented MNNG induced toxicity in hepatocytes. It has been suggested that MNNG induced cytotoxicity is a result of cytotoxic nitric oxide and peroxynitrite formation (Niknahad and O'Brien, 1995). The protective effect of hydrogen sulfide possibly arises from the reduction of peroxynitrite.

All our findings suggest that hydrogen sulfide or its metabolites are protective in hepatocytes by enhancing ROS scavenging activities in the cells.

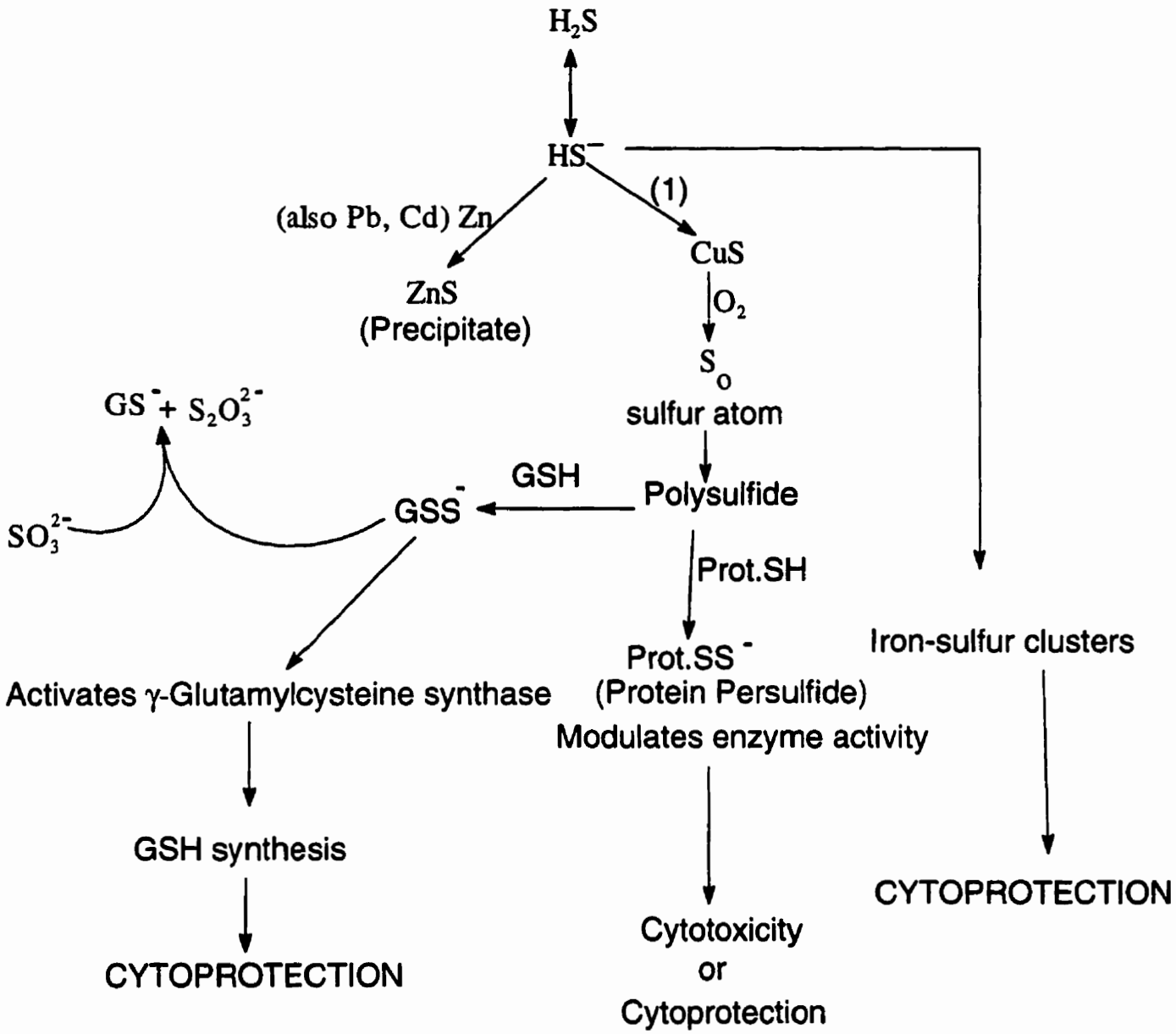
The three mechanisms suggested for the cytotoxic and cytoprotective actions of hydrogen sulfide have been summarizedin Figure 6.1 and are as follows:

1) Hydrogen sulfide can precipitate in the presence of metals such as Zn, Pb, and Cd. This mechanism is possible in both protective action of metals against hydrogen sulfide toxicity and protective effect of hydrogen sulfide against metal toxicity.

2) Hydrogen sulfide can be oxidized to atomic sulfur. This reaction can be catalyzed by metals. Atomic sulfur can form polysulfide which in turn can react with protein thiols or disulfides and form protein persulfides. This reaction can reactivate some enzymes such as xanthine oxidase and can be cytotoxic or cytoprotective. Alternatively, the interaction of polysulfide with GSH forms glutathione persulfide and decreases GSH levels which in turn will activate γ-glutamylcysteine synthase and increase GSH synthesis, thus, being cytoprotective. Glutathione persulfide can also be reduced to GSH by the action of sulfite.

3) Hydrogen sulfide activates the assembly of iron-sulfur clusters and this action may control expression of proteins involved in the adaptation of cells to changes in redox states.

Figure 6.1 SUGGESTED CYTOTOXIC/CYTOPROTECTIVE MECHANISMS



(1) Redox Transition Elements(Cu, Fe)

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