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Role Of Metallothionein In Chemical Toxicity Mediated By Reactive Intermediates Generated From Xenobiotic And Oxygen Metabolism

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ROLE OF METALLOTHIONEIN IN CHEMICAL TOXICITY MEDIATED BY REACTIVE INTERMEDIATES GENERATED FROM XENOBIOTIC AND OXYGEN METABOLISM

by

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ABSTRACT

This research was concerned with the role of metallothionein (MT) in chemical toxicity mediated by reactive intermediates generated from xenobiotic and oxygen metabolism. Metallothionein(s) are ubiquitous, low molecular weight, cysteine-rich (30% of total amino acids), metal binding proteins. These proteins have been shown to be important in the regulation of essential metal metabolism and in the detoxication of toxic metals. In the present reasearch it was hypothesized that i) because of its high cysteinyl thiolate concentration, MT is reactive towards intermediates generated from xenobiotic metabolism; and ii) the metal (Zn, Cu) released from MT concomitant with the inactivation of metal binding sites would influence the modulating role of MT in chemical toxicity.

To determine the role of MT thiolate groups in scavenging free radicals generated from xenobiotic metabolism, experiments were carried out to characterize the reaction between CCl_4 and purified Cd,Zn-MT, focusing on MT thiols as potential sites of interaction. Incubation of MT with CCl_4 resulted in time-dependent depletion of MT thiols with concurrent reduction in the metal binding sites of the protein; this was due to CCl_4-linked oxidation of MT, rather than the covalent binding of $^{14}CCl_4$ metabolites, indicating an antioxidative property of MT thiol groups.

Because Zn and Cu ions by themselves have antioxidant and prooxidant properties, respectively, the influence of Zn-MT and Cu-MT in oxidative stress was examined to

determine the role of metal complement of MT in chemical toxicity. For this, Ehrlich cells with different concentrations of Zn-MT or Cu-MT were exposed to H_2O_2. In vitro toxicity testing revealed that the H_2O_2 toxicity was negatively correlated with cellular Zn-MT concentrations but directly related to cellular Cu-MT concentrations. H_2O_2 treatment resulted in oxidation of MT thiolate groups, loss of its metal-binding capacity, and translocation of MT-bound Zn or Cu to other cellular sites. Study with Cu and Fe chelating agents as well as antioxidant showed that Cu-MT enhanced sensitivity to H_2O_2 by a Cu-dependent Fenton chemistry. The direct effect of Zn or Cu in H_2O_2 was also examined in control cells. Zn and Cu produced inhibition and enhancement of H_2O_2 toxicity, respectively, indicating the inherent antioxidative and prooxidative properties of Zn and Cu, respectively.

To investigate the toxicological significance of the prooxidative property of Cu-MT, the influence of Cu-MT in vivo was also investigated. Neonatal guinea pigs were used as the biological model because copper and Cu-MT are known to exist in high concentrations in the livers of 3-day-old guinea pigs but declined to low adult levels by day 7 of life. Comparison of the hepatotoxic responses to iron nitrilotriacetate (FeNTA) in the three age groups revealed heightened sensitivity in the 3-day-old guinea pigs but not in 7-day-old and adult animals. FeNTA treatment resulted in oxidation of MT thiolate groups, loss of its metal binding capacity and translocation of MT-bound Cu to other cellular sites. Results of in vitro studies confirmed the prooxidative function of Cu-MT and indicated involvement of Cu released from MT.

In conclusion, the data of these studies showed that MT thiolate groups possess antioxidative property and that MT can act either as an antioxidant or a prooxidant, a property related to the metal complement of the metalloprotein.

Dedicated with my deepest admiration

to my parents

Emmanuel and Helen

and my brother George

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ABBREVIATIONS

ADP	adenosine 5-diphosphate
ATP	adenosine 5-triphosphate
Ag	silver
AST	aspartate aminotransferase
b.wt	body weight
Ca^{+2}	calcium
CAT	catalase
Cd-MT	cadmium-metallothionein
Cd,Zn-MT	cadmium, zinc-metallothionein
CCl_4	carbon tetrachloride
Cu-MT	copper-metallothionein
$CuSO_4$	copper sulphate
DEF	desferoxamine
DTT	1,4-dithiothreitol
E	1x10
EDTA	ethylenediaminetetraacetic acid
Fe	iron
g	gram
GSH	glutathione (reduced form)
GSH-Px	glutathione peroxidase
GSSG	glutathione (oxidized form)
h	hour
H_2O_2	hydrogen peroxide
HMW	high molecular weight
ip	intraperitoneal
kg	kilogram
l	litre
M	molar
MAN	mannitol
MDA	malondialdehyde
μCi	microcurie
μg	microgram
μl	microlitre
μmole	micromole
mCi	millicurie
mg	milligram
min	minute
ml	millilitre
mM	millimolar

mmole	millimole
NADPH	nicotinamide adenine dinucleotide 3-phosphate (reduced form)
NbS_2	5,5-dithiobis-2-nitrobenzoic acid
NTA	nitrilotriacetic acid
OH	hydroxyl radical
O_2	superoxide anion
PEN	D-penicillamine
PBS	phosphate buffered saline
sc	subcutaneous
SEM	standard error of mean
SH	sulphydryl
SOD	superoxide dismutase
TBA	2-thiobarbituric acid
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl)aminomethane
Zn-Def	zinc-deficient
Zn-MT	zinc metallothionein
$ZnSO_4$	zinc sulphate

CHAPTER ONE

1.1. TOXICITY MEDIATED BY REACTIVE INTERMEDIATES GENERATED FROM XENOBIOTIC AND OXYGEN METABOLISM

It has long been recognized that the toxicological effects of xenobiotics may be caused not only by the xenobiotic itself but also by one or more of its metabolites (Mason, 1982; Anders, 1985; Slater, 1984). Research conducted in some laboratories has been able to characterize the nature of these reactive metabolites and classify them into general groups: a) electrophilic species; b) organic free radicals; and c) reactive oxygen species (Mason, 1982; Anders, 1985; Mitchell et al., 1984).

1.1.1 Reactive metabolites generated from xenobiotic metabolism.

A substantial proportion of xenobiotics are bioactivated to reactive intermediates through interactions with the NADPH-cytochrome P-450 (cyt P-450) electron transport chain, which is located in the endoplasmic reticulum in many type of cells and involves the flavoprotein NADPH-cyt P-450 reductase and cyt -P-450 together in a phospholipid environment. Although the cyt P-450 monooxygenase system is mainly located in liver, small amounts of cyt P-450 can be detected in many other tissues (Parke, 1987; Anders, 1985). The NADPH- cyt P-450 reductase flavoprotein is also widely distributed in significant amounts. Examples of xenobiotics that have been shown to be metabolically-

activated to reactive intermediates are halogens such as carbon tetrachloride, trichlorobromomethane, chloroform, and halothane; nitro compounds; aromatic amines and nitrosamines; quinones; and polycyclic hydrocarbons (Mason, 1982; Slater, 1984; Parke, 1987; Anders, 1985).

A chemically-reactive intermediate can be an electrophilic intermediate or a free radical species. An electrophilic metabolite is one that possesses a low electron density. These electrophiles can form covalent adducts non-enzymatically with a wide variety of nucleophilic sites (electron-rich entities) in cellular macromolecules such as peptides, lipids, RNA and DNA. Metabolism of chemicals to electrophilic reactants appear to play important roles in the mutagenicity and carcinogenicity of many chemicals, the hepatotoxicity of acetaminophen, furosemide, isoniazid and iproniazid, the bone marrow aplasia induced by benzene, methemoglobinemia and hemolysis associated with aromatic amines and renal and pulmonary toxicity induced by derivatives of furan and thiophene (Anders, 1985; Mitchell et al., 1984; Mason, 1982).

Free radicals can also be produced in the cell as a result of the metabolism of a number of xenobiotics. Free radical is any species capable of independent existence that contains one or more unpaired electrons. In living systems, free radicals are formed by the addition or removal of a single electron, in other words by one-electron oxidation or one-electron reduction (redox reactions). Redox reactions that produce free radical intermediates may occur either non-enzymatically, usually involving a transitional metal for catalysis or enzymatically, usually involving a metalloprotein or flavoprotein (Slater, 1984; Willson, 1988; Kappus, 1987).

Free radicals, because of their unpaired electrons, are highly reactive. As a result they may i) react with another free radical with whom they can share odd electrons (covalent binding or dimerization when both radicals are the same); ii) react with another free radical and donate or receive an odd electron (dismutation when both radicals are of the same type); iii) react with a normal molecule and either donate their odd electron or accept one; iv) add on to a normal molecule so forming a larger and chemically different species (for example, the rapid addition of oxygen to many organic radicals and the addition of the hydroxyl radical to unsaturated ethylenic and aromatic double bonds); and v) break down immediately as they are formed, as occurs with the electron adduct radicals of CCl_4 or H_2O_2. It is clear that once free radicals are formed in biological systems, a list of different free radical reactions can take place. As to which reaction(s) predominate will depend on the concentration of the solutes present and the respective rate constants of reaction with the particular free radical in question (Slater, 1984; Anders, 1985; Parke 1987; Willson, 1988).

1.1.2. Reactive metabolites generated from oxygen metabolism.

Under certain circumstances, chemically-reactive metabolites may lead to the activation of oxygen with the formation of reactive oxygen species which may also manifest cellular injury. Elevation in the cellular steady-state concentration of reactive oxygen species, such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) has been characterized as oxidative stress (Sies, 1985; Kappus, 1987).

Oxidative stress can lead to cellular injury because oxygen species are capable of reacting with a variety of cellular components including nucleic acids, proteins, free amino acids and carbohydrates; these may lead to initiation of lipid peroxidation, DNA damage, mutagenesis, inhibition of pathways of intermediate metabolism and gene expression as well as cell death (Halliwell and Gutteridge, 1984a,b; Sies, 1985; Balentine, 1982; Kappus, 1987). Oxidative stress has also been implicated in a wide range of biological and pathological conditions such as i) mediation of drug action such as adriamycin (Doroshow, 1986; Sinha and Mimnaugh, 1990); ii) as part of the host defense system such as release of reactive oxygen species associated with respiratory burst activity in neutrophils and macrophages (Sies, 1985; Halliwell, 1987); iii) mediation of drug and chemical toxicity such as in the case of ethanol-, gentamycin-, and acetaminophen-induced hepatotoxicity, hyperoxia, paraquat, and some carcinogens (Sun, 1990; Comporti, 1989; Sies, 1985; Mitchell et al., 1984); iv) causative agents in several pathological and disease conditions such as aging, diabetes mellitus, cancer, rheumatoid arthritis, muscular dystrophy, inflammation, and myocardial ischemic/reperfusion injury (Sies, 1985; Balentine, 1982; Grisham and McCord, 1986; Halliwell and Gutteridge, 1984b, Halliwell, 1987; Halliwell, 1989; Shina and Mimnaugh, 1990; Grisham and McCord, 1986).

Reactive oxygen species are generated from the incomplete reduction of molecular oxygen. A large number of drugs and other xenobiotics (eg. adriamycin, paraquat, metronidazole and other quinone and nitroaromatic compounds) can generate reactive oxygen species by redox cycling. These agents undergo reversible one electron-transfer

from an electron transfer component to molecular oxygen thereby producing the potentially toxic superoxide anions and subsequently other more reactive oxygen radicals, such as hydroxyl radical (Sies, 1985; Mitchell et al., 1984; Kappus, 1987).

Although the oxygen molecule has two unpaired electrons, each located in a different orbital but both spinning in the same direction, O_2 is not considered as a free radical (Halliwell and Gutteridge, 1984a,b). If O_2 attempts to oxidize another atom or molecule by accepting a pair of electrons from it, both new electrons must be of parallel spin as to fit into the variant spaces in the orbitals. According to Pauli's principle, a pair of electrons in an orbital would have anti-parallel spins. This imposes a restriction on oxidations by O_2 which tends to make O_2 accept its electrons one at a time and slows its reaction with non-radical species (most biomolecules) (Halliwell and Gutteridge, 1984b, 1985; Di Guiseppi and Fridovich, 1983).

Acceptance of a single electron by the oxygen molecule forms the superoxide anion radical, $O_2^{\cdot -}$, with one unpaired electron. Living cells contain multiple potential sources of superoxide anion, most of them being small molecules which are known to autooxidize at appreciable rates such as hydroquinones, catecholamines, thiols, pyrogallols, and reduced hemoprotein. Several enzymes have also been shown to produce the superoxide anion including aldehyde oxidase, mitochondrial NADH dehydrogenase, cytochrome P-450 reductase and xanthine oxidase. Subcellular organelles such as mitochondria, nuclei and chloroplasts release $O_2^{\cdot -}$ as do microsomes. Whole cells such as granulocytes and macrophages have been shown to produce $O_2^{\cdot -}$ during the respiratory burst when they contact foreign particles or immune complexes

(DiGuiseppi and Fridovich, 1983).

Superoxide anion is highly reactive in hydrophobic environments. Indeed, the interior of biological membranes is well known to be hydrophobic, and $O_2^{\cdot-}$ produced in this environment could be extremely damaging, destroying phospholipids by a nucleophilic attack upon the carbonyl groups of the ester bonds linking fatty acids to glycerol. Much of the $O_2^{\cdot-}$ generated within cells comes from membrane-bound systems and is certainly possible that some of it is formed in the membrane interior, especially as O_2 is much more soluble in organic solvents than it is in water (Fridovich, 1975, 1978). Superoxide anions, even though are poorly reactive in aqueous solution, are known to act as reducing agents; for example, $O_2^{\cdot-}$ has been shown to reduce Fe (III) ions at the active site of cytochrome c and Cu (II) ions at the active site of plastocyanin. However, any reaction undergone by $O_2^{\cdot-}$ in aqueous solution will be in competition with the dismutation reaction since in aqueous solutions the basic properties and nucleophilicity of $O_2^{\cdot-}$ are greatly reduced as is its oxidizing capacity (Fridovich, 1983; Halliwell, 1989; DiGuiseppi and Fridovich, 1983; Halliwell and Gutteridge, 1984 a,b).

Cellular processes that yield superoxide anion will also produce hydrogen peroxide, a non-radical, as an $O_2^{\cdot-}$ dismutation by-product. Dismutation reactions, which involves the addition of a second electron to $O_2^{\cdot-}$, may be spontaneous or enzymatic and is represented by the equation:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \text{---}> H_2O_2 + O_2.$$

The spontaneous dismutation of $O_2^{\cdot-}$ has a rate constant at pH 7.4 of approximately 2 x

10^5 $M^{-1}sec^{-1}$, whereas the reaction catalyzed by superoxide dismutase is about 10^4 times faster (Halliwell and Gutteridge, 1984 a, b; Fridovich, 1983).

Hydrogen peroxide has been shown to be produced in vivo and is poorly reactive in aqueous solutions at physiological concentrations but it can cross biological membranes, whereas $O_2^{\cdot-}$ cannot unless there is an "anion channel' for it (Halliwell and Gutteridge, 1986). Although H_2O_2 does not quilify as a radical it has been shown to be toxic to many cells. Cytotoxicity occurs when H_2O_2 comes into contact with the reduced forms of certain metal ions such as iron Fe(II) or Cu(I). These have been shown to decompose the H_2O_2 to the highly reactive hydroxyl radical in the following reactions:

$$H_2O_2 + Fe(II) \text{---} > Fe(III) + {}^{\cdot}OH + {}^{-}OH$$

$$H_2O_2 + Cu(I) \text{---} > Cu\ (II) + {}^{\cdot}OH + {}^{-}OH$$

Iron bound to transport proteins (ferritin, transferrin) or functional (haemoglobin) proteins react slowly with H_2O_2 to form hydroxyl radicals. Low-molecular-weight iron complexes such as Fe-citrate or Fe-ADP are probably the major catalysts in the generation of hydroxyl radicals in vivo. Similarily, protein-bound copper ions are not good catalysts of hydroxyl generation via H_2O_2 decomposition; only low-molecular-weight copper complexes cause free hydroxyl radical to be formed (Autor et al., 1979; Aust et al., 1985; Halliwell and Gutteridge, 1984b; Chevion, 1988; Winterbourn, 1981).

The hydroxyl radical is an exceptionally reactive species. It reacts at a great speed with almost every molecule found in living cells, including DNA, proteins, and

carbohydrates. The damage done to DNA by added H_2O_2 is probably due to hydroxyl radical formation at the sites where metal ions are bound to DNA. Other damaging effects of hydroxyl radical is its action upon membrane lipids initiating the process of lipid peroxidation (Winterbourn, 1981; Chevion, 1988; Halliwell, 1987; 1989; Halliwell and Gutteridge, 1984 a, b).

1.1.3. Cellular defense systems against chemically reactive metabolites.

Maintenance of cell integrity depends on the balance between the cellular activational and defense system. Imbalances may occur when increased generation of reactive metabolites overwhelms the defense system, or when the defense system is severely compromised and incapable of detoxifying the normal flux of reactive metabolites, or when some combination of increased production and decreased detoxication occurs

In view of the cellular and functional distubances which reactive intermediates can cause, it is perhaps not surprising that the cell seems to have developed a number of different protective measures to combat any chemically-induced cellular damage. These can be divided into four main types: i) those aimed at reactive metabolite preventation including the maintenance of cellular structure, its compartmentalization and the preventation of geographical association between substances that might react to form free radicals; the effective control of iron distribution and the destruction of peroxides by catalase of by glutathione peroxide are included in this category; ii)

those aimed at reactive metabolite scavenging including the maintenance of effective levels of anti-oxidants such as vitamin E, vitamin C, ß-carotene and glutathione, as well as the enzyme superoxide dismutase; iii) those aimed at free radical repair in particular the maintenance of effective levels of glutathione; and iv) those involved in nucleic acid repair such as the polymerase enzymes (Willson, 1988).

1.1.4 Mechanisms of irreversible cell injury.

Extensive studies with model systems have clearly shown that reactive metabolites are able to produce chemical modifications of, and damage to, proteins, lipids, carbohydrates and nucleotides. Therefore, if such reactive metabolites are produced in vivo, or in cells in vitro, in amounts sufficient to overcome the normally efficient protective mechanisms, metabolic and cellular disturbances may occur in various ways.

Whether reactions with reactive metabolites results in biological damage will depend of how vital a particular damaged molecule is and the subsequent reactions the new radical derived from it can undergo. Damage to some molecules such as DNA or perhaps a key protein, be it an enzyme, and enzyme inhibitor or another cellular factor whose normal rate of synthesis is comparatively slow, may be significant. On the other hand, the destruction of a few molecules of glucose is hardly likely to be noticed unless the products of such reactions are in themselves toxic. Some of these toxic products may be able to diffuse greater distances than their free radical

forebears, resulting in an increase in the overall damage as in the case of some products of lipid peroxidation (i.e. hydroxynonenals) which have been shown to be extremely cytotoxic (Slater, 1984).

Highly reactive, toxic metabolites of xenobiotics may interact with cells in numerous potentially detrimental ways, including interaction with DNA, depletion of essential reducing equivalents (NADH, NADPH, and GSH) and nucleotides (ATP), modification of protein and non-protein thiols, covalent binding with essential cellular constituents, stimulation of the peroxidative decomposition of cellular lipids or disturbances in Ca^{+2} homeostasis, leading to irreversible cell injury and death (Comporti, 1989; Boobis et al., 1989; Mitchell et al., 1984). Covalent binding, lipid peroxidation and disturbances in calcium homeostasis are mechanisms of irreversible cell injury generally recognized to be of most importance and these will be discussed in more detail in the following sections.

1.1.4.1. Covalent binding

The hypothesis of covalent binding of reactive metabolites to macromolecules as a fundamental mechanism for chemical-induced lethal cell injury has been examined for many years (Recknagel and Glende, 1973; Mitchell et al., 1984). The studies of liver necrosis induced by carbon tetrachloride, bromobenzene, acetaminophen and furosemide have shown that the magnitude of covalent binding correlated with the hepatotoxic effects caused by these agents. Treatment of rats with phenobarbital, an agent that increases cyt-P-450 concentration, prior to carbon

tetrachloride and bromobenzene intoxication increased both the covalent binding of their metabolites to liver protein and lipids and the incidence of liver necrosis. On the other hand, pretreatment with SKF-525A, an agent that inhibits NADPH-cytochrome-P-450 activity, reduced metabolism, covalent binding and extent of liver necrosis. However, the causal role of covalent binding as a mechanism of toxicity is largely circumstantial and only based on correlations between the extent of binding and the severity of liver cell necrosis. In particular, the molecular targets which interact with reactive metabolites leading to cell death have not been identified. In addition, the functional consequences of such interactions between the chemical reactive metabolites and key cellular targets have rarely been considered. Nevertheless, covalent binding remains a widely accepted hypothesis and it has been suggested to be a convenient means of measuring the presence of reactive intermediates (Slater, 1984; Mitchell et al., 1984; Comporti, 1989).

1.1.4.2. Lipid peroxidation.

Lipid peroxidation represents a degradative process which is the consequence of the production and propagation of free radical reactions primarily involving membrane polyunsaturated fatty acids. The peroxidative breakdown of polyunsaturated fatty acids has been implicated in the pathogenesis of many types of tissue injury (Poli et al., 1987; Comporti, 1989; Girotti, 1985).

The peroxidation of unsaturated fatty acids within biological membranes results in a complex series of biochemical and biophysical events which include i) structural

derrangement of the bilayer and altered fluidity (Richter, 1987); ii) increased permeability of cytosolic constituents; iii) release of lysozomal enzymes; iv) inactivation of intrinsic and transporting enzymes such as Na^{+}-K^{+}-ATPase, succinic dehydrogenase, cytochrome P-450); v) covalent cross-linking of lipids and proteins; vi) loss of the Ca^{+2} sequestration capacity; vii) DNA damage and mutagenesis; and viii) depletion of NADPH due to antioxidant activity of the glutathione peroxidase\ glutathione reductase system (Dianzani, 1987; Poli et al., 1987; Ungemach, 1987).

The process of lipid peroxidation may be initiated by direct attack of a foreign free radical by abstracting a hydrogen atom from polyunsaturated fatty acids; examples of such free radicals include the trichloromethyl radical, a metabolite of CCl_4, (Comporti, 1989; Dianzani, 1987; Ungemach, 1987; Recknagel, 1983) or singlet oxygen or hydroxyl radicals generated by foreign compounds (Halliwell and Gutteridge, 1986; Halliwell and Gutteridge, 1984; Halliwell, 1989). Since a hydrogen atom has only one electron, this leaves behind an unpaired electron on the carbon atom from which it was abstracted. This carbon radical tends to be stabilized by a molecular rearrangement to produce a conjugated diene that then reacts with molecular O_2 to give a peroxy radical. The peroxy radicals can abstract a hydrogen atom from another lipid molecule to continue the process, converting itself into a lipid hydroperoxide; this step has been considered as the propagation stage of lipid peroxidation. The lipid hydroperoxides are stable complexes at physiological temperatures when present by themselves but in vivo their decomposition can be catalyzed by copper or iron salts and other complexes (e.g. haem, methaemoglobin,

cytochromes). The decomposition products include radicals that can abstract further hydrogen atoms, various lipid alcohols and aldehydes, hydrocarbon gases and very-short chain products such as malondialdehyde. Some of these are toxic to the cells and distribute the toxic effects away from the initial point of attack (Halliwell, 1985, 1987, 1989; Girotti, 1985; Mitchell et al., 1984).

A number of techniques are available for measuring the peroxidation of membrane lipids or fatty acids. Each technique used relies on measurement of products generated in the process of lipid peroxidation or processes involved in lipid peroxidation and these include measurement of malondialdehyde, conjugated dienes, hydroperoxide content, hydrocarbon gases (ethane, pentane), loss of fatty acids or uptake of oxygen; however, no one method by itself can be said to be an accurate measure of lipid peroxidation (Girotti, 1985).

1.1.4.3. Disturbances of calcium homeostasis.

It was well noted by pathologists decades ago that there was a correlation between calcification and tissue necrosis, and most early mechanistic studies were based on proposed role for Ca^{+2} in the process of cell death (Farber and El-Mofty, 1975; Reynolds, 1963). It was believed that this accumulation was causally related to cell death, and the hypothesis was proposed that it represented an influx of extracellular Ca^{+2} from interstitial spaces into injured cells (Farber and El-Mofty, 1975). This influx of extracellular Ca^{+2} was considered to be the convergent point in cell

toxication in which, regardless of mechanism of initial toxic insult, the cell accumulated Ca^{+2} across a damaged plasma membrane and became irreversibly injured. This extracellular calcium-dependent mechanism was termed the "final common pathway" to cell death (Schanne et al., 1979).

Accumulating evidence, however, implicates the disturbance of intracellular calcium homeostasis as a major event that triggers cell death and not so much the presence of extracellular calcium (Orrenius, 1985; Todd et al., 1984). It has been demonstrated that during chemically-induced oxidative stress in isolated hepatocytes, the depletion of intracellular GSH and modification of key protein thiols perturb the normal flux and storage of intracellular Ca^{+2} at internal organelle sites, particularly the mitochondrial and endoplasmic reticular membranes (Orrenius et al., 1983; Bellomo and Orrenius, 1985; Richter and Frei, 1988). This results in the release of free Ca^{+2} into the cell cytosol. The thiol containing plasma membrane pumps are also damaged, which may contribute to a decreased ability of the cell to extrude the increased Ca^{+2} load. The net result of this disruption in calcium homeostasis is increased cytosolic Ca^{+2} concentration. This reasoning has been extended to earlier studies on the role of extracellular Ca^{+2} in cell death (Smith and Sandy, 1985; Smith et al., 1981) whereby an intracellular disturbance in calcium homeostasis, rather than an influx of extracellular Ca^{+2}, is considered to have been responsible for cell death. The influx of extracellular Ca^{+2}, however, has been considered to be partly responsible for the chemically-induced sustained increases in $[Ca^{+2}]i$.

The observation of a relationship between a sustained increase in cytosolic Ca^{+2}

concentration and the toxicity of several chemicals in mammalian cells has led to a search for mechanism(s) by which the increased cytosolic Ca^{+2} could trigger cytotoxicity (Boobis et al., 1989; Orrenius et al., 1989). It has been proposed that sustained increase in Ca^{+2} concentration may cause abnormal stimulation of physiological functions, including various Ca^{+2}-dependent degradative processes (Orrenius et al., 1989). These degradative processes have been recently reviewed (Boobis et al., 1989; Orrenius et al., 1989) and include the calcium activated-phospholipases, proteases and endonucleases whose activities may contribute to irreversible cell damage.

An immediate consequence of raised cytosolic Ca^{+2} is the appearance of surface protrusions known as blebs, which can rupture, compromising plasma membrane integrity and possibly precipitating cytotoxicity. Cell blebbing could be due to disruption of cytoskeletal organization, which is known to be regulated by the intracellular Ca^{+2} concentration through its effects on the formation of actin bundles, actin-myosin interactions and α-tubulin polymerization. Additional mechanisms, perhaps of equal importance, involved in chemical-induced cytoskeletal alterations that lead to plasma membrane blebbing include ATP depletion and thiol modification (Boobis et al., 1989; Orrenius et al., 1989).

1.2. METALLOTHIONEIN

1.2.1. Occurrence

In 1957, Margoshes and Vallee first isolated an unusual cadmium (Cd) binding

protein from equine renal cortex. This protein was subsequently further purified and characterized by Kagi and Vallee (1960,1961) who reported that this protein had the capacity to bind cadmium (Cd), zinc (Zn) and copper (Cu) and had an exceptionally high content of cysteinyl sulfur. These characteristics prompted Kagi and Vallee (1960) to name the protein "metallothionein" (MT).

Metallothionein(s) and MT-like proteins have now been isolated from a large variety of organisms including prokaryotes (bacteria and a blue green algae), yeast, fungi, green plants, molluscs, crustacea and vertebrates (Kagi and Nordberg [eds,], 1979; Hamer, 1986; Kagi and Schaffer, 1988). Metallothionein have also been detected in tissues from humans, monkey, sheep, pig, cow, rat, chicken, fish, bird and guinea pig (Hamer, 1986; Lui, 1987). In addition, MT-like proteins have been isolated from bacteria Pseudomonas putida, yeast Schizosaccharomyces pombe, and marine mussel Mytilus edulis (Hamer, 1986; Stone and Overnell, 1985).

It has been proposed that for proteins to be classified as MTs, the following chemical and physical properties have to be met: (Kagi and Nordberg, 1979; Elinder and Nordberg, 1985) i) high content of heavy metals bound exclusively by clusters of thiolate bonds; ii) high content of cysteine (30% of total amino acids); iii) absence of disulfide bonds, aromatic amino acids and histidine; iv) low molecular weight (typically less than 10 000); and v) heat stability.

In animals, the protein is most abundant in parenchymatous tissues, ie., liver, kidney, pancreas and intestines. There are wide variations in concentration on different species and tissues, reflecting effects of age, stage of development, dietary

regimen and other not yet fully identified factors (Kagi and Schaffer, 1988; Andrews et al., 1987; Bakka and Webb, 1981; Riordan and Richards, 1980). Tissues of the developing organism contains elevated levels of MT which decline rapidly after birth to attain the low adult levels (Lui, 1987; Andrews et al., 1987; Brady et al., 1982). Synthesis of MT in tissues of adult animals can be induced by injection of certain metals and by various other stress conditions (Hamer, 1986; Kagi and Schaffer, 1988; Karin, 1985). Although MT is a cytosolic protein, it can also accumulate in lysosomes (Bremner, 1987) and during development it has been observed in the nucleus (Nartey et al., 1987).

1.2.2. Metal composition and binding characteristics.

The metal composition of native MTs has been demonstrated to vary between species, tissues and the age of the animal studied. MT isolated from fetuses and/or neonates of the rat, rabbit, mouse, sheep and human contain much more Zn than Cu (Bakka and Webb,1981; Riordan and Richards, 1980) whereas that isolated from neonatal guinea pigs contains only Cu (Lui, 1987; Srai, 1986); adult animals, however contained relatively low levels of MT. MT isolated from livers of humans contains mainly Zn whereas that isolated from kidneys contained mainly Cd and Zn (Chung et al., 1986; Kagi and Nordberg, 1979).

In addition to Cd, Zn and Cu, mammalian MTs can also bind several other divalent cations including silver (Ag), mercury (Hg), lead (Pb), bismuth (Bi), tin

(Sn), cobalt (Co), and nickel (Ni) (Szymanska et al., 1983; Winge et al., 1975; Kagi and Schaffer, 1988; Scheuhammer and Cherian, 1986). The affinity of the metal ions for the binding sites of mammalian MTs follows the order Zn < Pb < Cd < Cu < Hg < Ag (Nielson et al., 1985). Copper binds with an association constant which is approximately 100-fold greater than that for Cd, which in turn is approximately 1000-fold greater than that for Zn. Mercury and silver both bind with greater affinity than does Cu (Nielson et al., 1985; Scheuhammer and Cherian, 1986).

Amino acid analysis has shown that mammalian MT is a 61- or 62-amino-acid peptide containing an unusually high concentration of cysteines (30% of total amino acids). Evidence indicates that the metals are associated exclusively through thiolate bonds to all cysteine residues. The metals in MT are contained in two distinct polynuclear clusters termed A and B (Kagi et al., 1984; Vasak and Kagi, 1983; Furey et al., 1985). The A cluster contains 11 cysteines, binds four atoms of Cd or Zn whereas the B cluster contains 9 cysteines and binds three atoms of Zn or Cd. The zinc and Cd atoms are tetrahedrally coordinated to four cysteinyl thiolate ligands (Kagi et al., 1984; Vasak and Kagi, 1983; Nielson and Winge, 1984; Nielson et al., 1985).

The binding stoichiometries and coordination geometry have not been extensively investigated for Ag (I) and Cu (I) (Winge et al., 1975; Nielson and Winge, 1984; Scheuhammer and Cherian, 1986). Copper-metallothionein, however, is one form of the protein that deviates from the usual coordination of 7 tetrahedrally-bound metals/

polypeptide (Nielson and Winge, 1984; Suzuki and Maintani, 1981). It was shown that 11 or 12 Cu ions were bound to metallothionein and that cluster B can coordinate 6 Cu (I) ions (Suzuki and Maitani, 1981). It has been suggested that copper atoms are trigonaly coordinated to three cysteinyl thiolate ligands (Neilson et al., 1985).

1.2.3. Biosynthesis and induction.

Metallothioneins exist in low levels in tissues of normal adult laboratory species; however, their synthesis can be induced markedly by administration of a number of metals, such as Zn, Cd and Cu (Onosaka and Cherian, 1981, 1982; Cousins, 1985; Bremner, 1987), glucocorticoids, interleukin-1 (Klaassen, 1981; Hagar and Palmiter, 1981; Cousins, 1985; Bracken and Klaassen, 1987), chemicals such as alkylating agents, CCl_4 and high-dose X-irradiation (Kotsonis and Klaassen, 1979; Koropatnick et al., 1989; Oh et al., 1983; Shiraishi et al., 1983) as well as stressful conditions such as starvation, physical and inflammatory stress, infection and exposure to low temperature (Oh et al., 1983; Bremner and Davies, 1974; Oh et al., 1978; Cousins, 1985). The expression of MT genes is also regulated during development (Brady et al., 1982).

The degree of induction of MT depends on the inducing agent used and varies from tissue to tissue. Metallothionein levels are much more higher following administration of divalent ions, such as Cd, Zn, or Cu than glucocorticoid hormones or adrenocortical steroids (Klaassen, 1981; Hager and Palmiter, 1981; Bracken and

Klaassen, 1987). Also, administration of Cd or Zn results in marked increases in MT levels being highest in liver followed by pancreas, intestine and kidney (Onosaka and Cherian, 1982). It is also known that the liver is more responsive to induction of MT as compared to the kidney or other tissues following metal administration (Sendelbach and Klaassen, 1988; Bremner, 1987). It has been suggested, however, that the main determinant in the induction of MT may be the distribution of inducing agents to different tissues (Onosaka and Cherian, 1982).

The induced synthesis of metallothionein in mammalian tissues and cell cultures by metals and glucocorticoids can be inhibited by cycloheximide and actinomycin-D. These findings support that the induction of MT required de novo synthesis of a specific MT-mRNA which is controlled by metals or glucocorticoids at the transcriptional levels. (Bremner and Davies, 1976; Premakumar et al., 1975; Hagar and Palmiter, 1981; Karin et al., 1981).

1.2.4. Functions.

i) Detoxication of cadmium and other heavy metals.

The ability of MT to act as a Cd detoxifying agent against Cd has been extensively investigated. Pretreatment of animals with low doses of Zn or Cd protected the animals against a subsequent challenge of a lethal dose of Cd (Nordberg, 1971; Webb,1972; Shaikh, 1982; Goering and Klaassen, 1984; Yoshikawa, 1970). These results suggested that the metal pretreatment induces the synthesis of MT which

is then capable of detoxifying high Cd doses. The ability of MT to act as a detoxication agent in cultured mammalian cells has been demonstrated by three observations: i) cell lines that fail to produce MT due to gene hypermethylation are unusually sensitive to Cd poisoning; ii) cell lines selected for cadmium resistance overproduce MT due to gene amplification, and iii) cell lines that contain additional MT genes are also highly resistant to Cd due to MT overproduction (Hamer, 1986).

The ability of MT to sequester metals such as Cu, Hg, Pb, Ag, Au and Pt (Webb, 1987) has been claimed to be responsible for the development of resistance toward Cu (Hamer, 1986; Kagi and Schaffer, 1988; Bremner, 1987) and Au-and Pt-containing drugs in cultured cells and for the selective protection of some tissues from such agents in animals following preinduction of MT (Naganuma, 1987; Kelley et al., 1988; Freedman and Peisach, 1989; Freedman et al., 1986; Thiele et al., 1986).

ii) Homeostasis of essential metals.

The induction of metallothionein by zinc and copper as well as the changes in its concentration during early development and physiological stress have been accepted as evidence for the implication of metallothionein in the homeostasis of Zn and Cu. As a homeostatic mediator, MT could donate metal ions in the biosynthesis of Zn- and Cu- containing metalloenzymes and metalloproteins and other metabolic processes (Webb, 1972; Winge et al., 1975; Bremner 1976; Bremner et al., 1978). The emergence of Cu-MT in N. crassa prior to the formation of the Cu-containing enzymes tyrosinase and laccase would be in concert with such role (Kagi and

Schaffer, 1988; Karin, 1985). In addition in vitro experiments have demonstrated that Cu and Zn can be transferred from MT to the apoforms of a number of Cu and Zn proteins, respectively (Li et al., 1980; Geller and Winge, 1980; Schechinger et al., 1986).

It has also been proposed that the presence of MT in the intestine plays an important role in regulating absorption of zinc. Acute changes in dietary zinc increase the expression of MT gene in intestinal cells as measured by changes in mRNA activity (Menard et al., 1981) and this is accompanied by enhanced binding of zinc to the protein. The size of intracellular zinc pools determines the content of metallothionein in the mucosal cell (Cousins, 1985). The mutual antagonism between intestinal Cu and Zn absorption has also been explained to occur, at least in part, by the sequestration of Cu by mucosal MT whose synthesis has been induced by the high Zn intake (Hall et al., 1979).

Studies have also shown that metallothionein is a normal fetal protein which functions in zinc and probably copper homeostasis during gestation and early post-natal life. Zinc and copper are known to play an important role in cellular differentiation, growth and development of mammals (Prassad, 1984; Walravens, 1980) and that the liver is generally considered to be an important organ for the storage or homeostasis of zinc and copper during perinatal development before other regulatory mechanisms come into play (Brady and Webb, 1981; Terao and Owen, 1977). Studies have shown that the developmental profile of hepatic metallothionein concentration of mammalian species is closely related to those of hepatic Zn and/or

Cu (Bakka and Webb, 1981; Suzuki et al., 1983; Lui, 1987). Two major functions of hepatic MT in the developing organism have been proposed: firstly, to regulate Zn and/or Cu metabolism during perinatal development, thereby preventing tissues from exposure to excessive amounts of the essential, but potentially toxic Zn and Cu ions; and secondly, to supply the essential metals for various metabolic processes during periods of rapid growth and development (Bell and Waalkes, 1982; Bakka and Webb, 1981; Terao and Owen, 1977).

iii) Detoxication against reactive intermediates generated from xenobiotic or oxygen metabolism.

Evidence from several studies have postulated the involvement of metallothionein in the detoxication of electrophilic xenobiotics and reactive oxygen species. Thus, the toxic effects associated with the release of superoxide anions from macrophages following the exposure of cultured cells to bacterial endotoxin have been shown to be reduced in the presence of MT (Patierno et al., 1983). Cell lines expressing high levels of MT have been reported to be resistant to DNA damaging agents such as andriamycin (Naganuma et al., 1988; Satoh et al., 1988), and ionizing radiation (Bakka et al., 1982; Lohrer and Robson, 1989). Moreover, resistance to the alkylating agents chlorambucil, melphalan (Endersen et al., 1983; Kelley et al., 1988; Basu and Lazo, 1990) and cisplatin (Andrews et al., 1987; Basu and Lazo, 1990) is conferred by irreversible binding of the drug to intracellular MT. Similarly, studies have also demonstrated that Zn pretreatment that induces MT protected against CCl_4

hepatotoxicity (Cagen and Klaassen, 1979; Clarke and Lui, 1986). Scavenging reactions of MT protein with electrophilic anticancer drugs or hydroxyl radicals have been observed in vitro (Endersen et al., 1983; Thomas et al., 1986; Thornalley and Vasak, 1985; Abel and Ruiter, 1989).

Some investigators have attributed the protective role of MT in chemical toxicity to its high cysteine content. Alkylating agents have been shown to react quickly and specifically with thiolate groups of MT (Kelley et al., 1988; Kraker et al., 1985; Basu and Lazo, 1990). With regard to oxygen radicals, the thiolate clusters of MT have been shown to act in vitro as efficient intereceptors of reactive oxygen species radicals suggesting that the protein may have antioxidant function (Thornally and Vasak, 1985; Thomas et al., 1986; Abel and Ruiter, 1989). Oxidation and alkylation of cysteinyl thiolates of MT results in inactivation of metal binding sites and the release of metal ions (Thomas et al., 1986; Basu and Lazo, 1990). There is some evidence which suggests that the transfer of Zn ions to membrane during the oxidation of MT thiolate groups may play a role in the protective function of MT against the damaging effect of lipid peroxidation (Thomas et al., 1986). Zn ion is known to play an important role in the maintenance of membrane structure and function, which is independent of its function as a component of known Zn metalloenzymes (Bray and Bettger, 1990).

1.3. Essentiality of zinc (Zn) and copper (Cu) and their role in chemical toxicity.

Zinc has been proven to be an essential nutrient for a variety of animal species,

plants and microorganisms (Hambridge et al., 1986; Failla, 1977; Jackson, 1988; Mengel and Kirkby, 1987). The essentiality of Zn to living systems was first documented in 1896 by Raulins, who reported that the common bread mold Aspergillus niger required Zn for growth. Subsequent to this, many investigators have reported that Zn deficiency reduced the rate of growth of animals, plants, microorganisms and tumour cells. Although the biochemical basis for the essentiality of Zn is not completely understood, Zn has been postulated to have roles in protein metabolism, synthesis of nucleic acids, cell division and differentiation and gene expression (Golden, 1988; Keen and Hurley, 1988). This is consistent with the observation that Zn deficiency perhaps is responsible for the disturbances of embryonic, fetal and postnatal development (Keen and Hurley, 1988).

More than 100 enzymes have been demonstrated to require Zn for maximal catalytic activity and these Zn metalloenzymes are found in every known class of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases)(Vallee and Falchuk,1983; Williams, 1988; Disilvestro and Cousins, 1983). Zinc can confer structural integrity and/or participate directly in catalysis. The finding that Zn is a component of extremely diverse classes of enzymes has led to the hypothesis that the pathologies induced by a deficiency of this metal are strictly a function of altered enzyme activity. However, activities of only a few of the Zn metalloenzymes have been shown to be decreased in Zn deficiency states. As well, it has not been possible to correlate specific lesions to the lack of a particular enzyme (Clegg et al., 1988).

Zinc has also been shown to play a critical role in membrane structure and integrity in that it has a general stabilizing effect (Chvapil, 1973; Bettger et al., 1981; Bray and Bettger, 1990). Indeed, Zn has been shown to be an integral component of plasma, microsomal and lysosomal membrane systems (Bettger et al., 1981) and protect these systems against CCl_4- or oxidative stress-induced toxicity (Bray and Bettger, 1990; Chvapil et al., 1973; Thomas et al., 1986). Zn has been shown to preserve the pattern of membrane packing in acid-swollen peripheral myelin (Inouye and Kirschner, 1984). Microtubules formed from rat brain extracts in the presence of Zn show enhanced cold stability (Hesketh, 1984). Zn stabilizes the structure of keratins in the tail of mammalian sperm and protects the structure from oxidative/peroxidative damage (Williams, 1984). In certain studies, Zn has been shown not to prevent membrane lipid peroxidation, but rather to stabilize peroxidized membranes so that they retain function (Bettger et al., 1978).

The antioxidant properties of Zn have been demonstrated in organelle-based systems, cell-based systems and in vivo. Treatment of erythrocyte membranes, microsomes and mitochondria with Zn resulted in protection against CCl_4 or oxidative stress-induced injury (Thomas et al., 1986; Bray and Bettger, 1990; Chvapil et al., 1973; Cousins and Coppen, 1987; Clarke and Lui, 1986). Zn has been shown to decrease superoxide anion radical production in isolated, activated human neutrophils (Beswick et al., 1986) and decrease MDA and free radical production in cultured hepatocytes (Coppen et al., 1988). Administration of pharmacological doses of Zn to animals protected against CCl_4, ethanol, and whole body radiation (Clarke and Lui,

1986; Cagen and Klassen, 1979; Shiraishi et al., 1983).

It has been suggested that zinc exerts its antioxidant effects by at least two mechanisms, namely the protection of sulphydryl groups against oxidation and the inhibition of the production of reactive oxygen species by transition metals. Zinc has been shown to protect the activity of the enzymes δ-aminolevulinate dehydratase and dihydroorotase against oxidative stress by virtue of its ability to maintain an essential sulphydryl group (Gibbs et al., 1985; Kelley et al., 1986).

The second mechanism by which Zn may function as an antioxidant involves the blockade of reactive oxygen species production. Studies have shown that cysteine-bound Fe can transfer electrons to oxygen and produce OH radicals; Zn has been shown to decrease the amount of OH trapped by the free radical spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) probably by competing with Fe for chelation by the organic ligand cysteine (Searle and Tomasi, 1982). It has also been suggested that Zn inhibits oxygen centered free radical generation from other common oxygen-free radical generating systems such as Fe-ADP-ascorbate and Fe-ADP-NADPH due to its ability to bind to ADP (Taqui Khan and Martel, 1962) and NADPH and inhibit oxidation of NADPH (Ludwig et al., 1980).

Copper is an essential trace element whose presence in biological systems was first reported over 150 years ago. Early suggestions that the metal may be involved as a catalyst in living cells has been confirmed. It is a cofactor in several mammalian enzymes including cytochrome c oxidase, lysyl oxidase, tyrosinase, copper/zinc

superoxide dismutase, dopamine ß-hydroxylase and ceruloplasmin (Hochstein et al., 1980; DiSilvestro and Cousins, 1983).

Exposure of biological systems to excessive amounts of copper are highly toxic. Elevated and toxic levels of copper may be encountered as a result of accidental exposure (such as in the case of ingestion of copper salts, after application of copper compounds to large areas of burned skin), genetic defects in copper metabolism (such as those found in Wilson's disease), in certain diseases (such as Indian childhood cirrhosis, cholestatic syndromes and primary biliary cirrhosis) and in certain neoplastic diseases (Sternlieb, 1980; DiSilvestro and Cousin, 1983; Guigui et al., 1988; Lefkowitch et al., 1981). Copper toxicity will result from the presence of copper in amounts excess of the capacity of specific intracellular and extracellular proteins to adequately sequester the metal (Hochstein et al., 1980; Bremner, 1987).

Several theories have been proposed to explain the mechanism of cellular copper toxicity. Copper, being a redox transition metal, can function like Fe in certain respects. In fact, Cu(I) is a better catalyst than Fe(II) in the formation of highly toxic hydroxyl radicals via a Fenton reaction. It has been stated that if both H_2O_2 and Cu(I) are available in vivo, then OH radicals will form in biologically-significant amounts. Hydroxyl radicals can then inactivate essential enzymes and/or initiate lipid peroxidation reactions thereby disrupting membrane organelles (Halliwell and Gutteridge, 1984b; Hochstein et al., 1980; McBrien, 1980).

It has also been proposed that copper can exert its toxic effects by oxidizing cellular sulphydryl groups to disulphides. This oxidation of sulphydryls is

accompanied by the formation of cuprous [Cu(I)] copper, which may rapidly oxidize with molecular oxygen to form superoxide radicals at membrane sites; these radicals may either directly or indirectly (after dismutation to form peroxide or after interaction with peroxide to form hydroxyl radicals) initiate the peroxidation of unsaturated fatty acids in membrane phospholipids. The capacity of cupric [Cu(II)] copper to react with sulphydryl groups has been postulated to be the mechanism by which copper enhances the autooxidation of reduced GSH. A reaction between copper and GSH would not only form hydrogen peroxide but would also deplete cells of the reduced glutathione necessary for the detoxication of both hydrogen peroxide and organic hydroperoxides. Reactions such as these, which may involve the autooxidation of other heme proteins and constituents such as ascorbate, phenols and phenolic amines, may well be the basis for the cytotoxicity of copper in cells and tissues (Hochstein et al., 1980; McBrien, 1980; Kumar et al., 1978; Wallin et al., 1977).

CHAPTER TWO

2.1. OVERALL OBJECTIVE.

This research was concerned with the role of metallothionein (MT) in chemical toxicity mediated by reactive intermediates generated from xenobiotic and oxygen metabolism.

2.2. HYPOTHESES.

i) Since sulphydryl compounds are known to act as nucleophiles or reductants in biological systems, it was hypothesized that because of its high cysteinyl thiolate concentration, MT would be reactive towards intermediates generated from xenobiotic metabolism.

ii) All cysteinyl thiolate groups of MT are known to participate in the binding of metals (Zn and Cu). Inactivation of thiolate groups of MT by reactive intermediates from xenobiotic and oxygen metabolism has been shown to release metal ions from MT. Moreover, zinc and copper ions, by themselves, are known to have direct influence in oxidant toxicity. Therefore, it was hypothesized that the metal released from MT concomitant with the oxidant-induced inactivation of metal binding sites would also contribute to the modulating role of MT in oxidant toxicity.

2.3. SPECIFIC AIMS AND EXPERIMENTAL APPROACH.

i) To determine the role of cysteinyl thiolate groups of MT in scavenging

chemically-reactive intermediates generated from xenobiotic metabolism, the interaction of purified Cd,Zn-MT and carbon tetrachloride (CCl_4) was studied in vitro. Carbon tetrachloride was used as the model compound because its metabolism and toxicity have been well defined. It is generally believed that CCl_4 exerts its toxicity by covalent binding of CCl_4 metabolites to lipids and proteins and via induction of oxidative injury. In this study, purified Cd,Zn-MT was used because it is less susceptible to oxidation during isolation and experimental procedures. The degree of interaction between MT and CCl_4 as measured by both the reduction of metal binding capacity and thiol content of MT. To examine the involvement of cysteinyl thiolate groups as scavengers against CCl_4 metabolites, the relationship between reduction of metal binding capacity or thiol content of MT and covalent binding of $^{14}CCl_4$ or CCl_4-induced oxidation of cysteinyl thiolate groups of MT was investigated under various experimental conditions.

ii) Since Zn or Cu ions by themselves are known to have antioxidant or prooxidant properties, respectively, the influence of Zn-MT or Cu-MT in oxidative stress was examined. If the hypothesis is correct, then Zn-MT or Cu-MT would have antioxidant or prooxidant properties, respectively. Ehrlich ascites tumour cells were chosen as the experimental model because these cells can be obtained in high yields and at >97% viability; the trace metal metabolism and oxidative stress-induced injury in these cells have been extensively investigated. The prooxidant model compound chosen in this study was hydrogen peroxide (H_2O_2). In addition, as being a commonly used model prooxidant, H_2O_2 is an intermediate in the reductive metabolism of oxygen and is actually involved in various oxidant-stress related cell injury. To investigate the role of Zn-MT

or Cu-MT in oxidative stress, the relationship between cellular Zn-MT or Cu-MT concentrations and the magnitude of H_2O_2-induced injury was examined. In addition, the effect of oxidative stress on cellular Zn-MT or Cu-MT, with respect to oxidation of cysteinyl thiolate groups, loss of metal binding capacity and distribution of MT-bound metal, was examined. Antioxidants and specific metal chelators were also used to investigate the possible mechanism(s) underlying the role of MT-bound Cu on oxidative stress induced toxicity. Oxidative stress-induced toxicity was assessed by measuring the changes in intracellular calcium concentration, lipid peroxidation, GSH concentrations and cell death.

iii) To determine whether or not the prooxidant properties of Cu-MT can be demonstrated in vivo, oxidant induced-injury was assessed in a neonatal guinea pig model. The developing guinea pig was used because hepatic Cu-MT levels are known to exist in high concentrations in the neonates but decline rapidly to the low adult levels by day 7 of life. Accordingly, susceptibility of 3-day-old, 7-day-old and adult animals to oxidative stress in vivo was examined. Moreover, the responsiveness of 7-day-old animals pretreated with copper sulphate ($CuSO_4$), an MT inducing agent, to oxidative stress was also examined to better define the role of Cu-MT in the modulation of oxidative stress. Iron-nitrilotriacetate (FeNTA), being one of the more potent iron prooxidant compounds was used in this study to induce oxidative stress. It, unlike other Fe complexes, produces a rapid and preferential deposition of Fe in hepatocytes. FeNTA exerts its toxicity by stimulating the generation of reactive oxygen species in liver by undergoing NADPH-cyt-P450-reductase-dependent cyclic oxidation and reduction

reactions that require molecular oxygen and reducing equivalents. To examine the involvement of Cu-MT in oxidative stress, the effect of oxidative stress on hepatic MT in vivo (with respect to the oxidation of cysteinyl thiolate groups, loss of metal binding capacity and distribution of MT-bound Cu) was also examined.

In vitro studies were carried out to examine the existence of possible correlation of hepatic Cu-MT concentrations in the different age groups and the magnitude of the observed oxidative stress-induced toxicity. Moreover, the role of MT-bound Cu on oxidative stress-induced injury was examined by the inclusion of D-penicillamine, a Cu chelating agent, in the experimental design.

CHAPTER THREE

3.1. INTRODUCTION

Endogenous thiols are known to be one of the most important defense systems against the toxic effects of chemically reactive metabolites. In particular, studies have clearly demonstrated that glutathione (GSH) acts as an intracellular interceptor of reactive electrophilic compounds, free radicals and reactive oxygen species because of the nucleophilic and reductant properties of its cysteinyl sulphydryl groups (Meister, 1984; Meister and Anderson, 1983; Anders, 1985). Accordingly, it was hypothesized that because of its high cysteinyl thiolate concentration, metallothionein (MT) would also be reactive towards intermediates generated from xenobiotic and oxygen metabolism. To investigate the role of cysteinyl thiolate groups of MT in scavenging chemically-reactive intermediates generated from xenobiotic metabolism, the interaction of purified Cd,Zn-MT and CCl_4 was examined in vitro.

Metallothionein exists in low levels in tissues of normal adult animals; however, its synthesis can be induced by a number of metals such as Cd, Zn and Cu (Kagi and Schaffer, 1988). Induction of MT synthesis by Zn or Cu treatment is usually accompanied by increases in the binding of both metals to MT, with the inducing agent being the predominant species (Weiner and Cousins, 1980; Bremner and Young, 1976; Winge et al., 1975). Induction of MT synthesis by Cd treatment is accompanied by increases in the binding of both Cd and Zn to MT, with Cd being the predominant species. Although many similarities exist among these different metalloproteins (Hamer,

1986; Kagi and Schaffer, 1988), in vitro studies have demonstrated that under aerobic conditions Zn,Cu-MT is much more labile to oxidation than Cd,Zn-MT (Minkel et al., 1980). Since the integrity of the cysteinyl thiolate groups of MT is important when examining their functional role in chemical toxicity, Cd,Zn-MT instead of Zn,Cu-MT was used in the present study.

Carbon tetrachloride was used as the model compound because its metabolism and toxicity have been extensively investigated. Carbon tetrachloride (CCl_4) is metabolized by the cytochrome P-450 monooxygenase system to the trichloromethyl radical (CCl_3) which reacts with unsaturated lipids to form chloroform with the initiation of lipid peroxidation (Recknagel and Glende, 1973; Cignoli and Castro, 1971; Comporti, 1989). Some investigators have postulated that CCl_4 causes its hepatotoxic effects by inducing lipid peroxidation (Recknagel and Glende, 1973; Comporti, 1989; Mourelle and Meza, 1990) while others have stressed that CCl_4 hepatotoxicity results from the covalent binding of the trichloromethyl radical to lipids and proteins (Gillette et al., 1974; Trudell et al., 1982; Comporti, 1989). Recent studies, however, have also implicated the trichloromethylperoxyl radical ($CCl_3OO\cdot$), formed from the reaction of CCl_3 and oxygen, to be the more likely damaging species in aerated systems (Packer et al., 1978; Mico and Pohl, 1983; Comporti, 1989).

3.2 MATERIALS AND METHODS

3.2.1 Animals

Male Sprague-Dawley rats (300-325 g) obtained from Charles River Breeding

Laboratories, Inc., Montreal, Quebec, were used throughout these studies. These animals were housed in polyethylene cages with free access to food (pelleted Purina Laboratory chow) and water. They were kept at room temperature (21-24° C) and exposed to alternate cycles of 12 h light and darkness.

3.2.2 Isolation of Metallothionein

Hepatic Cd, Zn-MT-II was isolated from adult male Sprague Dawley rats pretreated with $CdCl_2$ at 18 mg/kg body weight (s.c.) in six divided doses (Templeton and Cherian, 1984). The rats were sacrificed by decapitation and the livers were removed and homogenized in a 1.15% KCl - 50 mM Tris-HCl buffer, pH 7.4. After the homogenate was centrifuged (10,000 x g for 10 min at 4°C) to remove nuclear and mitochondrial fractions, the supernatant was heated to 80°C for 2 min and re-centrifuged (13,000 x g for 10 min at 4° C). The resultant supernatant was fractionated on a Sephadex G-75 column (2.5 x 100 cm) to isolate MT. The Cd, Zn-MT-I and -II isorforms were separated on a DEAE cellulose 23 anion exchange column with a Tris-HCl buffer gradient, pH 7.4 (10 - 250 mM). All of the fractionation procedures were performed at 4°C. The purified samples were desalted by dialysis against distilled deionized water at 4°C in Spectrapor membrane tubing (MW cutoff 6,000 - 8,000), lyophilized and stored at -80°C until used. The Cd and Zn content of MT was measured by atomic absorption spectrophotometry and the concentration of the protein was estimated by the Cd-heme saturation method as described in Section 3.2.4.1. For all experiments described in this chapter, only Cd, Zn-MT-II isoform was used. Each molecule of th purified Cd, Zn-

MT-II contained 4.20 ± 0.10 g. atom Cd and 1.90 ± 0.18 g. atom Zn as measured by atomic absorption spectrophotometry. Copper bound to MT was below the level of detection as measured by atomic absorption spectrophotometry (limit of detection 0.01 μg/ml). The thiol content of this MT was 16.97 ± 0.43 thiol residues measured following 5,5-dithiobis-(2-nitrobenzoic acid) (NbS_2) titration as described in Section 3.2.4.2.

3.2.3 Microsomal Preparation

Livers from control adult male rats were removed immediately and washed in ice cold saline to remove excess blood. All subsequent steps were carried out at 0-4°C. Following washing, livers were finely minced and a 3.0 g tissue sample was homogenized with a Potter-Elvehjem glass homogenizer and a loose-fitting Teflon pestle in a sufficient volume of 1.15%KCl-50 mM Tris-HCl buffer (pH 7.4) to produce a 20% homogenate. The homogenate was centrifuged at 9000 x g for 10 min in a refrigerated Sorval RB-5 centrifuge. The post-mitochondrial supernatant was decanted and re-centrifuged at 105000 x g for 60 min in a refrigerated Beckman L8-55 ultracentrifuge equipped with a Beckman 50.2Ti rotor. The resulting microsomal pellets were resuspended by hand in 150 mM Tris-HCl buffer (pH 7.4) using a Potter-Elvehjem glass homogenizer and a loose-fitting Teflon pestle and washed once by centrifugation at 105000 x g for 60 min. The washed microsomal pellet was resuspended in 150 mM Tris buffer, pH 7.4. The microsomal suspension (approximately 14 mg/ml) was stored at 4°C and used within 2 h for the in vitro study.

3.2.4 Biochemical and Chemical Analyses

3.2.4.1. Metallothionein concentration.

The concentration of metallothionein in hepatic cytosols or incubation mixtures from in vitro studies were estimated indirectly by the Cd-heme saturation method as described by Clarke and Lui (1986). One millilitre of a 10-ppm Cd solution was added to a solution containing 500 μl of sample and 2.0 ml of a 1.15%KCl - 30mM Tris-HCl buffer (pH 8.0). After 10 min, 200 μl of rat hemolysate was added, the samples were mixed for 30 sec, and then immersed in boiling water for 1 min. The solution was then centrifuged at 600 x g for 5 min in a Beckman TJ-6 centrifuge, and the supernatant removed. These hemolysate-heat precipitation-centrifugation steps were repeated three times and the Cd concentration of the final supernatant, representing MT-bound Cd, was determined by the atomic absorption spectrophotometry. The concentration of MT in the samples was calculated by assuming that 7 g.atom of Cd is bound to each mole of thionein which has a molecular weight of 6050 as determined by amino acid analysis (Onosaka and Cherian, 1982).

3.2.4.2 Thiol content of metallothionein

Thiol content of MT was determined following the addition of 0.2 ml sample to a 2.8 ml solution containing 0.27 mM NbS_2 0.10 mM EDTA, 300 mM Tris-HCl buffer, pH 8.0, 1 M guanidine hydrochloride in a total volume of 3.0 ml (Li et al., 1981). Guanidine hydrochloride was included in the reaction mixture because it has been shown

to increase the reaction of MT thiolate groups with NbS_2 (Li et al., 1981). All solutions were de-aerated and kept under nitrogen in sealed cuvettes for 1 h. The absorbance at 420 nm was read against a blank containing an equivalent sample of control incubation mixture containing no MT.

3.2.4.3 Measurement of lipid peroxidation.

Lipid peroxidation is a degenerative process implicated as a mechanism of toxicity for a large variety of chemicals and metals. In this process, polyunsaturated fatty acids of cell membranes are broken down by free radicals resulting in the disruption of cellular and subcellular processes. Quantitation of tissue levels of malondialdehyde (MDA), a product of lipid breakdown using the thiobarbituric acid assay (TBA) has been used to measure tissue peroxidation in vitro and in vivo. Thiobarbituric acid reacting material was measured as described by Buege and Aust (1978). Samples from the incubation mixtures (0.5 ml) were added to 10% trichloroacetic acid and centrifuged at 600 x g for 3 min in a Beckman TJ-6 centrifuge, and the resulting supernatants were added to 1% thiobarbituric acid solution and the mixture was incubated at 110°C for 10 min. The change in absorbance at 535 nm was read against a blank containing an equivalent sample from a control incubation mixture.

3.2.4.4 Metal analysis.

Metal analysis was performed by the use of a Varian Techtron Model AA6 spectrophotometer using an air/acetylene flame. The analytic wavelengths were 228.8 nm for Cd and 213.9 nm for Zn. Metal concentrations were calculated from calibration

curves of concentration of 0.5-2.0 ppm for both Zn and Cd. Precautions were taken to avoid external contamination of samples with metals by using distilled-deionized water for all samples and standard dilutions.

3.2.4.5 Protein determination.

Protein determinations were estimated by the method of Lowry et al., (1951) using crystalline bovine serum albumin (BSA) as the standard.

A volume of 5.0 ml of Reagent A (4 mM $CuSO_4$-5 H_2O, 9 mM potassium tartrate, 200 mM Na_2CO_3 in 0.1 N NaOH) was added to 100 μl of an appropriate dilution of protein suspension in distilled-deionized water. The solution was mixed thoroughly and allowed to stand at room temperature. After 10 min, 0.3 ml of Phenol reagent (Folin-Ciocalteau solution 2 N) was added and the resulting solution was mixed thoroughly. After 30 minutes, the absorbance at 750 nm was read on a Beckman DU-8 spectrophotemeter. Analyses were performed in duplicates using standards of known concentrations of bovine serum albumin (10 - 100 μg).

3.2.5 Reaction of CCl_4 with Cd,Zn-MT-II

The standard reaction between CCl_4 and Cd,Zn-MT-II was carried at 37°C in a 25 ml Erlenmeyer flask in a final volume of 5.0 ml incubation mixture, containing approximately 1.5 mg washed microsomal protein, 0.64 mM NADPH, 0.40 mM $NADP^+$, 0.40 mM glucose-6-phosphate, 0.2 units glucose-6-phosphate dehydrogenase, 9.0 mM $MgCl_2$ and 30mM Tris-HCl, pH 7.4. The incubations were performed with air or nitrogen as the gas phase in a metabolic shaker oscillating at 100 cycles/min.

Following a 3 min preincubation, the reactions were initiated by the addition of sufficient CCl_4 in 200 μl of methanol to give a final concentration of 10mM in the incubation mixture. Aliquots of the incubation mixture were removed at appropriate incubation times and the reaction was terminated by boiling for 30 sec. The samples were centrifuged at 600xg for 15 min in a Beckman TJ-6 centrifuge and the supernatants were used for the measurement of maximum Cd-binding capacity, thiol content of MT, and lipid peroxidation. Control aerobic incubations containing hepatic microsomes and an NADPH-regenerating system in the absence of CCl_4 were included to rule out the effect contributed by oxygen radicals produced by NADPH-dependent oxidase reactions.
To determine whether the effect of CCl_4 on MT was mediated by the chloroform-phosgene metabolic pathway, chloroform ($CHCl_3$) (10 mM final concentration) instead of CCl_4 was added to the standard incubation mixture.

To determine the effect of products of CCl_4-induced microsomal lipid peroxidation on MT, incubations were carried out in the presence of 25 μM promethazine, an inhibitor of lipid peroxidation (Slater and Sawyer, 1971).

3.2.6 In vitro covalent binding of $^{14}CCl_4$ to MT

In vitro covalent binding of $^{14}CCl_4$-derived radioactivity to MT was determined following the reaction of MT and $^{14}CCl_4$ (specific activity 4.5 mCi/mmole) in an incubation mixture as described in section 3.2.5. Non-MT bound radioactivity was removed by treating the heat treated incubation mixture with 80% acetone. The acetone treatment resulted in the precipitation of MT and other proteinaceous components of the incubation mixture. The precipitated pellet was then dried under nitrogen and

reconstituted to original volume with incubation buffer, followed by two washes with chloroform:methanol (2:1) mixture. Then, the aqueous phase was saturated with 50 ppm Cd and chromatographed on a Sephadex G-75 column (1.5 cm x 55 cm) equilibrated with 10mM Tris-HCl buffer (pH 8.6) as previously described (Clarke and Lui, 1986). Eluent fractions were collected (2ml) and radioactivity was measured by scintillation counting using a Beckman LS-6800 scintillation counter. The acetone precipitation-extraction-evaporation procedure removed 95 to 98% of the radioactivity in blank incubations. Moreover, this procedure did not alter the thiol content and maximum Cd-binding capacity of MT; recovery of MT was > 96%.

3.2.7 Reduction of CCl_4-treated MT with 1,4 dithiothreitol

To determine whether or not CCl_4 caused the oxidation of cysteinyl thiolate groups of MT, heat-treated supernatant fractions from control and CCl_4-treated incubations were incubated with the sulphydryl reductant 5 mM 1,4 dithiothreitol (DTT) under nitrogen at room temperature for 30 min. To determine the regeneration of Cd-binding sites, the samples were then treated with 50 ppm Cd and chromatographed on a Sephadex G-75 column (as described previously). The eluent fractions corresponding to the MT peak (V/Vo = 2) were pooled and dried under a stream of nitrogen and then reconstituted to original volume with 10 mM Tris-HCl buffer, pH 8.0 and used for Cd and thiol determination. Recovery of MT following this procedure was >95%.

3.2.8 Measurement of Cd and Zn release from CCl_4-treated MT in vitro

To measure the amounts of Cd and Zn released from MT during its reaction with

CCl_4, heat-treated samples from control and CCl_4-treated incubations were treated with Chelex 100 (50 mg/ml, final concentration) for 6 min on ice to remove free metals. The samples were centrifuged at 600xg for 15 min in a Beckman TJ-6 centrifuge and metal analyses were performed on the supernatant. This procedure itself did not affect the Cd-binding capacity of Cd, Zn-MT.

3.2.9 Statistical analysis

Statistical evaluation of data was performed by Student's t test (Steel and Torrie, 1960). The level of statistical significance was chosen at $p<0.05$.

3.3 RESULTS

As shown in Fig. 3.1, CCl_4 reduced the maximum Cd-binding capacity of MT as determined by the Cd-heme saturation method. The observed reductions were not due to oxygen radicals produced by NADPH-dependent oxidase reactions, since control incubation carried out in the absence of CCl_4 failed to show any effect (fig. 2A). It is apparent that the reduction required metabolic activation of CCl_4, since no significant decreases in binding were observed in the absence of microsomes. Furthermore, the rate of decrease in the maximum Cd-binding capacity of MT appeared to be a function of microsomal protein concentration (Fig. 3.1). The decrease in the maximum Cd-binding capacity of MT was linear with time during the first 15 min of incubation but thereafter no further reduction was observed; however, addition of fresh microsomes at 15 min of incubation resulted in a further decrease in the maximum Cd-binding capacity of MT at

Figure 3.1. Effect of CCl_4 on the maximum Cd-binding capacity of MT as a function of microsomal protein concentration. The reaction between CCl_4 (10 mM) and Cd,Zn-MT-II (30 μg/ml incubation mixture) was carried out at 37°C under air atmosphere in a final volume of 5.0 ml incubation mixture, containing 0 mg (□), 0.3 mg (○) and 1.2 mg (●) washed microsomal protein and a NADPH-regenerating system. The reactions were initiated by the addition of CCl_4 and aliquots of incubations were removed at 0, 7.5 and 15 min of incubation period. Values represent mean ± SEM from 3 experiments. * Significantly different from corresponding control values, $p < 0.05$

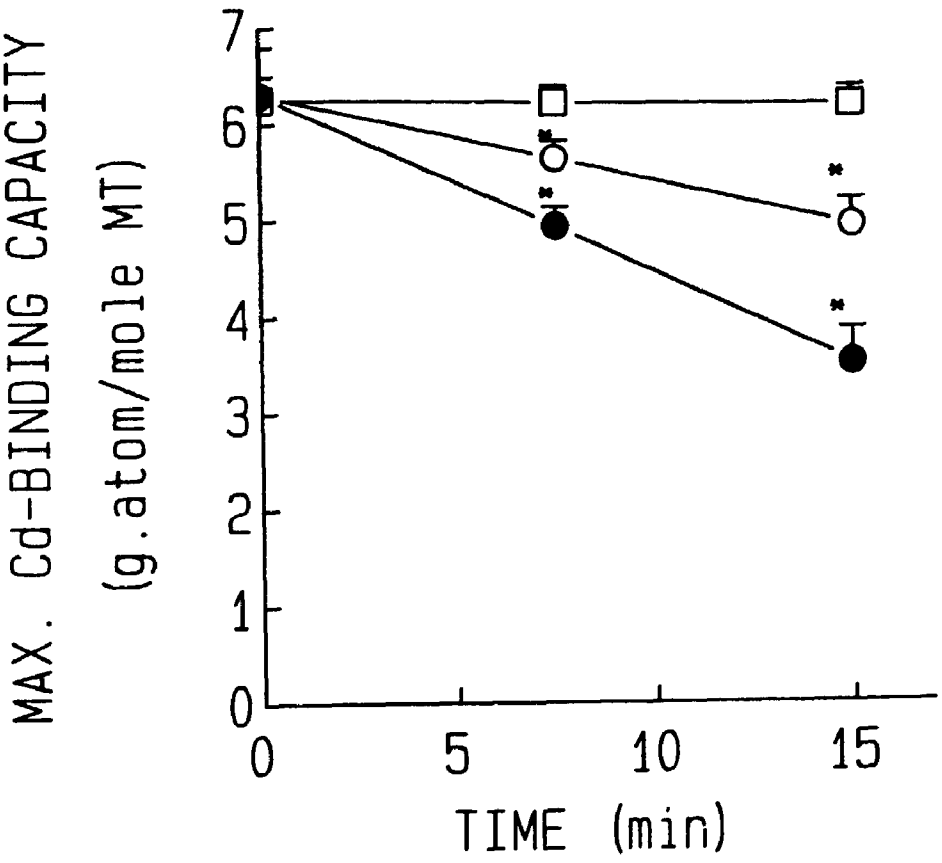

MAX. Cd-BINDING CAPACITY
(g.atom/mole MT)
7
6
5
4
3
2
1
0
0
5
10
15
TIME (min)

Figure 3.2. Effect of CCl_4 on the maximum Cd-binding capacity (A) and thiol content (B) of MT under air (●) or nitrogen (□). Control incubations (○) were performed under air in the absence of CCl_4. The reaction between CCl_4 (10 mM) and Cd,Zn-MT-II (30 μg/ml incubation mixture) were carried out at 37°C in a final volume of 5.0 ml incubation mixture, containing approximately 1.5 mg washed microsomal protein and an NADPH-regenerating system. The reactions were initiated by the addition of CCl_4 and aliquots were removed at 0, 7.5 and 15 min of incubation period. Values represent mean ± SEM from 3 experiments.

* Significantly different from corresponding control values, $p < 0.05$

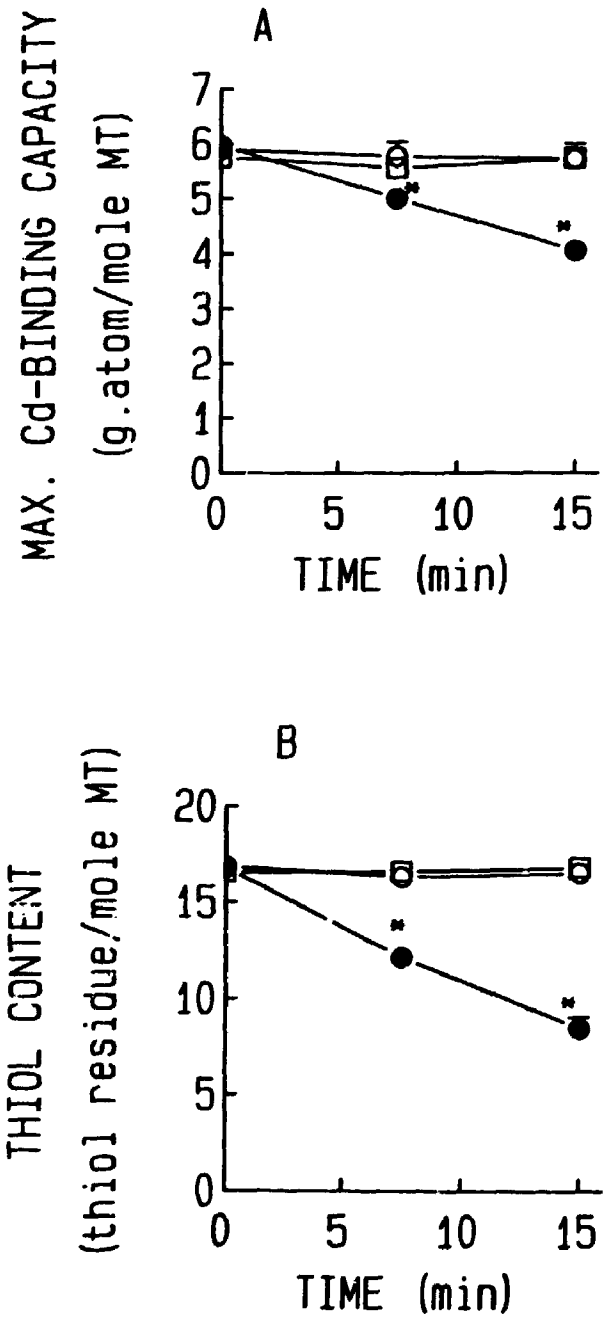

A
MAX. Cd-BINDING CAPACITY
(g.atom/mole MT)
7
6
5
4
3
2
1
0
0
5
10
15
TIME (min)
B
THIOL CONTENT
(thiol residue/mole MT)
20
15
10
5
0
0
5
10
15
TIME (min)

TABLE 3.1

The effects of CCl_4 and $CHCl_3$ on the maximum Cd-binding capacity of Cd,Zn-MT-II[a] in vitro[b]

EXPERIMENT	MAXIMUM Cd-BINDING CAPACITY[c] (μg MT bound Cd/ml incubation
CONTROL	1.54 ± 0.01
10 mM CCl_4	1.11 ± 0.04[d]
10 mM $CHCl_3$	1.47 ± 0.07

a. MT concentration in all incubations was 200μg in 5.0 ml incubation mixture.

b. The standard reaction between CCl_4 or $CHCl_3$ and Cd,Zn-MT-II was carried out at 37°C under air atmosphere in a final volume of 5.0 ml incubation mixture, containing 1.5 mg washed microsomal protein and an NADPH-regenerating system. The reactions were initiated by the addition of the test chemical and aliquots were removed 15 min later.

c. Values represent Mean ± SEM of three experiments.

d. Significantly different from control values, $p < 0.05$.

TABLE 3.2

CCl_4-mediated release[a] of Cd and Zn from MT[b]

		TIME OF INCUBATION			
		0 minutes		15 minutes	
Incubation condition	Treatment	Cd	Zn	Cd	Zn
Air	-	4.10±0.10	2.00±0.14	3.95±0.20	1.87±0.15
Air	CCl_4	4.05±0.15	2.00±0.10	3.55±0.25[c]	0.90±0.20[c]
Nitrogen	CCl_4	4.10±0.05	1.95±0.10	4.00±0.15	1.90±0.10

a. CCl_4-induced metal release from MT was measured with Chelex 100 (50 mg/ml)

b. Values are expressed in g.atom metal/mole MT and represent mean ± SEM from 3 experiments

c. significantly different from corresponding time zero values, $p<0.05$.

a rate that was similar to that of the initial reaction (data not shown). This may be due to the "suicidal" effect of CCl_4 metabolites on cytochrome P-450, thereby limiting further bioactivation of CCl_4 (Ugazio et al., 1972; Noguchi et al., 1982; Slater, 1984).

As shown in Fig. 3.2, the CCl_4-induced time-dependent reduction in the maximum Cd-binding capacity of MT (Figure 3.2A) was accompanied by concurrent decrease in thiol content (Figure 3.2B). A significant positive linear relationship was observed between the maximum Cd-binding capacity and thiol content of MT (correlation coefficient, $r = 0.825$) suggesting that these two parameters may be related. Since CCl_4 decreased the metal binding sites on MT, its effect on MT-bound Zn and Cd was also examined. As shown in Table 3.2, the presence of CCl_4 resulted in the loss of Cd and Zn from MT, the loss of Zn being greater than that of Cd within the 15 min incubation.

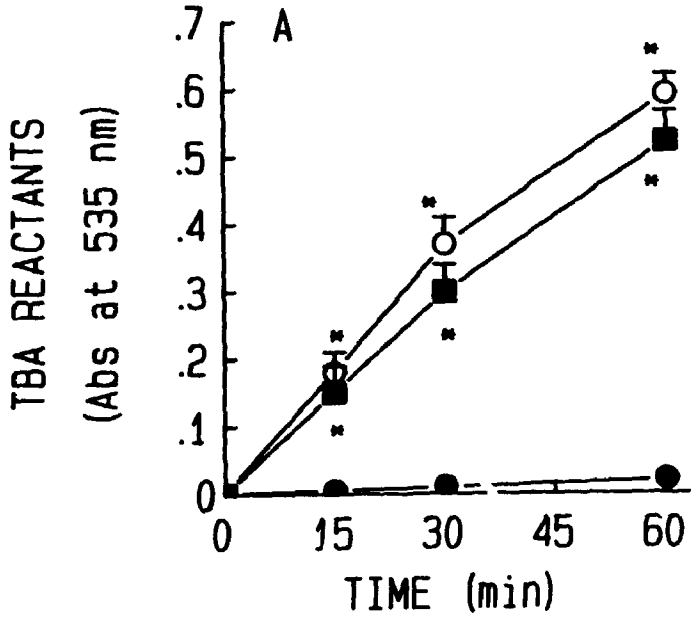
To examine whether or not the reduction in the thiol content and maximum Cd-binding capacity of MT was mediated by certain metabolites of CCl_4 the effect of $CHCl_3$ (10 mM), an intermediary metabolite of CCl_4 in the formation of phosgene (Pohl et al., 1979; Shah et al., 1979) was examined. Results showed that $CHCl_3$ in the presence of the microsomal activating system did not significantly alter the maximum Cd-binding capacity of MT (Table 3.1). Since the trichloromethyl radical is generated under both aerobic (Hanzlick, 1981; Recknagel and Glende, 1973; Cignoli and Castro, 1971) and anaerobic (Frank and Link, 1984) conditions, the examination of the effect of CCl_4 on the thiol content of MT under nitrogen should allow us to ascertain the role of the trichloromethyl radical. As shown in Fig. 3.2, no apparent changes were observed under anaerobic conditions suggesting that the trichloromethyl radical was not directly involved in the reduction of the thiol content and maximum Cd-binding capacity of MT.

Data concerning the effect of CCl_4-induced lipid peroxidation on MT is shown in Fig. 3.3. Carbon tetrachloride produced a time-dependent increase in microsomal lipid peroxidation as measured by the elevation of the absorbance of thiobarbituric acid reactants at 535 nm (Fig. 3.3A). Promethazine, which completely blocked the CCl_4-induced lipid peroxidation, did not alter the inhibitory effect of CCl_4 on the maximum Cd-binding capacity of MT (Fig. 3.3B).

It has been suggested that covalent binding of CCl_4 metabolites to proteins and lipids is a mechanism by which CCl_4 exerts its toxicity (Gillette et al., 1974; Trudell et al., 1982; Comporti, 1989). Accordingly, the role of covalent binding of CCl_4-derived radioactivity in the inhibitory effect of CCl_4 on MT thiol content was investigated. Data presented in Table 3.3, show the amount of covalent binding of $^{14}CCl_4$ metabolite(s) to MT occurring under aerobic conditions. To ascertain whether or not the covalent binding of $^{14}CCl_4$ occurred at the cysteinyl thiolate groups, MT that was pretreated with hydrogen peroxide (H_2O_2) to oxidize the thiolates to disulfides was used instead of the control MT. In these experiments H_2O_2 completely depleted the thiol groups and maximum Cd-binding capacity of MT but this did not apparently affect the covalent binding of $^{14}CCl_4$ to MT (Table 3.3). Moreover, anaerobiosis did not change the magnitude of covalent binding but prevented CCl_4-induced decreases in thiol content of MT, suggesting that the cysteinyl thiolate groups did not participate appreciably in the covalent binding of CCl_4 metabolites. To further investigate whether or not covalent binding of $^{14}CCl_4$-derived radioactivity to MT was sulfhydryl-specific, the binding to BSA was also examined. Although the covalently bound $^{14}CCl_4$-derived radioactivity per molecule of protein was higher in BSA than MT, no difference was apparent in the

FIGURE 3.3. The influence of promethazine on the CCl_4-mediated changes in microsomal lipid peroxidation (A) and maximum Cd-binding capacity of MT (B). The standard reaction between CCl_4 (10 mM) and Cd,Zn-MT-II (30 μg/ml incubation mixture) was carried out at 37°C under air atmosphere in a final volume of 5.0 ml incubation mixture, containing approximately 1.5 mg washed microsomal protein and an NADPH-regenerating system. The reactions were initiated by the addition of CCl_4 and aliquots of the incubation mixture were removed at 0, 15, 30 and 60 min of incubation period. TBA reactants were measured following the incubation of microsomes with 10 mM CCl_4 alone (■) and in the presence of MT (○) and MT plus 25 μM promethazine (●). Microsomal lipid peroxidation was not detected in the presence of MT or promethazine alone. The effect of microsomal lipid peroxidation on the maximum Cd-binding capacity of MT was measured in the absence of CCl_4 (□) and in the presence of 25 μM promethazine (■) or 10 mM CCl_4 (○) plus 25 μM promethazine (●). Values represent mean ± SEM from 3 experiments.

* Significantly different from corresponding control values $p < 0.05$


A
TBA REACTANTS
(Abs at 535 nm)
.7
.6
.5
.4
.3
.2
.1
0
0
15
30
45
60
TIME (min)

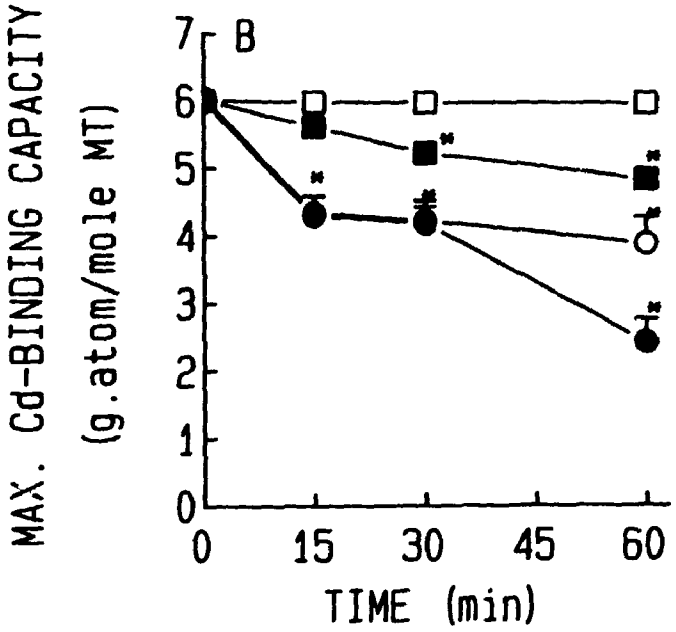

B
MAX. Cd-BINDING CAPACITY
(g.atom/mole MT)
7
6
5
4
3
2
1
0
0
15
30
45
60
TIME (min)

TABLE 3.3

Covalent binding of $^{14}CCl_4$ and the effect of CCl_4 on the thiol content and maximum Cd binding capacity[a]

Incubation condition[b]	Covalent binding		max. Cd-binding capacity[c] (g.atom/mol MT)	Thiol content[d] (thiol residue/mol MT)
	μmol CCl_4/mg MT or BSA	mol CCl_4/mol MT or BSA		
MT (control)				
Time zero	ND	ND	6.12 ± 0.30	16.00 ± 0.70
Omit NADPH	ND	ND	6.00 ± 0.25	16.00 ± 0.45
Omit microsomes	ND	ND	6.15 ± 0.15	16.10 ± 0.30
MT Air	1.65 ± 0.16	10.00 ± 1.10	3.67 ± 0.40[e]	10.80 ± 0.80[e]
N_2	1.70 ± 0.19	10.30 ± 1.25	5.99 ± 0.20	15.57 ± 0.60
H_2O_2-treated MT	1.52 ± 0.20	9.21 ± 1.40	ND	0.64 ± 0.10[e]
BSA	1.80 ± 0.28	107.00 ± 18.70[f]		

a. Values are means ± SEM from three experiments
b. A single 30-min incubation period was used unless otherwise stated (20 μg MT or BSA/ml incubation).
c. Estimated by the Cd-heme saturation method.
d. Estimated by titration with NbS_2.
e. significantly different from corresponding control values, $p<0.05$.
f. significantly different from the binding to MT under air atmosphere, $p<0.05$.
ND not detected

TABLE 3.4

1,4-Dithiothreitol-dependent regeneration of thiol content[a] and max. Cd-binding capacity[b] of CCl_4-treated MT[c]

		TIME OF INCUBATION			
		0 minutes		15 minutes	
Preincubation with CCl_4[d]	DTT Treatment	Thiol Residue/ mole MT	g.atom/mole MT	Thiol Residue/ mole MT	g.atom/mole MT
- Air	-	16.20±0.40	5.90±0.20	16.20±0.30	5.90±0.15
	+	19.00±0.80	6.70±0.30	19.10±0.55	6.73±0.25
+ Air	-	16.35±0.35	5.93±0.15	10.12±0.90[e]	3.57±0.40[e]
	+	18.88±0.20	6.60±0.25	18.50±0.75[f]	6.30±0.25[f]
+ N_2	-	16.30±0.25	5.98±0.20	16.28±0.40	5.96±0.15
	+	18.95±0.40	6.68±0.25	18.75±0.55	6.48±0.40

a. Estimated by NbS_2 titration
b. Estimated by Cd-heme saturation method
c. Values represent mean ± SEM from 3 experiments
d. Incubations were carried out in the absence (-) or presence (+) of CCl_4 (30 μg MT was used per ml of incubation mixture)
e. significantly different from corresponding time zero values, $p<0.05$.
f. Significantly different from corresponding non-DTT treated samples, $p<0.05$.

binding per unit weight of the two proteins reflecting perhaps the approximately 10 fold difference in their molecular weight (Table 3.3).

Data presented in Table 3.4 shows the effect of treatment of CCl_4-treated MT with the reducing agent 1,4-dithiothreitol (DTT) on the thiol content and maximum Cd-binding capacity of MT. Incubation of control MT with DTT resulted in increases in the thiol content and maximum Cd-binding capacity of MT presumably due to the restoration of thiolate groups that were oxidized during the initial isolation and purification steps. Incubation of CCl_4-treated MT with DTT restored its thiol content and maximum Cd-binding capacity to control MT levels.

3.4 DISCUSSION

The data obtained in the present study shows that treatment of hepatic Cd, Zn-MT-II with CCl_4 in vitro, resulted in reduction in the thiol content of MT (Fig. 3.2B). Since all thiolate groups are known to participate in the metal-binding of MT (Furey et al., 1985; Kagi et al., 1984), the reduction in the thiol groups of MT may explain the decreases in the maximum Cd-binding capacity of MT (Fig. 3.2A) and the release of Cd and Zn from MT (Table 3.2). Our in vitro data also corroborate with our previous observation of the loss of Zn from Zn-induced hepatic MT and the reduction in the maximum Cd-binding capacity of the protein following the administration of CCl_4 to Zn pretreated rats in vivo (Clarke and Lui, 1986). However, the in vitro effects on the metal binding of MT are not unique to CCl_4. Previous in vitro studies have shown that oxygen reactive species formed during radiolysis (Bakka et al., 1982; Thornalley and

Vasak, 1985) or other forms of oxidative stress (Patierno et al., 1983; Thomas et al., 1986) react with MT resulting in the oxidation of the protein thiols to disulfides with the subsequent loss of MT-bound metals. It is known that alkylation of the cysteinyl thiolate groups on MT by methyl bromide (Lui, 1987), iodoacetamide (Bernhard et al., 1986), or mercuribenzoate (Templeton et al., 1986) in vitro, also resulted in the release of MT-bound metals.

The mechanism(s) underlying the observed destruction of CCl_4 on MT thiols is not clear. The data in the present work suggest that this effect on MT required metabolic activation of CCl_4 (Fig. 3.1) which is consistent with the findings of the involvement of CCl_4 reactive intermediates in mediation of its toxicity (Recknagel and Glende, 1973; Cignoli and Castro, 1971; Packer et al., 1978). Electrophiles (Thorgeirsson et al., 1976) and other chemically-reactive intermediates (Shah et al., 1979) react with nucleophilic sites on endogenous and exogenous compounds; however, several of the known toxic metabolites of CCl_4, such as trichloromethyl radical, chloroform and phosgene, do not appear to be directly responsible for the CCl_4-induced depletion of MT thiolate groups. Nevertheless, the requirement for aerobic conditions in the induction of damage to MT would suggest the involvemnent of the highly reactive trichloromethylperoxyl radicals, which are generated from trichloromethyl radicals in the presence of oxygen (Packer et al., 1978; Mico and Pohl, 1983; Comporti, 1989).

Covalent binding of CCl_4-derived metabolites to tissue macromolecules has been implicated as a possible mechanism for CCl_4 to exert its toxicity (Gillette et al., 1974; Trudell et al., 1982; Comporti, 1989). On the basis of the observed covalent binding of $^{14}CCl_4$-derived radiolabel to MT, Cagen and Klaassen (1979) postulated that the

protective effect provided by Zn-induced hepatic MT against $^{14}CCl_4$ toxicity was due to the scavenging of $^{14}CCl_4$ metabolites by MT thiolate groups. Although covalent binding of $^{14}CCl_4$ to MT was detected in the in vitro study (Table 3.3), it does not account for the decreases in the reduced sulphydryl content for the following reasons: i) prior oxidation of the sulphydryl groups of Cd,Zn-MT by H_2O_2 did not alter the extent of covalent binding of $^{14}CCl_4$ to this MT; ii) anaerobiosis did not alter the covalent binding of $^{14}CCl_4$ to MT but obliterated the effect of CCl_4 on the maximum Cd-binding capacity and the total thiol content of MT; and iii) the extent of covalent binding per unit weight of protein to MT and BSA (a high molecular weight protein containing a single free sulphydryl group) was similar suggesting that covalent binding of $^{14}CCl_4$ metabolite(s) to MT was not sulphydryl-specific.

The ability of CCl_4 to induce microsomal lipid peroxidation is well established (Comporti, 1989; Cignoli and Castro, 1971) and this mechanism has also been implicated in its hepatotoxicity (Recknagel 1967; Benedetti et al., 1977; Mourelle and Meza, 1990). Carbon tetrachloride-induced lipid peroxidation has been shown to generate a variety of degradative products originating from membrane lipids and these chemically-reactive "secondary messengers" are known to be reactive toward sulphydryl compounds (Benedetti et al., 1981). As shown in Fig. 3.3, the CCl_4-induced reduction in the maximum Cd-binding capacity or thiol content of MT was not mediated by products of CCl_4-induced lipid peroxidation, as promethazine which was effective in inhibiting CCl_4-induced microsomal lipid peroxidation as demonstrated in Fig. 3.3A and other previous studies (Poli et al., 1981; Slater and Sawyer, 1971), did not modify the action of CCl_4 on MT.

The regeneration of the thiol content as well as the maximum Cd binding capacity of CCl_4-treated MT provide evidence supporting the CCl_4-linked oxidation of MT. Although the mechanism underlying the CCl_4-linked oxidation of MT thiolate groups is not known, it has been reported that protein and non-protein thiol groups are readily oxidized by many free radicals and the thiyl ($RS^{\cdot}$) radical produced may dimerize resulting in disulfide formation (Slater, 1984; Thomas et al., 1986). Since the loss of MT thiols does not appear to be due to lipid peroxidation, and since it did not occur under anaerobic conditions, it is likely that the trichloromethylperoxyl radicals are involved in the oxidation of MT sulphydryl groups.

In summary, our data suggest that CCl_4-induced oxidation of MT rather than the covalent binding of CCl_4 metabolite(s) is responsible for the CCl_4-induced loss of metal binding sites of Cd,Zn-MT with the concurrent release of Zn and Cd. The results of this study support our hypothesis that thiolate groups of MT are reactive towards chemically-reactive intermediates and interaction between CCl_4 and MT revealed that the MT thiolate groups possess antioxidant properties.

CHAPTER FOUR

4.1 INTRODUCTION

The research presented in Chapters four and five is concerned with the role of MT as a modulator of chemical toxicity induced by oxidative stress, a condition characterized by elevation in tissue concentration of reactive oxygen species (Sies, 1985; Balentine, 1982; Halliwell, 1989, 1987).

Mammalian MT contains 61 amino acids including 20 cysteines which participate in the binding of metals (Zn and Cu). Each MT molecule can bind up to 12 g.atoms of Cu and 7 g.atoms of Zn or Cd (Furey et al., 1985; Kagi et al., 1974; Nielson and Winge, 1984; Nielson et al., 1985). Results presented in Chapter 3 and elsewhere (Thomas et al., 1986; Thornalley and Vasak, 1985; Basu and Lazo, 1990) showed that chemically-induced oxidation or alkylation of MT thiolate groups resulted in inactivation of metal binding sites and release of MT-bound metals. However, the precise role of metal released during the inactivation of MT thiolate groups in chemically-induced toxicity is poorly understood. Since Zn and Cu ions, by themselves, are known to have direct influence in oxidative stress-induced toxicity, it was hypothesized that the metals released from MT concomitant with the oxidant-induced inactivation of metal binding sites would also contribute to the modulating role of MT in oxidant toxicity.

Zinc has been shown to have antioxidant properties by protecting sulphydryl groups against oxidation and inhibiting the production of reactive oxygen species by transition metals (Bray and Bettger, 1990; Kelley et al., 1980; Ludwig et al., 1980). Moreover,

Zn has been shown to be important in maintaining membrane function and integrity (Bettger et al., 1981; Chvapil, 1973). In contrast, Cu ions are known to have prooxidant properties. If our hypothesis stated in the above is correct, then Zn-MT would have antioxidant properties while Cu-MT would have prooxidant properties.

To investigate the role of metal complement of MT in oxidative stress, the influence of Zn-MT or Cu-MT on oxidative stress was compared in Ehrlich cells. Ehrlich cells were chosen as the experimental model because they can be obtained in high yields and at > 97% viability. Normal Ehrlich cells contain high levels of zinc, low levels of Cu and substantial amounts of MT; the metal complement of this MT is exclusively that of zinc. Hydrogen peroxide (H_2O_2) was chosen as the prooxidant compound because in addition as being a commonly used model prooxidant, it is an intermediate in the reductive metabolism of O_2 and is actually involved in various oxidant stress-related cell injury. To investigate the role of Zn-MT in oxidative stress, the relationship between cellular Zn-MT concentrations and the magnitude of H_2O_2-induced injury was examined. Study with Cu-MT will be presented in Chapter five.

4.2 MATERIALS AND METHODS

4.2.1 Animals.

Male Swiss albino CD-1 mice (approximately 30 g body weight) obtained from Charles River Breeding Laboratories Inc., Quebec were housed in groups of six to eight animals in polyethylene cages with free access to pelleted Purina laboratory chow and

deionized water. The animals were kept at room temperature (22-24°C) and were exposed to alternate cycles of 12 h light and darkness.

4.2.2 Preparation of cells.

Ehrlich ascites tumour cells were grown in the peritoneal cavity of mice and transferred weekly to new hosts. Ehrlich cells [mammary adenocarcinoma tumour cells] (DCT tumour repository) were harvested 6 days after inoculation, washed 4-5 times with 0.9% saline and resuspended in Dulbecco's phosphate buffered saline (PBS). Ehrlich cells were counted microscopically using a Spencer Bright-Line hemacytometer (American Optical Corp., Buffalo, NY) at 100-X magnification. Cell viability was confirmed by exclusion of 0.25% trypan blue dye and was routinely found to be >97%.

4.2.3. Tissue preparation.

Livers were excised immediately after removal of Ehrlich cells from the peritoneal cavity of host mice and washed in ice cold saline to remove excess blood and contaminating Ehrlich cells. All sbsequent steps were carried out at 0-4 C. Following washing, livers were finely minced and approximately 3.0 gram of tissue was homogenized with a Potter-Elvehjem glass homogenizer and a loose-fitting Teflon pestle in a sufficient volume of 1.15% KCl-50 mM Tris-HCl buffer, pH 7.4, to produce a 20% homogenate. To prepare Ehrlich cells for subcellular fractionation, washed cells (1x10E8 cells/ml) were sonicated for 30 sec with a W385 sonicator (Heat systems Ultrasonics, NY) with sufficient volume of PBS buffer containing 5 mM 1,4 dithiothreitol (DTT) (to

prevent oxidation of MT thiolates) to produce a 20% homogenate. To attain the cytosolic and particulate fractions, homogenates of Ehrlich cells and livers were centrifuged at 105,000 x g for 60 minutes in a refrigerated Beckman L8-55 ultracentrifuge equipped with a Beckman 50.2Ti rotor.

4.2.4. Treatment protocols to manipulate Zn and/or Zn-MT concentrations in mice and Ehrlich cells.

Studies have shown that normal Ehrlich cells contain relatively high concentrations of Zn and Zn-MT (Kra[illegible] et al., 1988). Zn-deficiency was induced in host mice to reduce Zn-MT concentrations in Ehrlich cells. For this, groups of mice were placed on either Zn-deficient or normal diets and housed in stainless-steel cages over woodchip bedding and fed and watered from stainless steel and glass containers. All animals had free access to their respective diets and distilled-deionized water in drinking bottles fitted with low Zn-Nalgene stoppers. Four weeks later, host mice were inoculated with Ehrlich cells and killed six days later to obtain the ascites cells as described in Section 4.2.2. These cells were designated as Zn-deficient cells. Preliminary data from our laboratory has shown that although Ehrlich cells showed a 25% reduction in total Zn concentration following the placement of host mice in Zn-deficient diet for 2 weeks, this treatment protocol did not result in significant changes in Zn-MT concentrations.

Elevation of cellular Zn and Zn-MT concentrations was achieved by administering a single dose of $ZnSO_4$ (10 mg Zn/kg, ip) to host mice 5 days following inoculation of Ehrlich cells. Ehrlich cells were harvested 24 h following Zn administration; these cells

were refered to as Zn-pretreated cells.

To increase the cellular non-MT-bound Zn concentration, Ehrlich cells isolated from control host mice were incubated with 2.5 ppm of $ZnSO_4$ for 30 min at 37°C under air atmosphere and thereafter washed twice with PBS buffer to remove extracellular Zn ions. This procedure yielded Ehrlich cells with total Zn concentrations that were comparable to those of Zn-pretreated cells (10 mg Zn/kg); these cells were designated as Zn-preincubated cells.

In this study, Zn and Cu metabolism was also examined in hepatic tissues of host mice in order to ascertain the effectiveness of treatment protocols.

4.2.5. Experimental design.

To examine the influence of Zn-MT on cellular susceptibility to H_2O_2-induced toxicity, dose toxicity relationship was established in control, Zn-pretreated and Zn-deficient Ehrlich cells. For this, cells from the three experimental groups were incubated with different concentrations of H_2O_2 (15, 30, 60, 120 and 200 μM) at 37°C; cell viability was measured 60 min after addition of H_2O_2 and LC_{50} values were estimated. To characterize the H_2O_2-induced oxidative stress in control, Zn-pretreated and Zn-deficient cells, additional toxicity parameters such as $[Ca^{+2}]i$, GSH, lipid peroxidation and cell blebbing, were monitored at different time intervals following exposure of cell preparations to 60 μM H_2O_2. Since Zn pretreatment is known to increase the binding of Zn to cellular components in addition to MT, toxicity studies were also carried out with Zn-preincubated cells to examine the influence of non-MT-bound Zn on H_2O_2 toxicity.

To investigate the involvement of Zn-MT in cellular response to oxidative stress, the effect of oxidant stress on Zn-MT was investigated. For this, the effects of H_2O_2 on the metal binding capacity, thiol content and metal content of MT of control, Zn-pretreated and Zn-deficient Ehrlich cells was examined at different incubation times. To examine whether or not thiolate groups of MT were prone to oxidation by H_2O_2, cytosols isolated from Ehrlich cells, with and without prior H_2O_2 treatment, were treated with the sulphydryl reductant 1,4-dithiothreitol (DTT) to examine possible regeneration of metal binding capacity of MT. To examine the effect of oxidative stress on Zn metabolism, cell suspensions were removed at different incubation times following addition of H_2O_2 and the cytosolic distribution of Zn was analyzed by Sephadex-G-75 chromatography study.

To further study the mechanism underlying the effect of Zn-MT on H_2O_2 toxicity, the effects of H_2O_2 in control and Zn pretreated cells were measured in the presence of either mannitol, a hydroxyl radical scavenger, or desferoxamine, a specific Fe(III) iron ion chelator.

To examine the validity of Ehrlich cells as a model for studying the role of Zn-MT in oxidative stress, cellular parameters that are known to influence cellular response to oxidative stress such as SOD and catalase activities as well as GSH, α-tocopherol and Fe concentrations were also measured in Ehrlich cells from all treatment groups.

4.2.6 Cytotoxicity study of H_2O_2 in Ehrlich cells.

Freshly isolated Ehrlich cells were used throughout this study. All incubations were

performed in 10-ml Erlenmeyer flasks containing 1x10E7 cells/ml PBS buffer, pH 7.4, at 37°C under air atmosphere in a shaking water bath oscillating 80 cycles/min. Control incubations were carried out in the absence of H_2O_2. Following a 3-min preincubation period, the reactions were initiated by the addition of H_2O_2 dissolved in distilled-deionized water. Aliquots of the incubation were removed at appropriate incubation times for measurement of cell viability, blebbing and other toxicity parameters. For the measurement of the other toxicity parameters, aliquots of the incubation were washed once with ice cold PBS buffer and resuspended to original volume with the same buffer. Cell suspensions or their subcellular fractions were used for the measurement of lipid peroxidation, GSH levels, metal binding capacity, thiol content and metal content of MT where appropriate.

Preincubation of Ehrlich cells with 30 mM mannitol or 500 μM desferoxamine was carried out at 37°C under air atmosphere for 60 or 45 min, respectively. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer prior to challenge with H_2O_2.

4.2.7 Biochemical analyses and procedures.

4.2.7.1 Measurement of cell viability.

Cytotoxicity was measured by loss of cell viability determined by the trypan blue exclusion test. Samples (50 μl) of cell suspension were mixed with 200 μl of 0.25% trypan blue and cell viability was determined with the use of a hemacytometer; four 1

mm^2 areas were counted per sample.

4.2.7.2 Measurement of cell blebbing.

The appearance of cell blebbing was observed under light microscopy. Control cells appeared uniformly rounded but following exposure to H_2O_2, "blebbed" processes or protrusions could be observed on cell surface. The number of cells exhibiting such changes were subjectively determined by the same investigator during the experiment. Since blebbing of viable cells was observed prior to cell death under the present experimental conditions, dead cells were also included in the measurement of cell blebbing to give the total blebbing. Thus, measurement of cell blebbing was expressed as a percentage of "blebbed" viable cells plus dead cells to total cell population.

4.2.7.3 Measurement of lipid peroxidation

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactants as described in section 3.2.4.3. Briefly, 1.0 ml of cell suspension (1x10E7 cells/ml) was added to 10% trichloroacetic acid and centrifuged at 600xg for 3 min; 0.5 ml of 1% thiobarbituric acid (TBA) solution was added to the resulting supernatant and the mixture was incubated at 110°C for 10 min. The absorbance of the solution was measured 535 nm and was read against the value of time zero sample.

4.2.7.4 Measurement of intracellular free calcium concentration ($[Ca^{+2}]i$).

A disruption in calcium homeostasis, leading to a sustained increase in intracellular

free calcium concentration has been associated with cytotoxicity in response to a variety of agents, including prooxidants (Orrenius et al., 1988; Boobis et al., 1989; Farber, 1990). Oxidant-induced inhibition of Ca^{+2}-dependent regulatory enzymes, particularly the sulphydryl-containing ATPase-dependent enzymes located in the plasma membrane and in the endoplasmic reticulum (Nicotera et al., 1985), may be responsible for the rapid and sustained rise in $[Ca^{+2}]i$ during oxidant stress. In the present study, $[Ca^{+2}]i$ was measured fluorometrically using the Ca^{+2}-sensitive probe Indo-1 (Grynkiewicz et al., 1985). Indo-1 is not toxic to the cell at loading concentration and no apparent quenching was observed in cells exposed to Zn, Cu or H_2O_2. In general, Indo-1 is loaded into intact cells by incubating them with a membrane-permeant ester derivative (Indo-1/AM). Cytosolic esterases split off the ester groups and leave the membrane-impermeant Indo-1 tetra anion trapped into the cytosol. Increases in Indo-1 fluorescence then signal increased $[Ca^{+2}]i$ (Grynkiewcz et al., 1985).

In the present study, Ehrlich cell suspensions (0.5 to 1.0 x10E6 cells/ml) in PBS buffer were loaded with 1 μM indo - 1 acetoxymethyl ester at 37°C for 15 min, sedimented at 200xg for 5 min and resuspended to original volume with PBS buffer. Prior to $[Ca^{+2}]$ determination, each sample was sedimented and resuspended in 2 ml of Na^{+2} buffer (containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1mM $CaCl_2$, 10 mM glucose and 20 mM Hepes) with pH adjusted to 7.4. Fluorescence was monitored using a HITACHI F-4010 Fluorescence Spectrophotemeter (Tokyo, Japan) at the following wavelengths: 331 excitation (3 nm slit width) and 398 emission (20 nm slit width) as described previously (Grynkiewcz et al., 1985). Cells were maintained in suspension in

continuous stirring and allowed to equilibrate for 5 min at which time fluorescence intensity (F) of the sample was determined. To obtain the fluorescence intensity of Ca^{+2}-saturated indo-1 (Fmax) and the autofluorescence intensity (Fauto), cell suspensions were treated with 75 nM digitonin and 2 mM $MnCl_2$, respectively (Grynkiewcz et al., 1985; Gelfand et al., 1986). The fluorescence of Ca^{+2}-free indo-1 (Fmin) was calculated using Fmin = (1/12)(Fmax-Fauto) + Fauto, where 1/12 is the ratio of the Ca^{+2}-saturated indo-1 fluorescence to the Ca^{+2}-free indo-1 fluorescence at wavelengths used (Gelfand et al., 1986). Cytosolic free calcium was obtained using the relation $[Ca^{+2}]i$ = kd (F-Fmin)/(Fmax-F) with an estimated dissociation constant (Kd) for the indo-1-Ca^{+2} complex of 250 nM (Grynkiewcz et al., 1985).

4.2.7.5 Measurement of superoxide dismutase activity.

Copper/zinc superoxide dismutase (SOD) in cytosols of Ehrlich cells was measured using the technique based on inhibition of pyrogallol (1,2,3-benzenetriol) autooxidation as described by Marklund (1985). Pyrogalloi autooxidizes rapidly in alkaline solution with the formation of a product that absorbs at 240 nm and superoxide anion which then participates as a chain propagating species in the process. Autooxidation of pyrogallol is highly dependent on superoxide anion and is inhibited 97.5% by SOD.

Pyrogallol autooxidation was initiated by addition of 25 μl of 24 mM pyrogallol in 10 mM HCl to a cuvette containing 50 mM Tris-HCl buffer, pH 8.2 with 0.1 μM catalase and 1 mM DTPA. The buffer was equilibrated with air by rapid stirring for 20 min. Maximum repeatability was obtained when pyrogallol autooxidation resulted in a

change in absorbance A_{420} = 0.02 /min. Inhibition of autooxidation was linearly related to concentration of purified bovine Cu/Zn SOD up to about one unit of activity. A unit is defined as the amount of SOD that inhibits the reaction by 50% and is equivalent to about 400 μg bovine Cu/Zn SOD (Marklund, 1985). The total protein concentration was maintained below 60 μg/ml since higher concentrations affected the assay.

4.2.7.6 Measurement of catalase activity.

Catalase activity in sonicated homogenates was determined spectrophotometrically by following the disappearance of hydrogen peroxide at 240 nm as described by Claiborne (1985). Aliquots of 20% homogenate (25 μl) were added to a cuvette containing 0.05 M potassium phosphate buffer, pH 7.0. The reaction was initiated by the addition of 0.02 M H_2O_2 prepared from 30% (v/v) solution (analytical grade Fisher Scientific). The concentration of H_2O_2 used gave an absorbance of approximately A = 0.5 before the suspension was added. Decrease in absorbance was measured for the first 30-40 secs when the rate was linear. One Sigma unit of activity was defined as the amount of catalase required to decompose one μmole of H_2O_2 per min at 25°C. Activity was expressed as units per mg protein.

4.2.7.7 Protein determination.

Protein determinations were estimated by the methods of Lowry et al. (1951), using crystalline bovine serum albumin (BSA) as the standard as described in section 3.2.4.5.

4.2.7.8 Reduced and total glutathione concentrations.

Decrease in GSH/GSSG ratio is generally recognized as a sensitive index of oxidative stress (Mitchell et al., 1984). Glutathione is present in high concentrations in most mammalian cells as reduced glutathione (GSH), with minor fractions made up of oxidized glutathione (GSSG), mixed disulfides of GSH and other cellular thiols and minor amounts of other thioethers. GSH in biological systems acts both as a nucleophilic scavenger of numerous compounds and their metabolites, via enzymatic and chemical mechanisms, forming thioether bonds with electrophilic centers, and as a cofactor in the GSH peroxidase-mediated destruction of H_2O_2 (Pascoe and Reed, 1989).

Reduced and total glutathione, more precisely non-protein sulfhydryl, in Ehrlich cell cytosols was determined as described by Kuo and Hook (1982). To measure the concentration of reduced glutathione (GSH), approximately 4x10E8 cells were sonicated in 3.0 ml of ice-cold 6% TCA and centrifuged at 10000 x g for 20 min in a refrigerated Sorval RB-5 centrifuge. Aliquot of the resulting deproteinized supernatant (0.5 ml) was added to 2.0 ml of 0.3 M Na_2HPO_4 solution. A solution (0.5 ml) of 0.04% NbS_2 in 10% sodium citrate was then added and the absorbance at 412 nm was measured immediately after mixing.

To measure total glutathione (GSH and GSSG), 1.0 ml of deproteinized supernatant fraction was incubated at 45°C for 60 min with 1.0 ml sodium borohydride (5% $NaBH_4$), a potent reducing agent. The mixture was then neutralized with 0.5 ml of 2.7 N HCl and sulphydryl groups were assayed as described previously. Total and reduced glutathione concentrations were calculated from the standard curve of absorbance (at 412

nm) vs reduced GSH concentration; absorbance was linear with respect to reduced GSH concentration. The oxidized concentrations of GSH was calculated after subtraction of reduced GSH concentrations from the total GSH concentrations.

4.2.7.9 α-Tocopherol determination.

α-Tocopherol is the most biologically active and abundant of the four (α, ß, γ, and δ) tocopherol compounds collectively refered to as vitamin E. This membrane-associated chain-breaking phenolic antioxidant is generally recognized as the major antioxidant present within biological systems. α-Tocopherol functions as a trap for lipid peroxy (LOO) and other radicals, effectively inhibiting the peroxidation of celluiar membranes (Pascoe and Reed, 1989; Freeman and Crapo, 1982).

α-Tocopherol was determined as described by Zaspel and Saari-Csallany, (1983) with modifications. Ehrlich cells (1x10E8 cells) were sonicated in 5 volumes of acetone for 30 seconds and the homogenate was centrifuged at 9,000xg for 10 min; the resulting supernatants obtained were bubbled with nitrogen and used within 2 h for α-tocopherol determination. For this, 15 μl of supernatant was injected into a high-performance liquid chromatograph which included a Waters M45 and 510 solvent-metering pumps (Millipore Waters,Toronto), a Waters U6K solvent injector, and a Kratos FS 970 LC fluorescence spectrophotometer. The analytical column used was a Partisphere C18, 5μm, 110x4.7 mm (i.d). Elution time of α-tocopherol was 13.5min in 95% methanol, 5% water delivered at a flow rate of 1.0ml/min. Cellular α-tocopherol was detected by fluorescence at an excitation wavelength of 290 nm (slitwidth, 5 nm) and an emission

wavelength of 340 nm (slitwidth, 5nm).

4.2.7.10 Metallothionein determination.

Metallothionein concentrations from cytosols were estimated indirectly by measuring the metal binding capacity of the protein by the Ag-heme saturation method as described by Scheuhammer and Cherian (1986). For this, 1.0 ml of a 20 ppm Ag solution was added to a solution containing 500 μl of sample and 2.0 ml of a 0.5 M glycine-NaOH buffer, pH 8.5. After 10 min, the hemolysate-heat precipitation-centrifugation steps were carried out as described in section 3.2.4.1.

4.2.7.11 Measurement of thiol content of MT.

To determine the thiol content of MT, 0.5 ml of heat-treated supernatants isolated from cytosols of incubation mixtures was added in a 2.8 ml solution containing 0.27 mM 5,5-dithiobis-(2-nicrobenzoic acid) (NbS_2), 0.10 mM EDTA, 30 mM Tris buffer, pH 8.0, 1 M guanidine hydrochloride (has been shown to increase the reaction of MT thiolate groups with NbS_2 (Li, et al, 1981)) in a total volume of 3.30 ml. All solutions were deaerated and kept under nitrogen until used. Reactions were carried out for 60 min in sealed cuvettes under a nitrogen atmosphere and read at 420 nm. The total absorbance values obtained under these assay conditions represented the thiol concentration of MT and GSH. Because the reaction of NbS_2 with sulphydryl groups of GSH is very fast and occurs within minutes (Kuo and Hook, 1982) but with those of MT is very slow (Li et al, 1981), the concentration of MT sulphydryl groups was determined by subtracting the

absorbance values obtained for GSH (at 10 min) from the total. In addition, the contribution of other protein thiol groups present in the samples was insignificant because most of them were eliminated by heat-treatment.

4.2.7.12. Sephadex G-75 gel filtration study.

To study the chromatoghraphic separation profile of cytosolic Cu and Zn, an aliquot (2ml) of cytosol was chromatographed at room temperature on a calibrated Sephadex G-75 column (1.5 cmx 55cm) equilibrated with 10 mMTris-HCl buffer(pH 8.6), containing 5mM DTT to prevent air oxidation of Cu-MT, and eluted with the same buffer at a rate of 1.0 ml/min. Two milliliter fractions were collected and used directly for the quantitation of Cu and Zn by atomic absorption spectrophotometry.

4.2.7.13. Regeneration of metal binding sites of MT by 1,4 dithiothreitol (DTT).

To determine whether H_2O_2-induced loss of metal binding capacity of MT was due to oxidation of MT thiolates, heat-treated supernatant fractions of control and H_2O_2-treated samples were treated with the reducing agent DTT under nitrogen at room temperature for 30 min to determine the regeneratability of binding. The metal binding capacity of MT was estimated by the Ag-heme saturation method; the DTT residue present in the sample did not interfere with the binding assay because it was eliminated during the heat-treatment procedure.

4.2.7.14 Metal analysis.

Estimation of total, cytosolic and particulate Cu, Zn and Fe concentrations from tissues was performed following wet tissue digestion as described by Clarke and Lui (1986). An aliquot of homogenate, cytosolic and particulate fraction (0.5 ml) was added to 1.0 ml of a nitric acid:sulphuric acid (1:2 solution). The solution was heat treated at 60°C for 1 h and then was diluted with 2.0 ml of distilled-deionized water. Metal concentrations were measured with a Varian AA-475 atomic absorption spectrophotometer, using an acetylene/air flame as described in section 3.2.4.3.

4.2.7.15 MT-I mRNA measurement

Total cellular RNA was purified using the guanidinium isothiocyanate/hor phenol method. Two to ten milligrams of total RNA was immobilized on nylon filters by RNA dot plotting as described by Koropatnick et al., 1990. RNA dot blots were hybridized to a ^{32}P-labelled MT-I cDNA probe (400 bp) under conditions that allow less than 5% cross-hybridization between mRNA for MT-I and MT-II. Densitometer tracings of autoradiographs derived from hybridized filters provided equivalent data to measurement of radioactivity by scintillation counting. Rehybridization of the same filters to ß-actin cDNA probe (2.1 Kb in pBR 322) allowed comparative quantitation within the same microsample of RNA. Regression analysis of a plot of probe hybridized vs μg RNA dotted yielded values for MT-I and ß-actin mRNA accumulated. Relative MT-I mRNA expression was defined as MT-I mRNA accumulation/ß-actin mRNA accumulation.

4.2.8. Statistical analysis.

Statistical evaluation of data was performed by paired Students t-test and two way analysis of variance (Gad and Weil, 1982). The level of significance was chosen at $p < 0.05$.

4.3 RESULTS

i) Manipulation of Zn/Zn-MT status.

Total hepatic Zn and MT concentrations of normal mice were increased by 45% and 90%, respectively, 6 days following inoculation of Ehrlich cells while no significant changes in Cu concentrations were observed (fig. 4.1). Subcellular fractionation of hepatic tissues revealed that increases in hepatic Zn concentrations were mostly due to increases in cytosolic Zn concentrations (fig. 4.1). As shown in table 4.1, cytosolic Zn of hepatic tissues was associated only with HMW component in untreated mice and the observed increases in hepatic cytosolic Zn following inoculation of Ehrlich cells were due to additional binding of Zn to MT fraction (table 4.1).

Maintenance of control host mice on Zn-deficient diet for 5 weeks resulted in significant decreases in total hepatic Zn and MT concentrations; the decrease was mostly associated with the reduction in cytosolic Zn (fig. 4.1). The gel filtration profile of cytosols isolated from Zn-deficient host mice showed a significant decrease in Zn associated with MT fractions when compared to that of control host mice (Table 4.1). Zn-deficient conditions did not significantly change the subcellular distribution of copper (fig. 4.1).

Twenty-four hour pretreatment of control host mice with $ZnSO_4$ (10 mg Zn/kg) resulted in marked increases in hepatic concentrations of Zn and MT but not that of Cu (fig. 4.1); the increases were detected as early as 4 h after treatment with maximal levels being measured by 24 h (fig. 4.2). Increases in Zn concentrations were noted in both the cytosolic (73%) and particulate (59%) fractions 24 h following Zn treatment; moreover, elevation in cytosolic Zn concentrations were mostly due to the increases in MT-bound Zn (Table 4.1).

Ehrlich cells isolated from control mice contained higher levels of Zn (216.00 ± 20.00 ng/1x10E6 cells) than Cu (9.10 ± 0.30 ng/1x10E6 cells) (fig. 4.3). The subcellular distribution profile showed that both Zn (60%) and Cu (80%) were mostly associated with the cytosolic fraction (fig. 4.3). Moreover, examination of the Sephadex G-75 elution profile of cytosols isolated from control Ehrlich cells revealed that Zn was associated with both HMW (78%) and MT (18%) fractions while Cu was associated only with the HMW fraction (fig. 5.5); it was apparent that MT of control cells consisted mostly of Zn.

Ehrlich cells isolated from mice placed on Zn-deficient diets for 5 weeks showed significant decreases in concentrations of both total Zn and MT (fig. 4.3) with no significant changes in those of Cu (fig. 4.3). The Sephadex G-75 elution profile showed that concentrations of Zn associated with HMW fraction were not significantly affected by the treatment; however, marked decreases (57%) in MT-bound Zn were observed (fig. 4.5).

Pretreatment of control host mice with 10 mg/kg $ZnSO_4$ 24 h prior to isolation of

cells resulted in time-dependent increases in the concentrations of total cellular Zn and MT (fig. 4.4). Elevations in both zinc and MT were detected as early as 4 h after Zn treatment and by 24 h maximal levels (2-fold and 4-fold of control, respectively) were reached (fig. 4.4). The cytosolic Zn concentrations, which accounted for approximately 75% of total, were 4-fold higher 24 h following Zn administration when compared to control cells (fig. 4.3); the elevation in cytosolic Zn could be accounted mostly by the increases in the binding of Zn to the MT fractions (fig. 4.5).

To examine whether or not the increases in MT concentrations in tissues or cells observed following administration of Zn were due to the induction of MT synthesis by a process involving increased transcription of MT gene, the accumulation of MT-I-mRNA levels in tissues was measured. Zn-pretreated Ehrlich cells showed rapid accumulation of MT-I-mRNA: 4-fold increase was measured by 4 h and maximal levels were attained at 8 h but thereafter declined to about 32% of the maximum 24 h following treatment (fig. 4.4). The levels of hepatic MT-I-mRNA were higher in host animals 6 days after innoculation of Ehrlich cells when compared to those of untreated animals (fig. 4.6). Moreover, marked elevations in MT-I-mRNA levels were detected 4 h following Zn treatment (fig. 4.6).

To further study the validity of the experimental model, some of the parameters that influence the response of Ehrlich cells to oxidative stress were also examined. As shown in Table 4.2, concentrations of Fe, which is required for H_2O_2-induced irreversible cell injury (Starke and Farber, 1985), and the major cellular antioxidant systems such as α-tocopherol and GSH concentrations as well as catalase activity were not significantly

Figure 4.1. Hepatic total and cytosolic Zn(A) and Cu (B) concentrations as well as MT concentrations (C) in host mice. Normal mice (N) were treated with Ehrlich cells (C) and 5 days later were injected with 10 mg $ZnSO_4$ dissolved in 0.9% saline; mice (ZnP) were killed 24 h later. Normal mice were placed on Zn deficient diets and 4 weeks later were treated with Ehrlich cells; host mice (ZnD) were killed 6 days later. Values represent mean $\pm$ SEM, ($n = 6$ animals).

a. significantly different from corresponding normal values, $p < 0.05$.

b. significantly different from corresponding control values, $p < 0.05$.

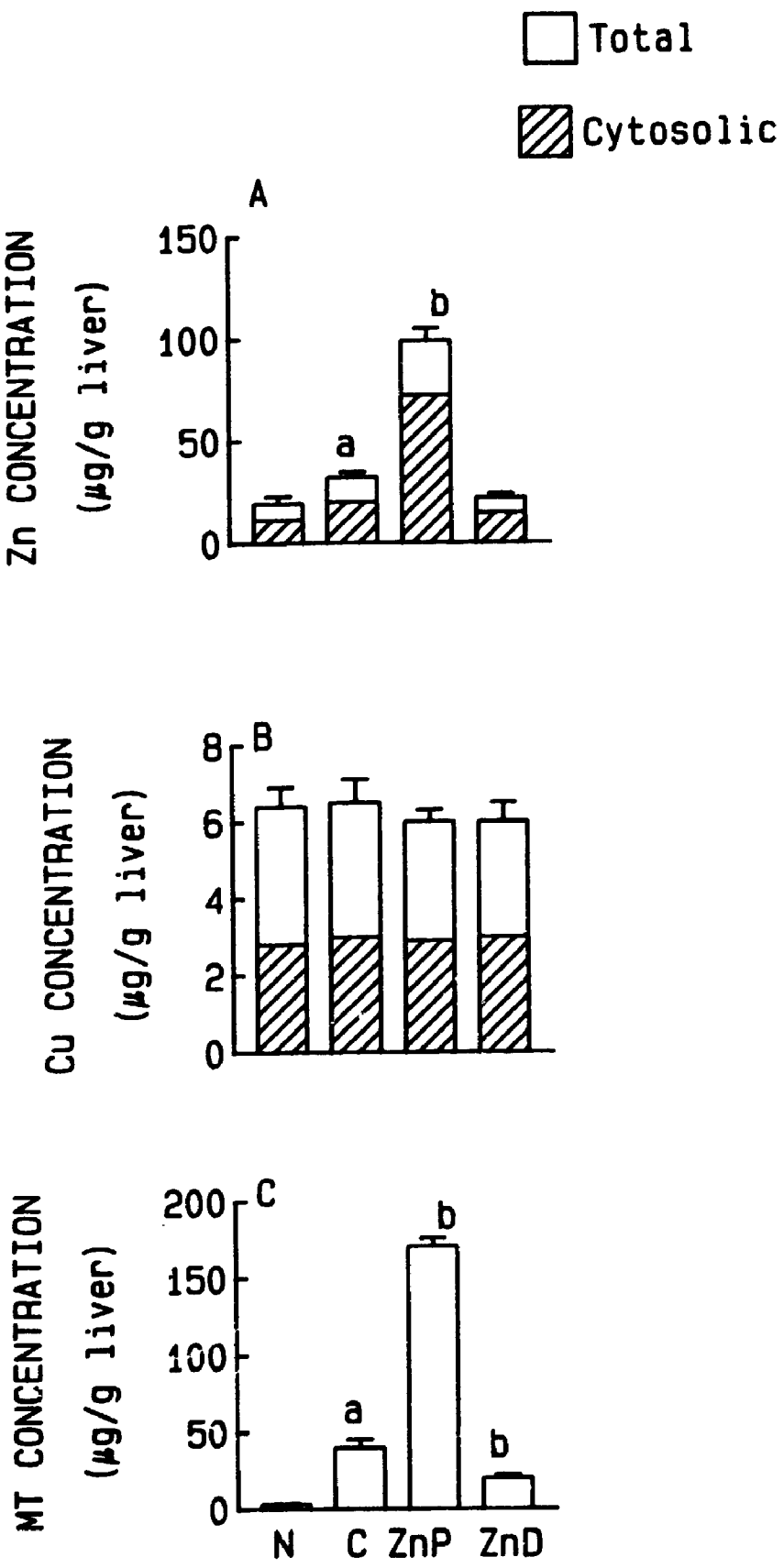

Total
Cytosolic
A
Zn CONCENTRATION (μg/g liver)
150
100
50
0
a
b
B
Cu CONCENTRATION (μg/g liver)
8
6
4
2
0
C
MT CONCENTRATION (μg/g liver)
200
150
100
50
0
a
b
b
N
C
ZnP
ZnD

TABLE 4.1

Metal contents of cytosolic components of host mouse liver following Sephadex G-75 chromatographic separationa

	METAL CONCENTRATION (μg/g liver)			
	HMW[d] COMPONENT		MT[e] COMPONENT	
TREATMENT	Zn	Cu	Zn	Cu
Normal mice	2.70 ± 0.35	2.70 ± 0.35	N.D.	N.D.
Control host mice[b]	2.51 ± 0.40	2.51 ± 0.40	6.25 ± 0.40	0.40 ± 0.05
10 mg $ZnSO_4$/kg[c]	2.35 ± 0.30	2.35 ± 0.30	43.10 ± 5.10[f]	1.15 ± 0.15[f]
Zn deficiency[c]	2.60 ± 0.25	2.60 ± 0.25	1.15 ± 0.30[f]	0.10 ± 0.02[f]

a. Values represent mean ± SEM, (n = 3 animals)
b. Mice were treated with Ehrlich cells for 6 days
c. Control host mice were injected with a single dose of $ZnSO_4$ (10 mg/kg, ip) 5 days following inoculation of cells; host mice were killed 24 h later. Normal mice were placed on Zn deficient diets and after 4 weeks were treated with cells; host mice were killed 6 days later.
d. High molecular weight component
e. Metallothionein component
f. Significantly different from corresponding control host values, $p<0.05$.

N.D. not detected

Figure 4.2. Time-course of the effect of Zn pretreatment on cellular Zn (A) and MT (B) concentrations in hepatic tissues of host mice. Control host mice (○) were injected with a single dose of $ZnSO_4$ (10 mg Zn/kg, ip) (●) dissolved in 0.9% saline 5 days following inoculation of Ehrlich cell; host mice were killed 4, 8, or 24 h later. Values represent mean ± SEM, (n = 6 animals).

* significantly different from corresponding control values, $p<0.05$.

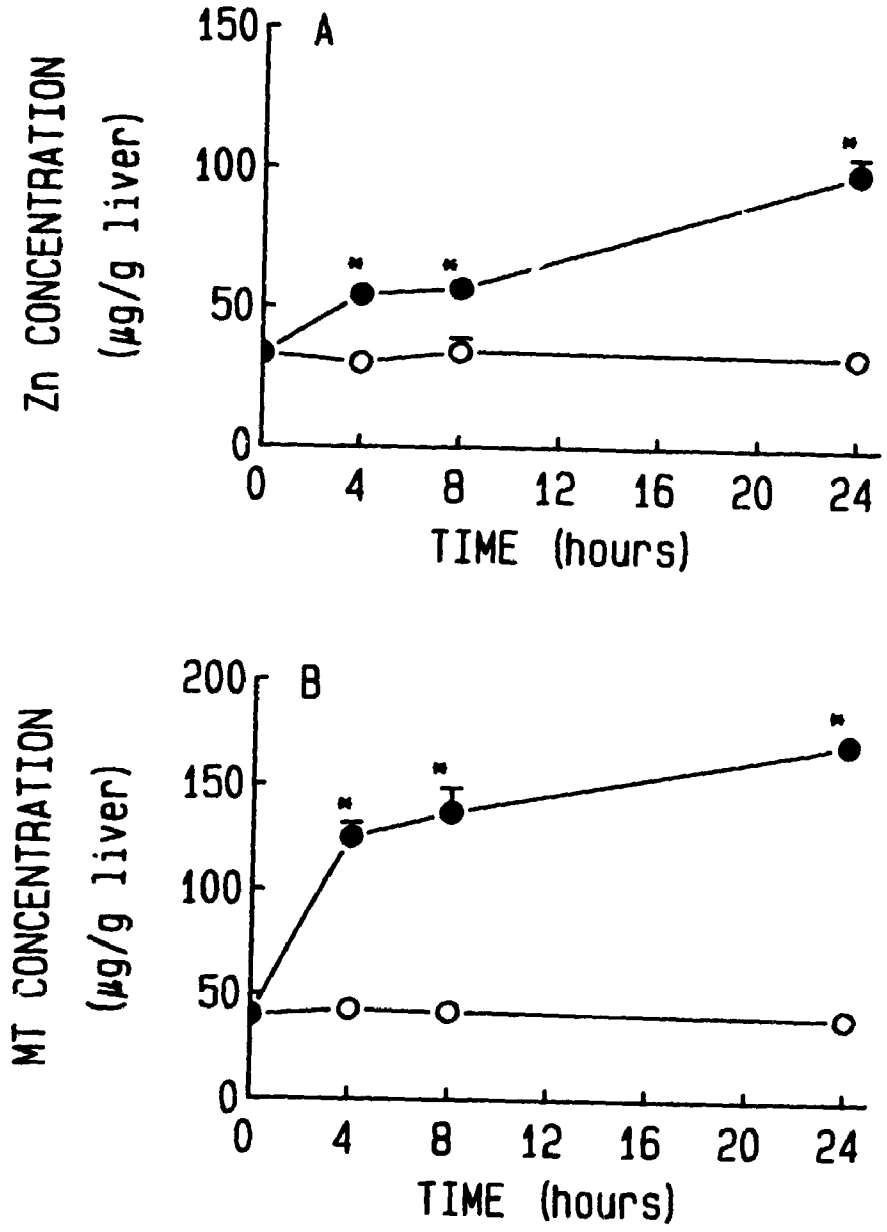

A
Zn CONCENTRATION
(µg/g liver)
150
100
50
0
0
4
8
12
16
20
24
TIME (hours)
B
MT CONCENTRATION
(µg/g liver)
200
150
100
50
0
0
4
8
12
16
20
24
TIME (hours)

Figure 4.3. Total and cytosolic Zn (A) and Cu (B) concentrations as well as MT concentrations (C) of Ehrlich cells obtained 6 days post-inoculation from control (C), Zn-pretreated (ZnP) and Zn-deficient (ZnD) host mice. To obtain Zn-deficient cells, control host mice were placed on Zn deficient diets; four weeks later host mice were inoculated with Ehrlich cells and killed 6 days later to obtain the ascites cells. To obtain Zn-pretreated cells, control host mice were injected with a single dose of $ZnSO_4$ (10 mg/kg, ip) dissolved in 0.9% saline 5 days following inoculation of Ehrlich cells; ascites cells were isolated 24 h after Zn treatment. Values represent mean $\pm$ SEM (n = 6 animals). * significantly different from corresponding control values, $p < 0.05$.

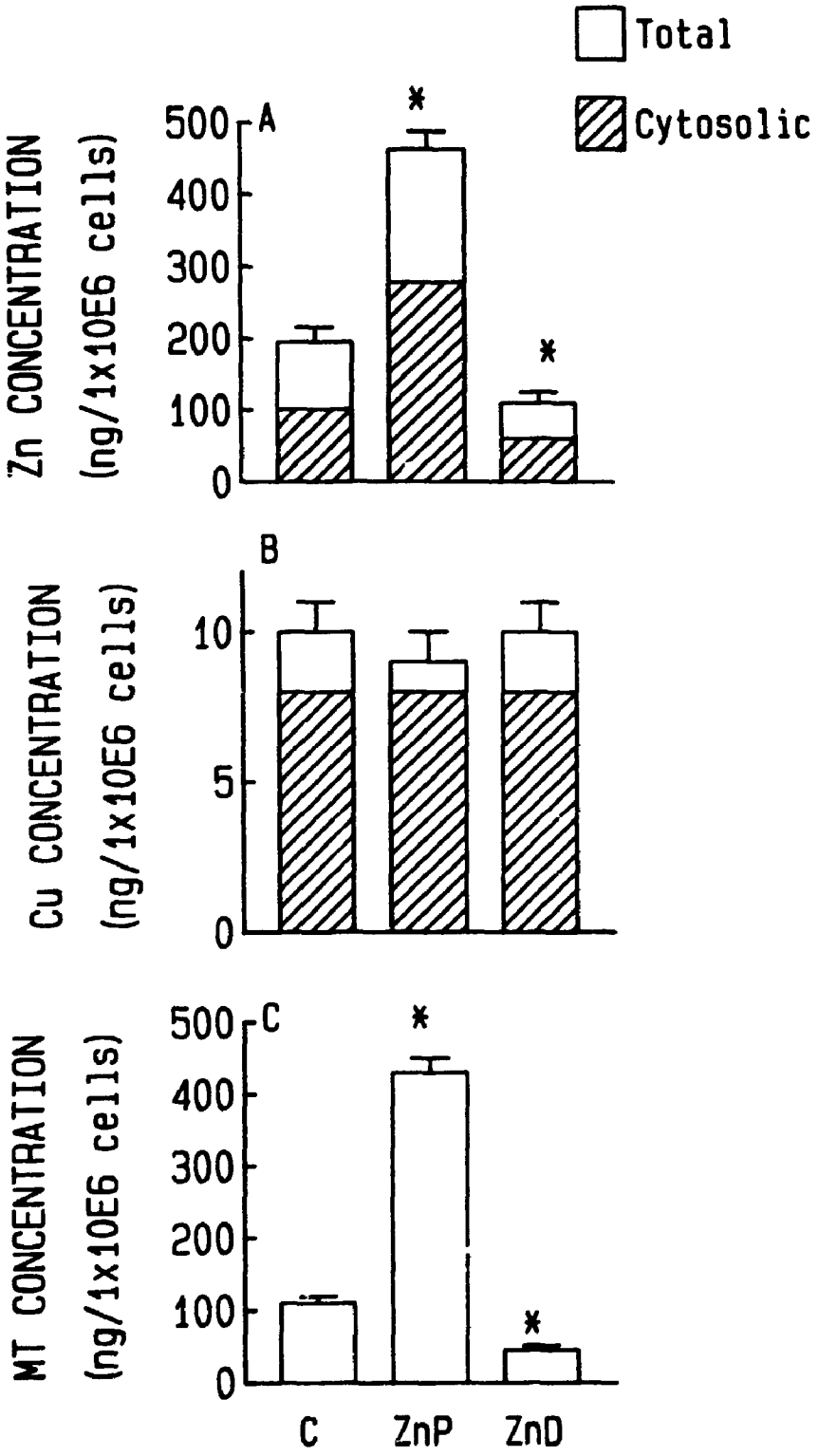

Total
Cytosolic
A
B
C
Zn CONCENTRATION
(ng/1x10E6 cells)
Cu CONCENTRATION
(ng/1x10E6 cells)
MT CONCENTRATION
(ng/1x10E6 cells)
500
400
300
200
100
0
10
5
0
*
C
ZnP
ZnD

Figure 4.4. Time-course of the effect of Zn pretreatment on cellular Zn (A) and MT (B) concentrations as well as MT-I-mRNA accumulation (C) in Ehrlich cells. Control host mice (○) were injected with a single dose of $ZnSO_4$ (10 mg/kg, ip) (●) dissolved in 0.9% saline 5 days following inoculation of Ehrlich cells; host mice were killed 4, 8, or 24 h later. Varying amounts of tissue RNA from Ehrlich cells were immobilized on nylon filters and hybridized to radiolabeled MT-I and ß-actin cDNA probes. Values represent mean ± SEM (n = 6 animals).

* significantly different from corresponding control values, $p < 0.05$.

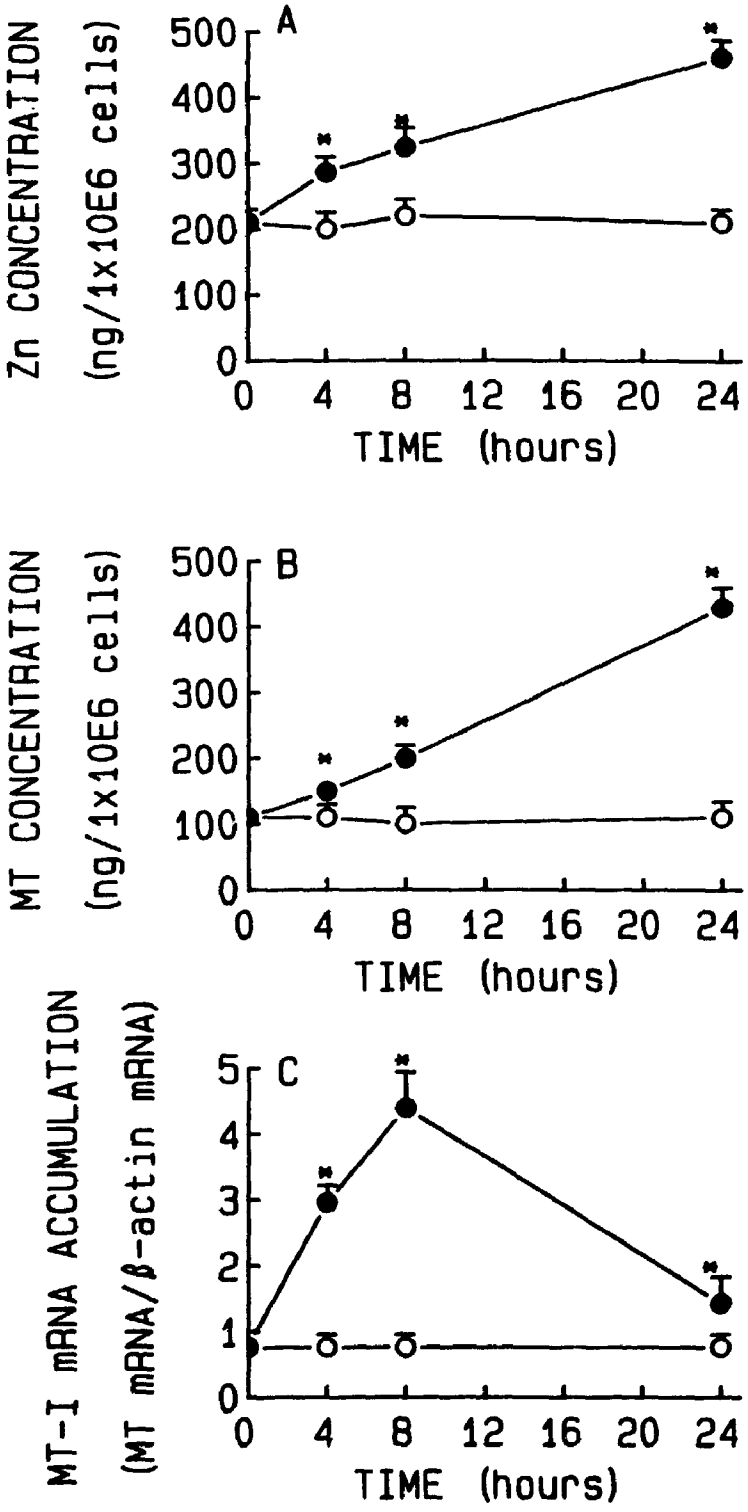

A
Zn CONCENTRATION
(ng/1x10E6 cells)
500
400
300
200
100
0
0 4 8 12 16 20 24
TIME (hours)
B
MT CONCENTRATION
(ng/1x10E6 cells)
500
400
300
200
100
0
0 4 8 12 16 20 24
TIME (hours)
C
MT-I mRNA ACCUMULATION
(MT mRNA/β-actin mRNA)
5
4
3
2
1
0
0 4 8 12 16 20 24
TIME (hours)

Figure 4.5. Cytosolic distribution of Zn in Ehrlich cells isolated from control (————), Zn-pretreated (····) and Zn-deficient (----) host mice. Cytosols were isolated from Ehrlich cell homogenates 6 days post-inoculation with Ehrlich cells and were chromatographed on a Sephadex G-75 column equilibrated with 10 mM Tris-HCl buffer, pH 8.6, at room temperature. Each cytosolic sample was analyzed chromatographically twice and the average values were used for the construction of the chromatogram.

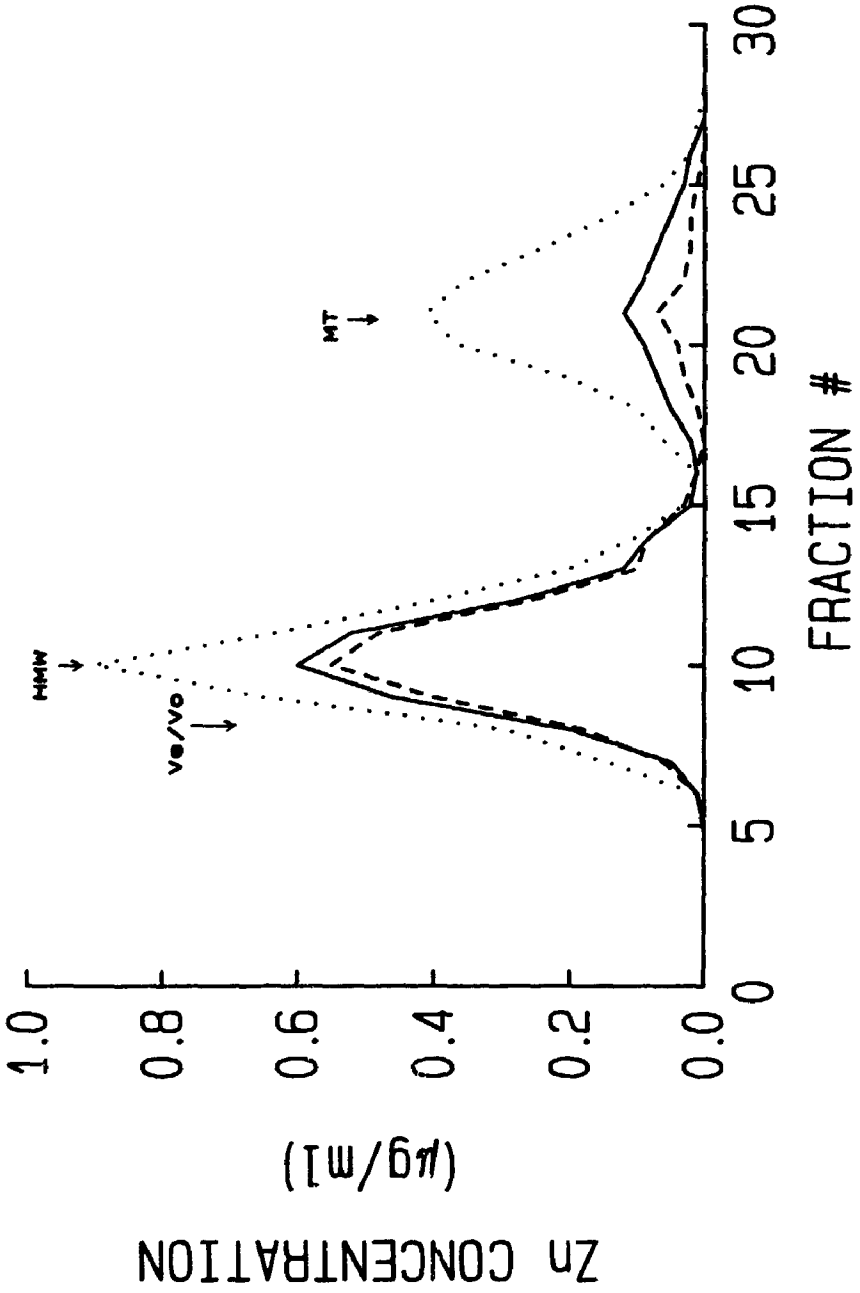

Zn CONCENTRATION
(µg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
FRACTION #
HMW
Ve/Vo
MT

Figure 4.6. MT-I mRNA accumulation in hepatic tissues following Zn pretreatment. Normal mice were treated with Ehrlich cells and 5 days later were injected with $ZnSO_4$ (10 mg Zn/kg, ip) dissolved in 0.9% saline; mice were killed 4 h later. Varying amounts of total tissue RNA from liver of mice were immobilized on nylon filters and hybridized to radiolabeled MT-I and ß-actin cDNA probes. Values represent mean relative MT-I mRNA accumulation $\pm$ SEM as determined in triplicate measurement of single RNA samples.

a. significantly different from normal value, $p<0.05$.

b. significantly different from value obtained from mice treated with Ehrlich cells, $p<0.05$.

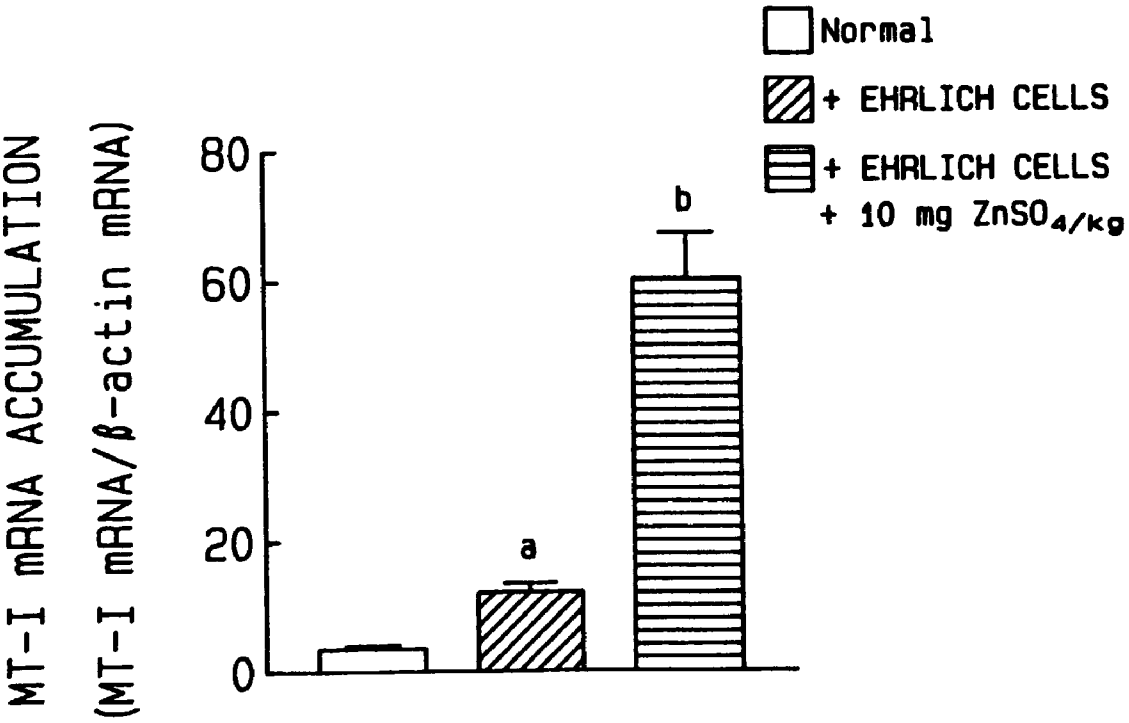

MT-I mRNA ACCUMULATION
(MT-I mRNA/β-actin mRNA)
80
60
40
20
0
a
b
Normal
+ EHRLICH CELLS
+ EHRLICH CELLS
+ 10 mg ZnSO4/kg

TABLE 4.2

Effect of treatment[a] of host mice with Zn on Fe concentration and antioxidant activities of Ehrlich cells[b]

Treatment	Fe Concentration (ng/1x10E6 cells)	SOD (U/mg protein)	Catalase[c] (U/mg protein)	Total GSH (nmole/1x10E6 cells)	α-tocopherol (nmole/1x10E6 cells)
Control	110 ± 10	N.D	0.12 ± 0.01	1.96 ± 0.18	0.10 ± 0.02
Zn pretreated	120 ± 14	N.D	0.14 ± 0.03	2.00 ± 0.16	0.11 ± 0.01
Zn deficient	106 ± 9	N.D	0.13 ± 0.02	3.13 ± 0.26[d]	-

a. Control host mice were pretreated with $ZnSO_4$ (10 mg Zn/kg, ip) 5 days post-inoculation with Ehrlich cells and killed 24 h later. To induce Zn deficiency, mice were placed on Zn-deficient diet for 5 weeks as described in Table 4.1.

b. Values represent mean ± SEM (n=6-8 mice)

c. One unit of catalase activity is defined as the amount of catalase required to decompose 1μmole of H_2O_2 per minute at 25°C.

d. Significantly different from corresponding control value, $p<0.05$.

N.D Not detected

affected following administration of Zn. The induction of Zn-deficiency in host mice resulted in significant increases in GSH levels in Ehrlich cells without affecting other parameters measured. Superoxide dismutase activity was not detected in any of the experimental groups examined.

ii) Influence of Zn/Zn-MT status on H_2O_2 toxicity.

In the present study, we have examined H_2O_2-induced toxicity in Ehrlich cells containing three different levels of this metalloprotein (fig. 4.3; fig. 4.5). Although Zn pretreatment increased cellular Zn concentrations in Ehrlich cells, it also resulted in increases in non-MT-bound Zn in addition to that of MT (Table 4.3). Therefore, this model alone would not be adequate to study the influence of Zn-MT in oxidative stress. To investigate whether or not non-MT bound Zn would also affect oxidative stress-induced injury, control cells were pre-incubated with 2.5 ppm $ZnSO_4$ in vitro for 30 min to obtain total cellular Zn concentrations similar to cells pretreated with $ZnSO_4$ but without induction of MT synthesis. These Zn-preincubated cells contained similar HMW-bound Zn concentrations, much higher concentrations in the particulate fraction and similar MT-bound Zn concentrations as compared to control cells (Table 4.3).

The result of the dose-toxicity relationship study gave estimate of LC_{50} values for the control, Zn-pretreated and Zn-deficient cells to be 60 $\pm$ 9, 130 $\pm$ 15 and 30 $\pm$ 5 μM, H_2O_2, respectively. The slopes of the log dose-toxicity lines among these three groups were not significantly different suggesting similar mechanisms of acute lethal injury (fig.

4.7). Experiments with Zn-preincubated cells showed that sensitivity (LC_{50} = 94 ± 6) to H_2O_2 was decreased as compared to control cells but was significantly smaller than that observed in Zn-pretreated cells (not shown).

The characteristics of H_2O_2-induced oxidative stress in control cells and the influence Zn-pretreatment and Zn-deficiency was also examined in this study. Exposure of control Ehrlich cells to H_2O_2 (60 μM) resulted in time-dependent decrease in cell viability (fig. 4.8), increase in lipid peroxidation (fig. 4.8) oxidation of GSH to GSSG (fig. 4.9) increase in $[Ca^{+2}]i$ (fig. 4.10) as well as the appearance of cell blebbing (fig. 4.10). The relative time-course of these H_2O_2-induced effects was depicted in the composite diagram (fig. 4.11). The increases in $[Ca^{+2}]i$ were rapid and this effect was mirrored by treatment-related oxidation of cellular GSH. These were followed by the appearance of cell blebbing, increases in lipid peroxidation and cell death (fig. 4.11). The relative-time course of these H_2O_2 effects was not affected by manipulation of Zn status. The magnitude of H_2O_2-induced changes in all of these parameters within the 60 minute exposure period were significantly lower in Zn-pretreated cells and higher in Zn-deficient cells as compared to control cells. The degree of protection conferred by Zn was not uniform among different toxicity parameters. Zn pretreatment was most effective in protecting against H_2O_2-induced lipid peroxidation; this was followed in decreasing order by $[Ca^{+2}]i$, cell blebbing and oxidation of GSH.

iii) Mechanism of Zn-pretreatment on H_2O_2 toxicity.

To investigate the mechanism(s) underlying the modulating role of Zn-MT in H_2O_2

TABLE 4.3

Subcellular distribution of Zn in control, Zn-pretreated (10 mg Zn/kg) and Zn-preincubated (2.5 ppm Zn) cells[a]

Zn concentration (ng/1x106 cells)

		Cytosolic		
Treatment	Total	HMW	MT	Particulate
Control	216.0±20.1	100.1±8.7	26.0±2.5	85.34±6.9
10 mg Zn/kg[b]	462.5±14.7[d]	172.0±12.8[d]	98.0±2.1[d]	175.0±15.7[d]
2.5 ppm Zn[c]	440.1±16.7[d]	150.1±8.5[d]	25.8±3.9[e]	263.5±18.8[de]

a. Values represent mean±SEM from 4 experiments

b. Ehrlich cells were isolated from host mice 24 h after 10 mg Zn/kg $ZnSO_4$ administration.

c. Ehrlich cells isolated from control host mice were treated with 2.5 ppm $ZnSO_4$ for 30 min at 37°C; cells were washed twice with PBS buffer and resuspended to original volume with the same buffer.

d. significantly different from corresponding control values, $p<0.05$.

e. significantly different from corresponding Zn-pretreated values, $p<0.05$.

Figure 4.7. Log dose-response of the killing of Ehrlich cells by H_2O_2. Ehrlich cells were challenged with 15, 30, 60, 120 and 200 μM H_2O_2 for 60 min and viability was assessed by the trypan blue exclusion method. Viability was expressed as probit units by converting percents of response to normal equivalent deviations (N.E.D.). A N.E.D. is the response increment brought about by increasing or decreasing the log dose by one standard deviation. Each point represents the mean $\pm$ SEM of 5 observations.

* significantly different from corresponding control value, $p < 0.05$.

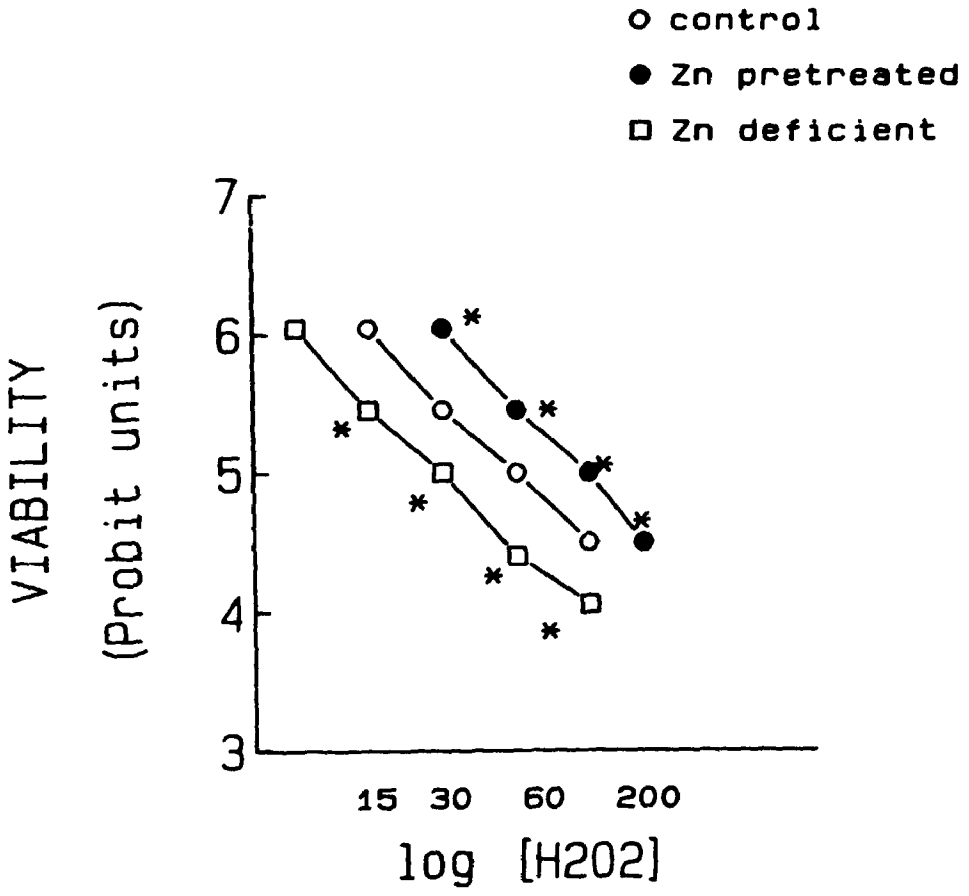
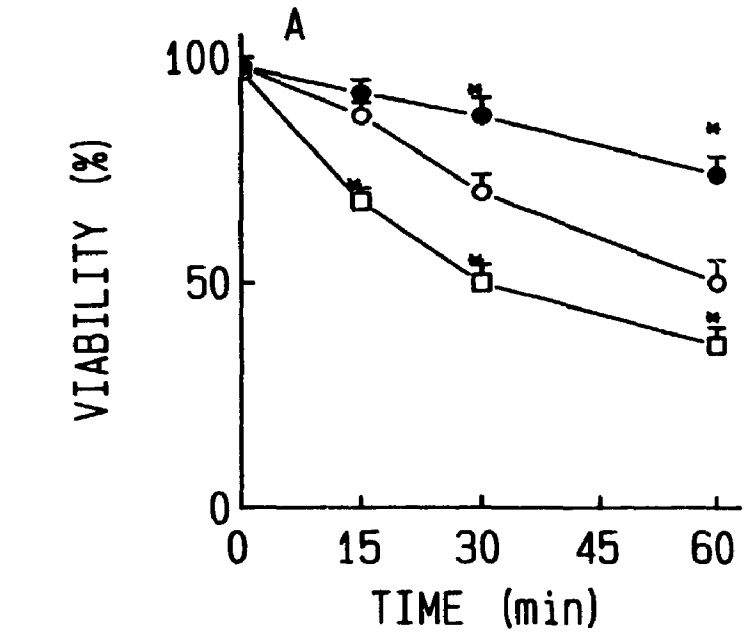

control
Zn pretreated
Zn deficient
VIABILITY
(Probit units)
7
6
5
4
3
15
30
60
200
log [H202]

Figure 4.8. Changes in cell viability (A) and lipid peroxidation levels (B) of Ehrlich cells isolated from control (○), Zn-pretreated (●) and Zn-deficient (□) host mice following treatment of cells with H_2O_2 (60 μM). Cell viability was determined by the trypan blue exclusion method. Formation of thiobarbituric acid reactants (abs at 535 nm) was taken as a measure of lipid peroxidation. Values represent mean $\pm$ SEM of 4 experiments. In the absence of H_2O_2, cell viability and lipid peroxidation values were not significantly different from their respective control time zero value.

* significantly different from corresponding control values, $p < 0.05$.


A
100
50
0
VIABILITY (%)
0
15
30
45
60
TIME (min)

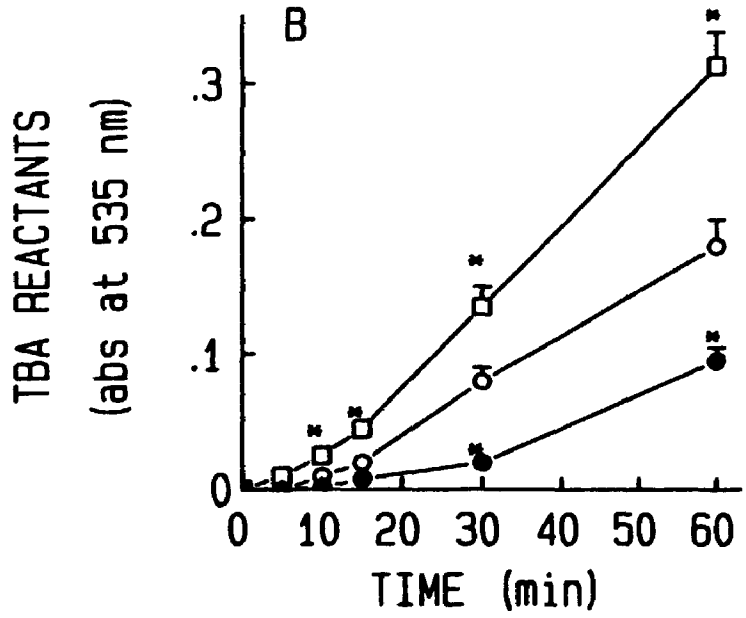

B
.3
.2
.1
0
TBA REACTANTS
(abs at 535 nm)
0
10
20
30
40
50
60
TIME (min)

Figure 4.9. Depletion of GSH in Ehrlich cells isolated from control (○) and Zn-pretreated (●) host mice following treatment of cells with H_2O_2 (60 μM). Control host mice were injected with a single dose of $ZnSO_4$ (10 mg Zn/kg, ip) 5 days following inoculation of Ehrlich cells; ascites cells were isolated 24 h after Zn treatment. Sulphydryls of GSH were measured by NbS_2 titration. Values represent mean ± SEM of 4 experiments.

* significantly different from corresponding control values, $p<0.05$.

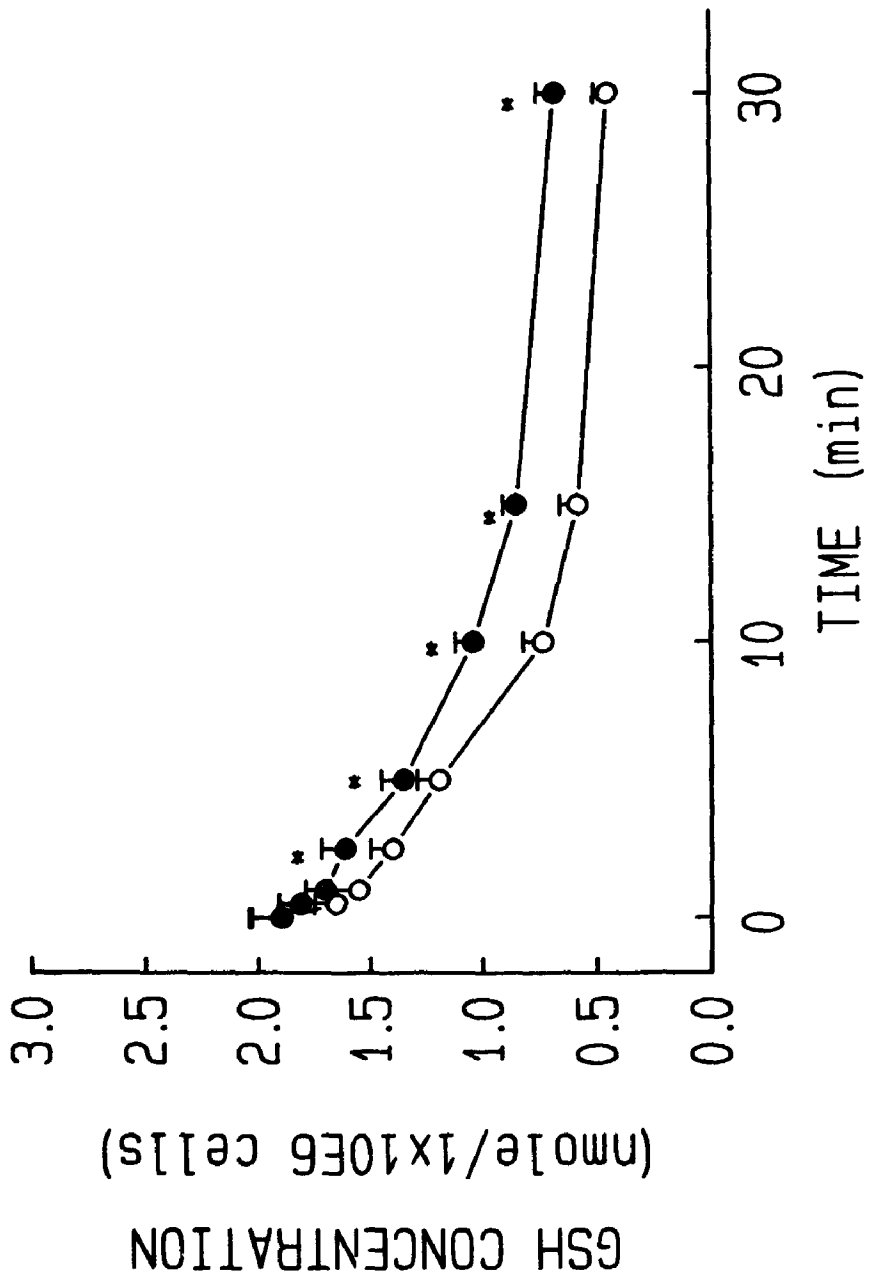
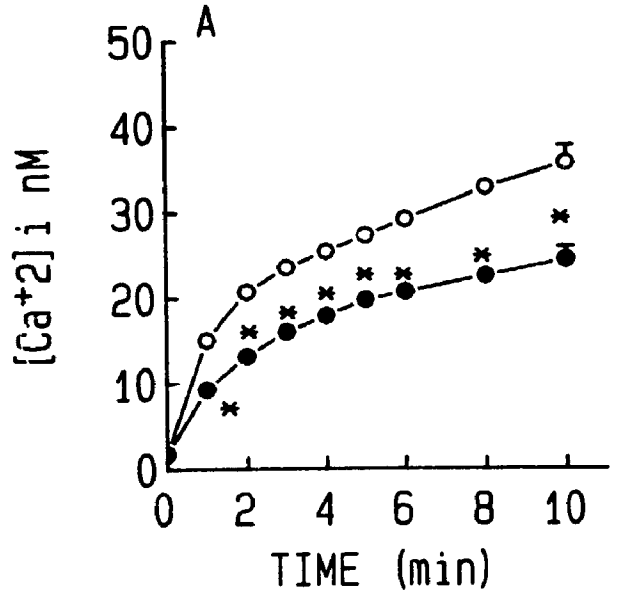

GSH CONCENTRATION
(nmole/1x10E6 cells)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
TIME (min)

Figure 4.10. Changes in $[Ca^{+2}]i$ (A) and cell blebbing (B) in Ehrlich cells isolated from control (○) and Zn-pretreated (●) host mice following treatment of cells with H_2O_2 (60 μM). Cell blebbing was assessed subjectively by light microscopy. $[Ca^{+2}]i$ was measured fluorometrically using the Ca^{+2}-sensitive probe Indo-1. Values represent mean ± SEM of 4 experiments.

* significantly different from corresponding control values, $p < 0.05$.


A
50
40
30
20
10
0
[Ca+2]i nM
0
2
4
6
8
10
TIME (min)

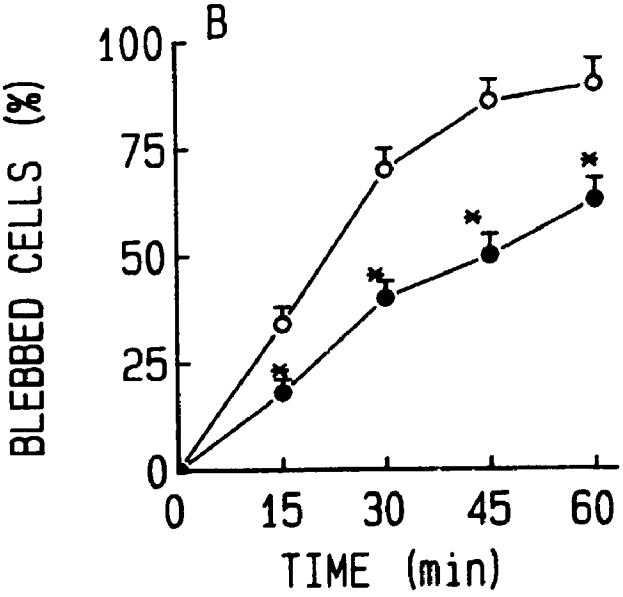
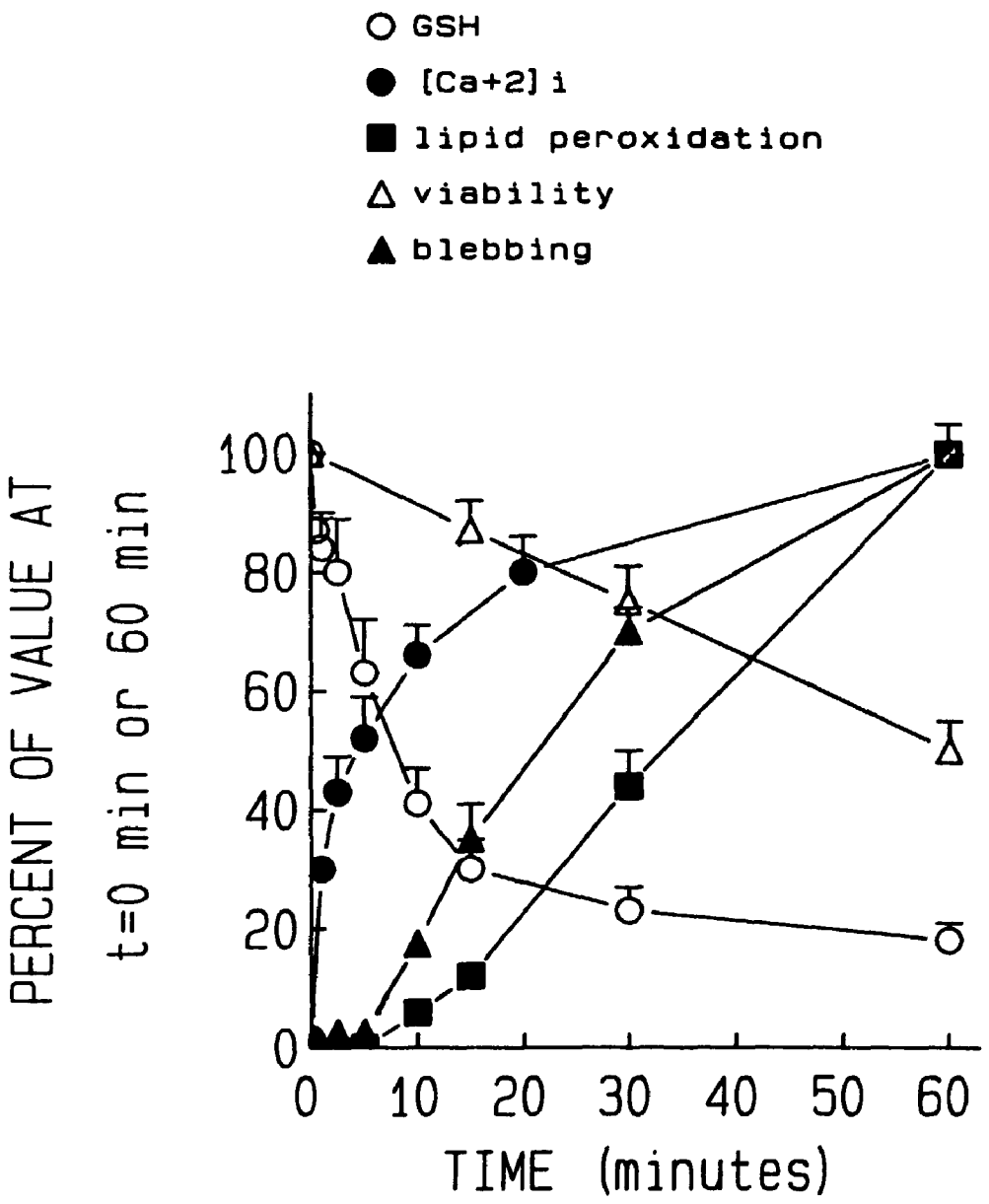

B
100
75
50
25
0
BLEBBED CELLS (%)
0
15
30
45
60
TIME (min)

Figure 4.11. Changes in cellular parameters of control Ehrlich cells following treatment of cells with H_2O_2 (60 μM). Values are expressed either as percentage of time zero control value (cell viability, GSH) or as a percentage of values measured at 60 min (lipid peroxidation, $[Ca^{+2}]i$, cell blebbing) of incubation. Values represent mean $\pm$ SEM of 4 experiments. Measurement of the parameters was determined as described in figures 4.8, 4.9, and 4.10.


GSH
[Ca+2] i
lipid peroxidation
viability
blebbing
PERCENT OF VALUE AT
t=0 min or 60 min
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME (minutes)

toxicity, evidence of interaction between Zn-MT and H_2O_2 was examined. As shown in figures 4.12 and 4.13, exposure of Zn-pretreated Ehrlich cells with 60 μM H_2O_2 resulted in paralleled reduction in both the metal binding capacity of MT as measured by the Ag-heme saturation method (fig. 4.12) and loss of MT sulphydryl groups (fig. 4.13). The H_2O_2-mediated loss in metal binding capacity was probably due to the oxidation of MT thiolate groups, since treatment with DTT, a reducing agent, restored the metal binding capacity of MT samples to pre-H_2O_2 treatment values (fig. 4.14). Results presented in figure 4.15 and Table 4.4 also showed that H_2O_2-induced oxidation and inactivation of metal binding sites of MT were accompanied by changes in the subcellular and cytosolic distribution of Zn. Exposure of control cells to 60 μM H_2O_2 resulted in decreases in MT-bound Zn (61%) 15 min after addition of H_2O_2 and this treatment effect was accompanied with concurrent increases in Zn concentration in the cytosolic HMW fraction (Table 4.3). Under the same experimental conditions, a 75% loss of MT-bound Zn from Zn-pretreated cells was accompanied with concurrent increases in Zn concentration in both the cytosolic HMW and particulate fraction (Table 4.3).

To examine the influence of non-MT-bound Zn on cellular susceptibility to H_2O_2 toxicity, study with Zn-preincubated cells was carried out. Preincubation of control cells with $ZnSO_4$ was most effective in protecting against $[Ca^{+2}]i$ (fig. 4.17), this was followed in decreasing order by cell blebbing (fig. 4.17) and lipid peroxidation (fig. 4.16). However, no apparent protective effect against GSH oxidation was observed (fig. 4.18). It should be noted that the protective effect of Zn pretreatment was greater than that for Zn-preincubation against cell death and lipid peroxidation while the reverse was true for

Figure 4.12. H_2O_2-induced reduction of MT concentration of Ehrlich cells isolated from control (A), Zn-pretreated (B) and Zn-deficient (C) host mice. Ehrlich cells were incubated at 37°C under air atmosphere and aliquots of incubation mixture were removed at 0, 0.5, 1, 2.5, 5, 10, 15 and 30 min after addition of 60 μM H_2O_2. Metallothionein concentration (or the metal binding capacity) was determined by the Ag-heme saturation method. Values represent mean $\pm$ SEM of 4 experiments.

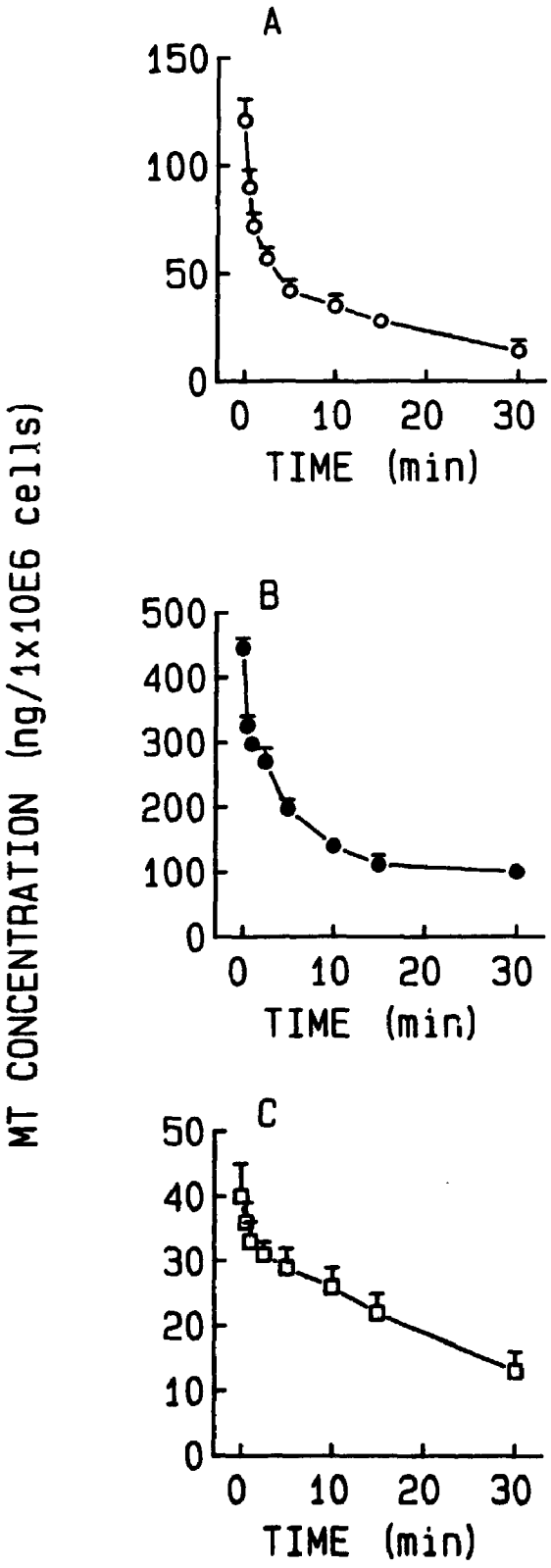

A
150
100
50
0
0 10 20 30
TIME (min)
B
500
400
300
200
100
0
0 10 20 30
TIME (min)
C
50
40
30
20
10
0
0 10 20 30
TIME (min)
MT CONCENTRATION (ng/1x10E6 cells)

Figure 4.13. H_2O_2-induced reduction of sulphydryl concentration of MT of Ehrlich cells isolated from control (A), Zn-pretreated (B) and Zn-deficient (C) host mice. Ehrlich cells were incubated at 37°C under air atmosphere as described in fig. 4.12. Thiol content of MT was measured by titration with NbS_2. Values represent mean $\pm$ SEM of 4 experiments.

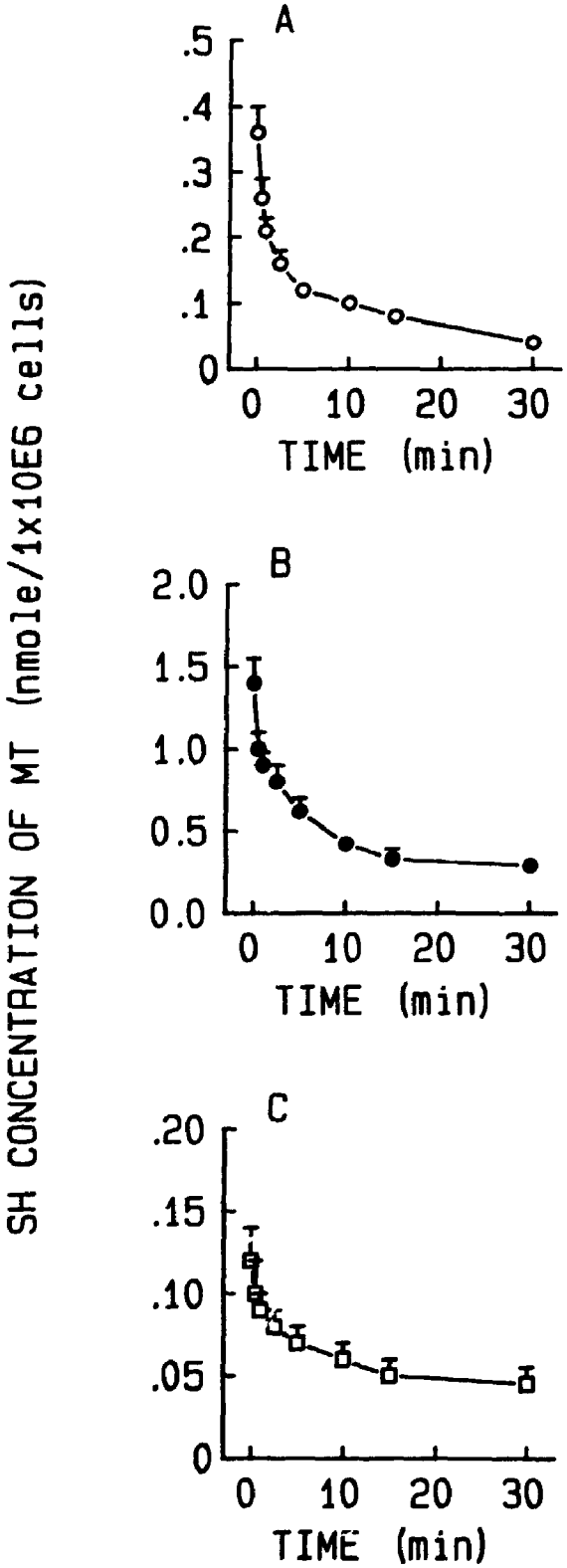

A
.5
.4
.3
.2
.1
0
0 10 20 30
TIME (min)
B
2.0
1.5
1.0
0.5
0.0
0 10 20 30
TIME (min)
C
.20
.15
.10
.05
0
0 10 20 30
TIME (min)
SH CONCENTRATION OF MT (nmole/1x10E6 cells)

Figure 4.14. In vitro 1,4-dithiothreitol (DTT)-dependent regeneration of metal binding capacity of MT in cytosols obtained from control (A) and Zn-prtreated (B) Ehrlich cells treated with H_2O_2 (60 μM) for 30 min. Heat-treated cytosolic supernatant fractions from control and H_2O_2-treated incubations were treated with 5 mM DTT under nitrogen at room temperature for 30 min and the metal binding capacity of MT was estimated by the Ag-heme saturation method. Values represent mean $\pm$ SEM of 3 experiments.

a. significantly different from corresponding time zero values, $p<0.05$.

b. significantly different from corresponding non-DTT-treated samples, $p<0.05$.

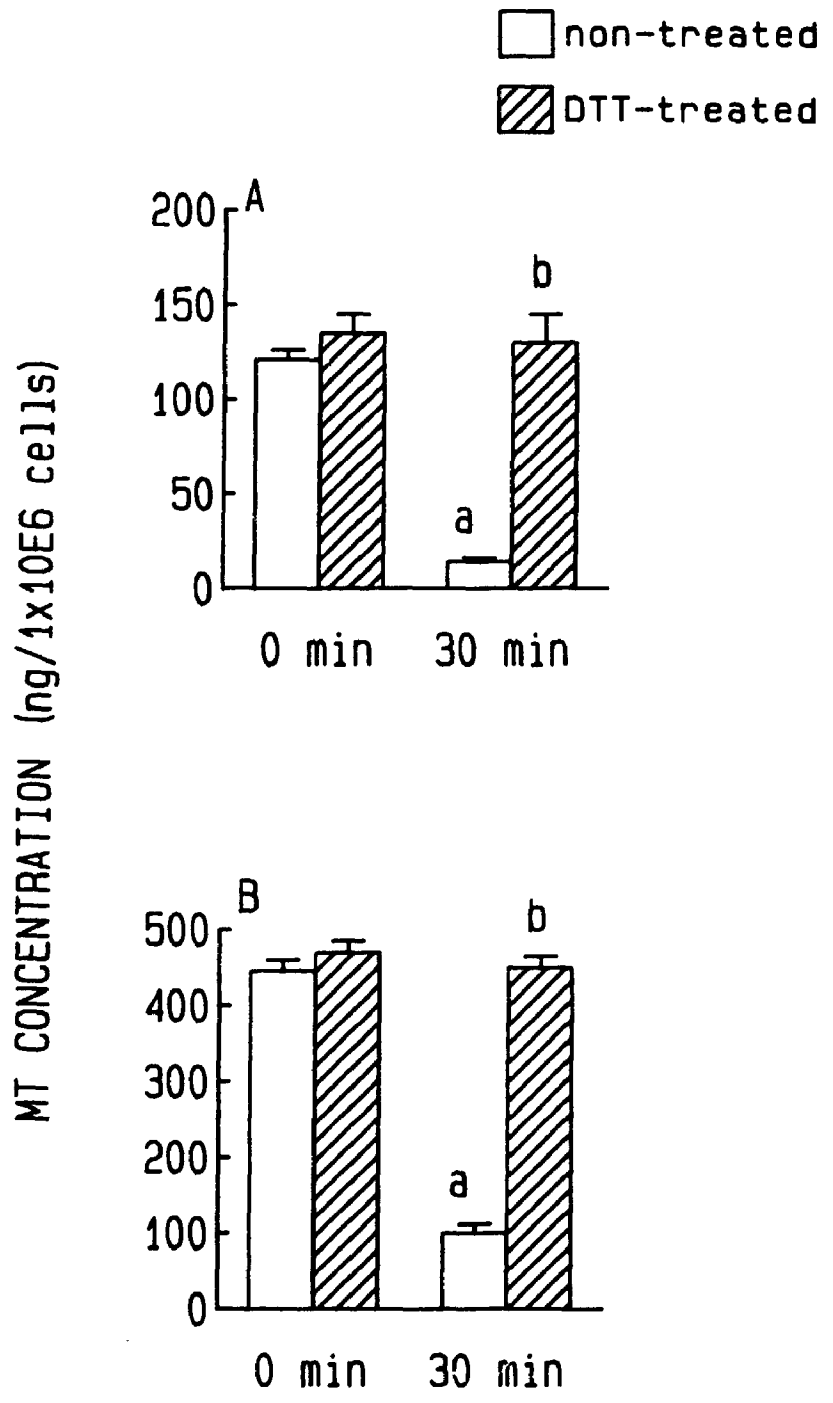
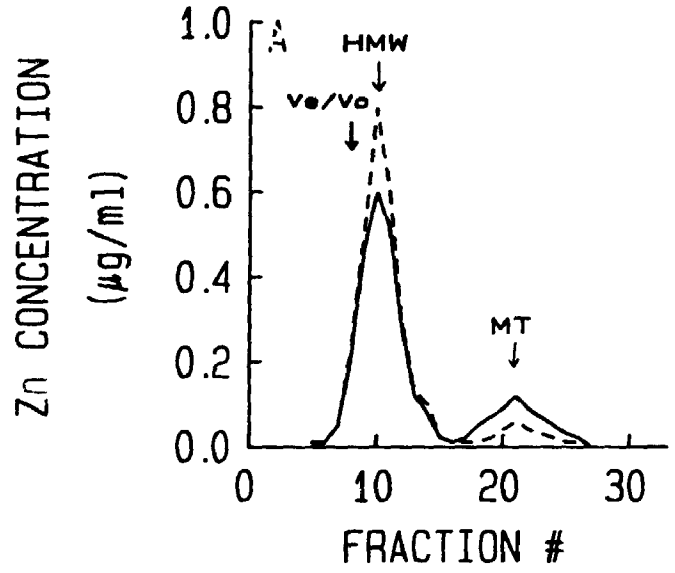

non-treated
DTT-treated
MT CONCENTRATION (ng/1x10E6 cells)
A
200
150
100
50
0
a
b
0 min
30 min
B
500
400
300
200
100
0
a
b
0 min
30 min

Figure 4.15. Distribution of Zn in cytosols of Ehrlich cells isolated from control (A) and Zn-pretreated (B) host mice in the absence (————) or presence (----) of H_2O_2 (60 μM). Exposure of Ehrlich cells to H_2O_2 was carried out for 15 min. Ehrlich cells obtained from 3 different incubations were pooled and then sonicated to obtain cytosolic fractions. Cytosols were then chromatographed on a Sephadex G-75 column equilibrated with 10 mM Tris-HCl, pH 8.6, at room temperature. Two milliliter fractions were collected at an elution rate of 1 ml/min and measured for Zn by atomic absorption spectrophotometry.


A
HMW
Ve/Vo
MT
Zn CONCENTRATION
(µg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
FRACTION #

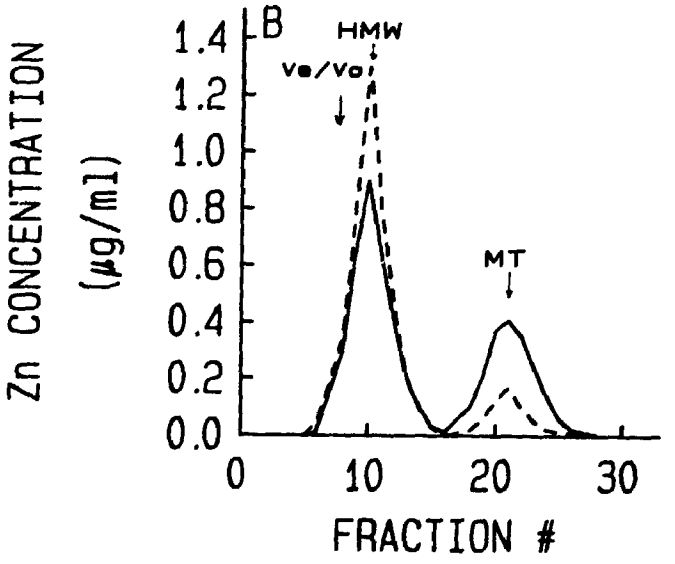

B
HMW
Ve/Vo
MT
Zn CONCENTRATION
(µg/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
FRACTION #

TABLE 4.4

Effect of H_2O_2 on the subcellular distribution of Zn in Ehrlich cells isolated from control and Zn pretreated host mice[a,b]

Zn CONCENTRATION (ng/1x10E6 cells)

Treatment	Total	HMW	MT	Particulate
Control	216.0±17.7	100.1±4.7	26.0±2.8	85.0±5.4
Control + H_2O_2	216.4±20.0	118.3±5.2[c]	10.0±1.8[c]	83.1±6.9
Zn-pretreated	462.0±25.8	172.9±9.8	98.91±3.8	175.4±9.5
Zn-pretreated + H_2O_2	470.7±14.4	200.0±11.0[c]	24.6±2.1[c]	216.1±12.5[c]

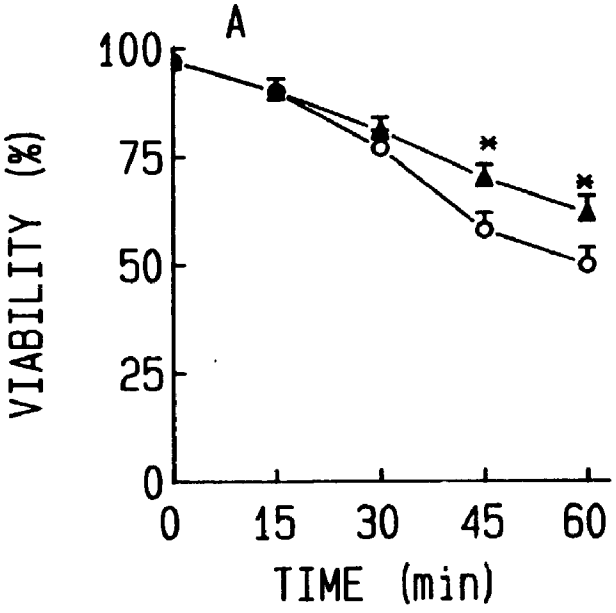
a. Values represent mean ± SEM from 3 experiments

b. Ehrlich cells (1x10E7 cells/ml incubation) isolated from control and Zn-pretreated (10 mg Zn/kg) host mice were challenged with 60 μM H_2O_2 at 37°C. Aliquots of cell suspensions were removed at 15 min after addition of H_2O_2 and washed once with PBS buffer and resuspended to original volume with the same buffer. Homogenate, cytosols and particulate fractions were obtained following sonication of cell suspensions. To obtain the high molecular weight (HMW) and metallothionein (MT) fractions, an aliquot of cytosol was chromatographed on a Sephadex G-75 column.

c. Significantly different from corresponding non-H_2O_2 treated values, $p < 0.05$. treated values, $p < 0.05$.

Figure 4.16. Changes in cell viability (A) and lipid peroxidation (B) in control (○) and Zn-preincubated (▲) Ehrlich cells exposed to H_2O_2 (60 μM). Ehrlich cells were preincubated with 2.5 ppm $ZnSO_4$ for 30 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2. Values represent mean ± SEM of 3 experiments.

* significantly different from corresponding control values, $p<0.05$.


A
100
75
50
25
0
VIABILITY (%)
0
15
30
45
60
TIME (min)

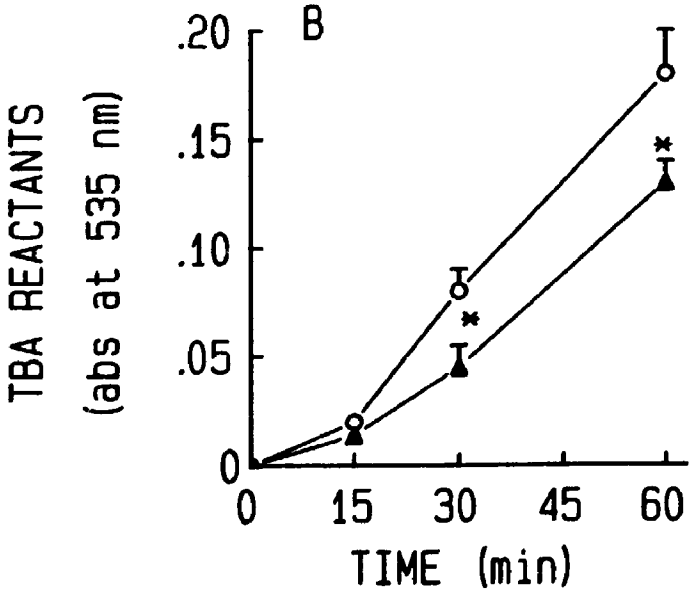
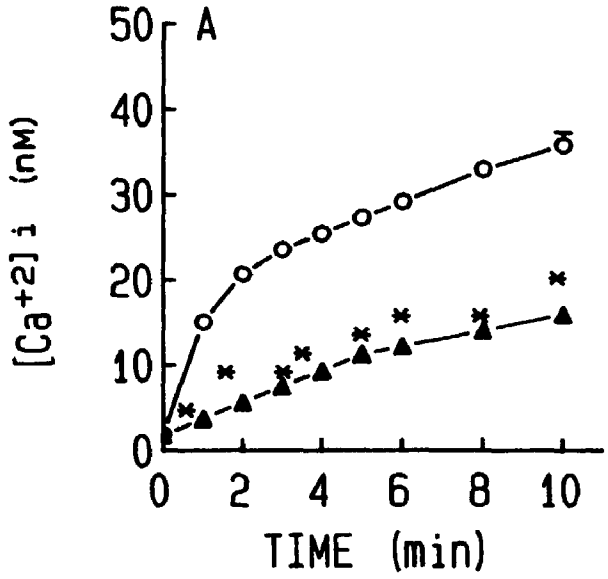

B
.20
.15
.10
.05
0
TBA REACTANTS
(abs at 535 nm)
0
15
30
45
60
TIME (min)

Figure 4.17. Changes in $[Ca^{+2}]i$ (A) and cell blebbing (B) in control (○) and Zn-preincubated (▲) Ehrlich cells exposed to H_2O_2 (60 μM). Ehrlich cells were preincubated with 2.5 ppm $ZnSO_4$ for 30 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2. Values represent mean ± SEM of 3 experiments.

* significantly different from corresponding control values, $p<0.05$.


A
50
40
30
20
10
0
[Ca+2]i (nM)
0
2
4
6
8
10
TIME (min)

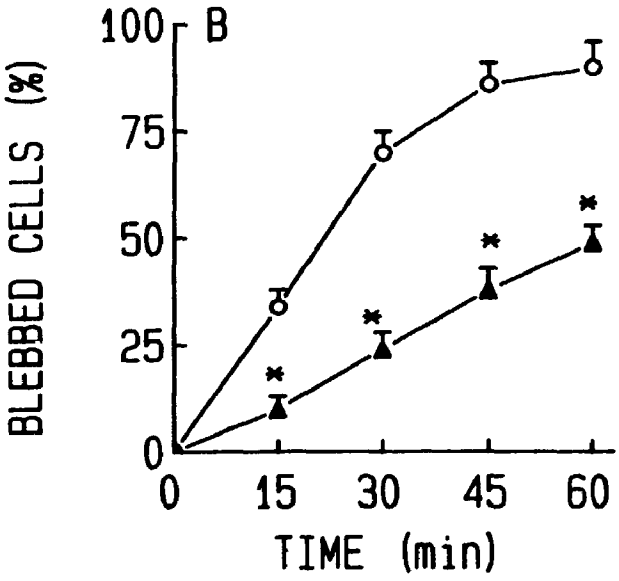

B
100
75
50
25
0
BLEBBED CELLS (%)
0
15
30
45
60
TIME (min)

Figure 4.18. Depletion of GSH in control and Zn-pretreated Ehrlich cells. Ehrlich cells were preincubated with 2.5 ppm $ZnSO_4$ for 30 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2. Valucs represent mean $\pm$ SEM of 3 experiments.

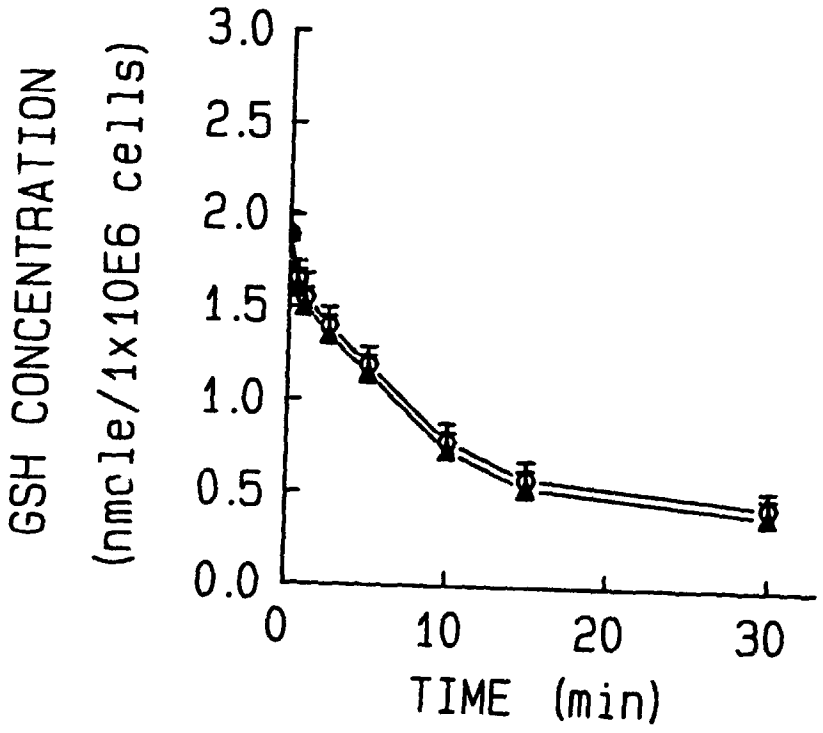

GSH CONCENTRATION
(nmole/1x10E6 cells)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
TIME (min)

Figure 4.19. Effect of mannitol (MAN) on H_2O_2-induced changes in cell viability (A) and lipid peroxidation (B) in Ehrlich cells isolated from control and Zn-pretreated (10 mg Zn/kg) host mice. Ehrlich cells were preincubated with 30 mM mannitol for 60 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2. Values represent mean ± SEM of 3 experiments.

* significantly different from corresponding non-mannitol treated groups, $p<0.05$.

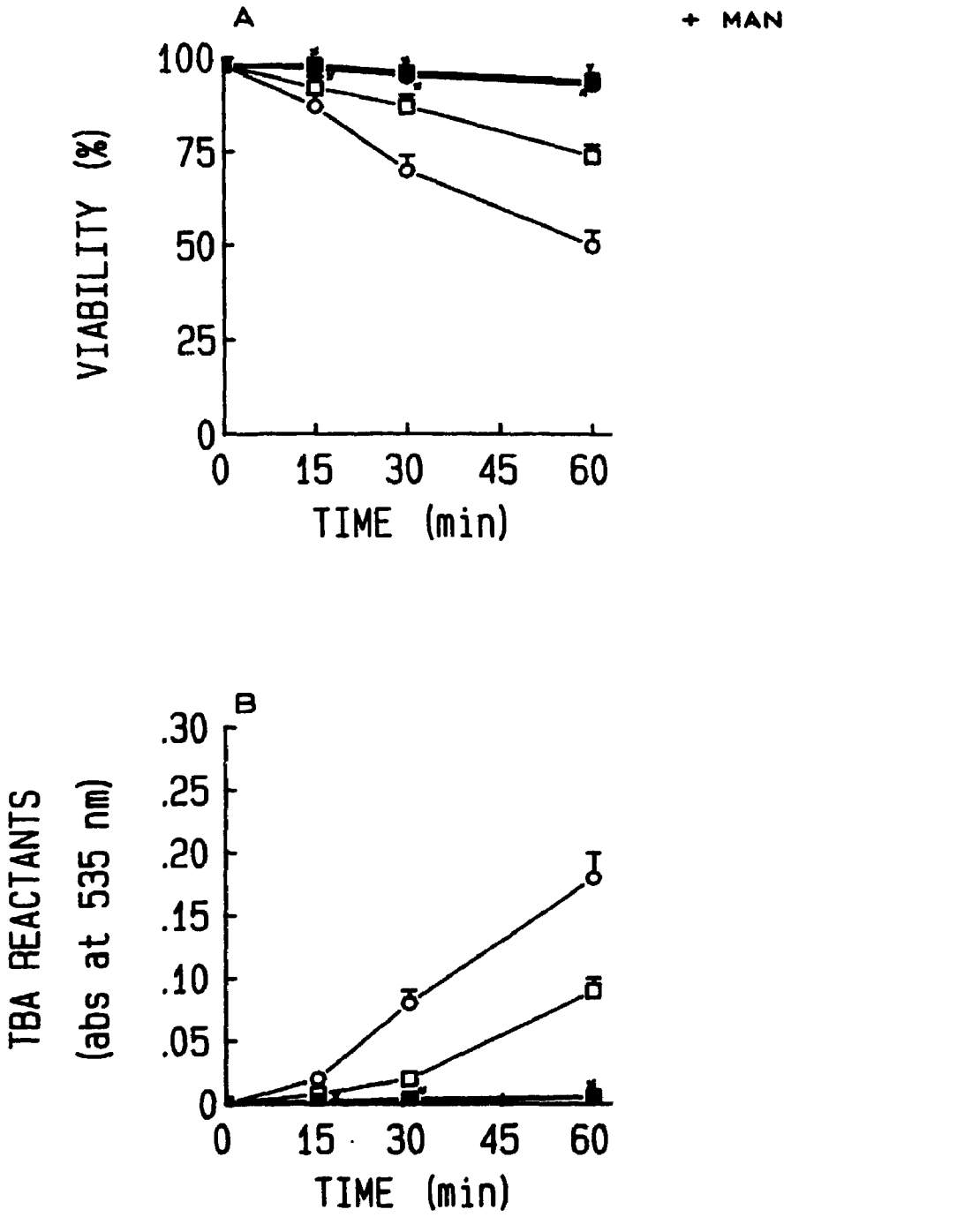
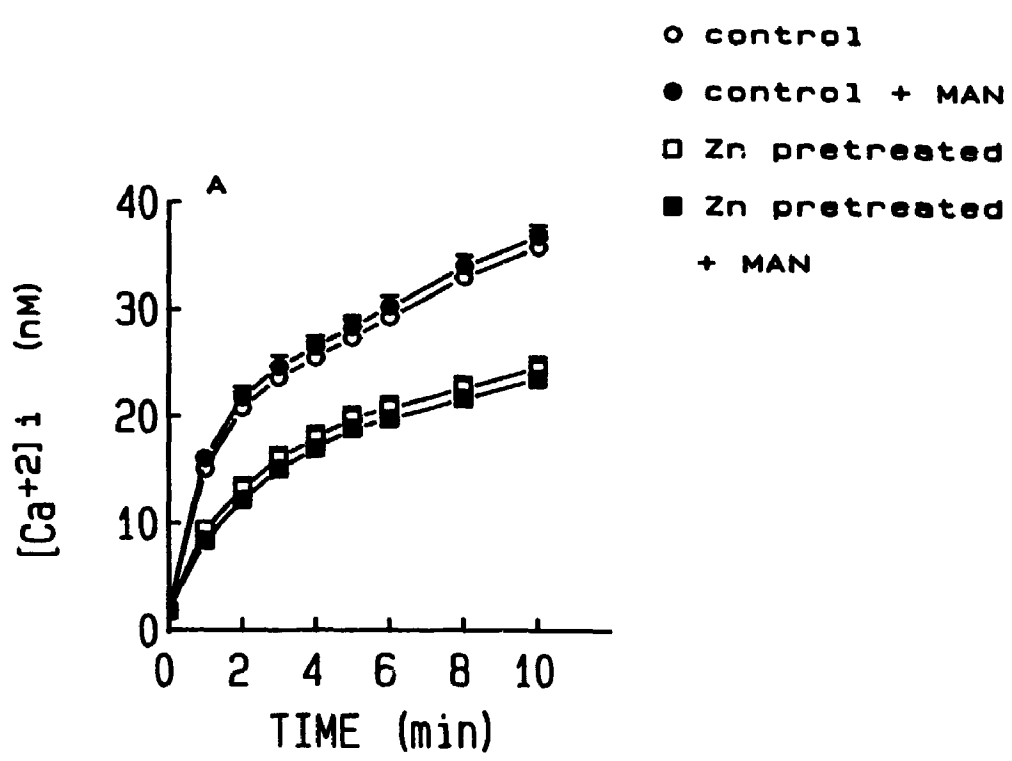

o control
● control + MAN
□ Zn pretreated
■ Zn pretreated + MAN
A
VIABILITY (%)
100
75
50
25
0
0
15
30
45
60
TIME (min)
B
TBA REACTANTS
(abs at 535 nm)
.30
.25
.20
.15
.10
.05
0
0
15
30
45
60
TIME (min)

Figure 4.20. Effect of mannitol (MAN) on H_2O_2-induced changes in $[Ca^{+2}]i$ (A) and GSH (B) concentrations in Ehrlich cells isolated from control and Zn-pretreated (10 mg Zn/kg) host mice. Ehrlich cells were preincubated with 30 mM mannitol for 60 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2 (60 μM). Values represent mean $\pm$ SEM of 3 experiments.


o control
● control + MAN
□ Zn pretreated
■ Zn pretreated + MAN
A
[Ca+2]i (nM)
40
30
20
10
0
0 2 4 6 8 10
TIME (min)

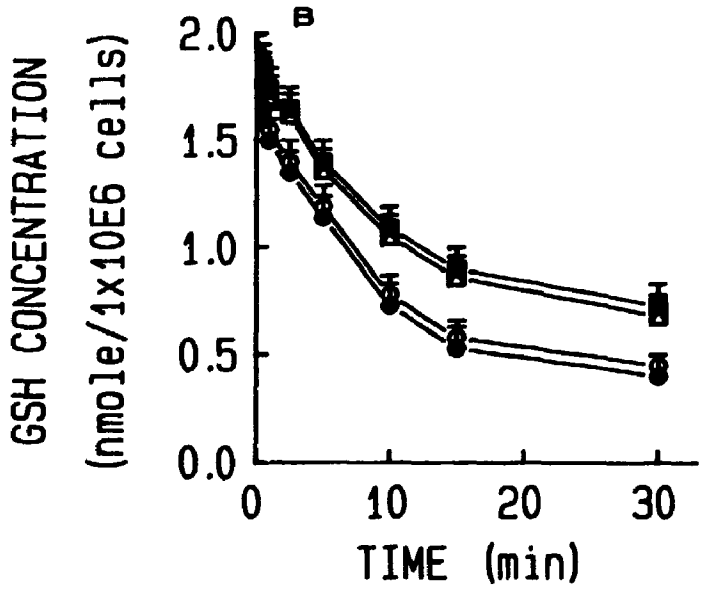

B
GSH CONCENTRATION
(nmole/1x10E6 cells)
2.0
1.5
1.0
0.5
0.0
0 10 20 30
TIME (min)

Figure 4.21. Effect of desferoxamine (DEF) on H_2O_2-induced changes in cell viability (A) and lipid peroxidation (B) in Ehrlich cells isolated from control and Zn-pretreated (10 mg Zn/kg) host mice. Ehrlich cells were preincubated with 500 μM desferoxamine for 45 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged to H_2O_2. Values represent mean $\pm$ SEM of 3 experiments.

* significantly different from non-desferoxamine treated groups, $p<0.05$.

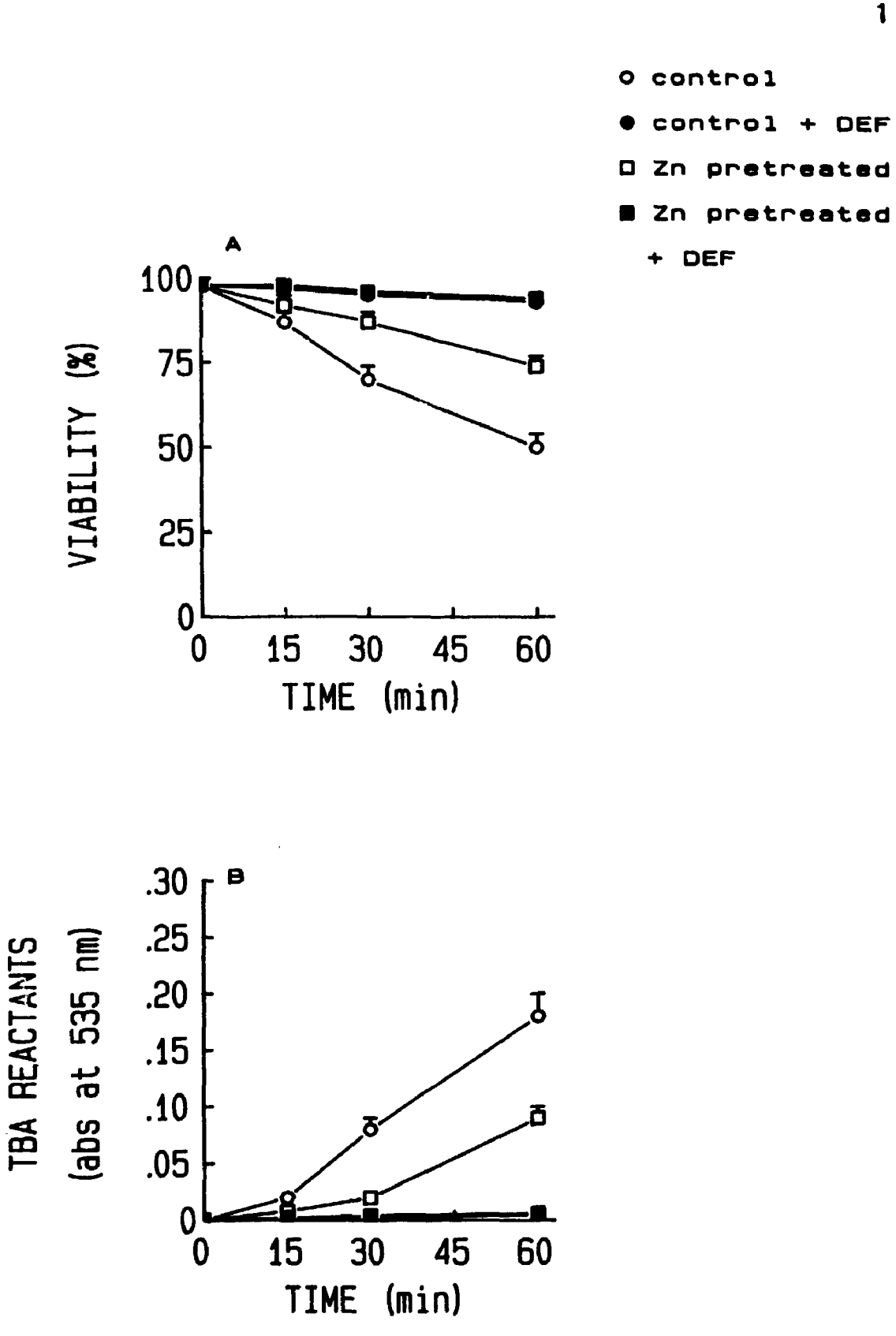

control
control + DEF
Zn pretreated
Zn pretreated + DEF
A
VIABILITY (%)
100
75
50
25
0
0
15
30
45
60
TIME (min)
B
TBA REACTANTS
(abs at 535 nm)
.30
.25
.20
.15
.10
.05
0
0
15
30
45
60
TIME (min)

Figure 4.22. Effect of desferoxamine (DEF) on H_2O_2-induced changes in $[Ca^{+2}]i$ (A) and GSH (B) concentrations in Ehrlich cells isolated from control and Zn-pretreated (10 mg Zn/kg) host mice. Ehrlich cells were preincubated with 500 μM desferoxamine for 45 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cell were the challenged with H_2O_2 (60 μM).

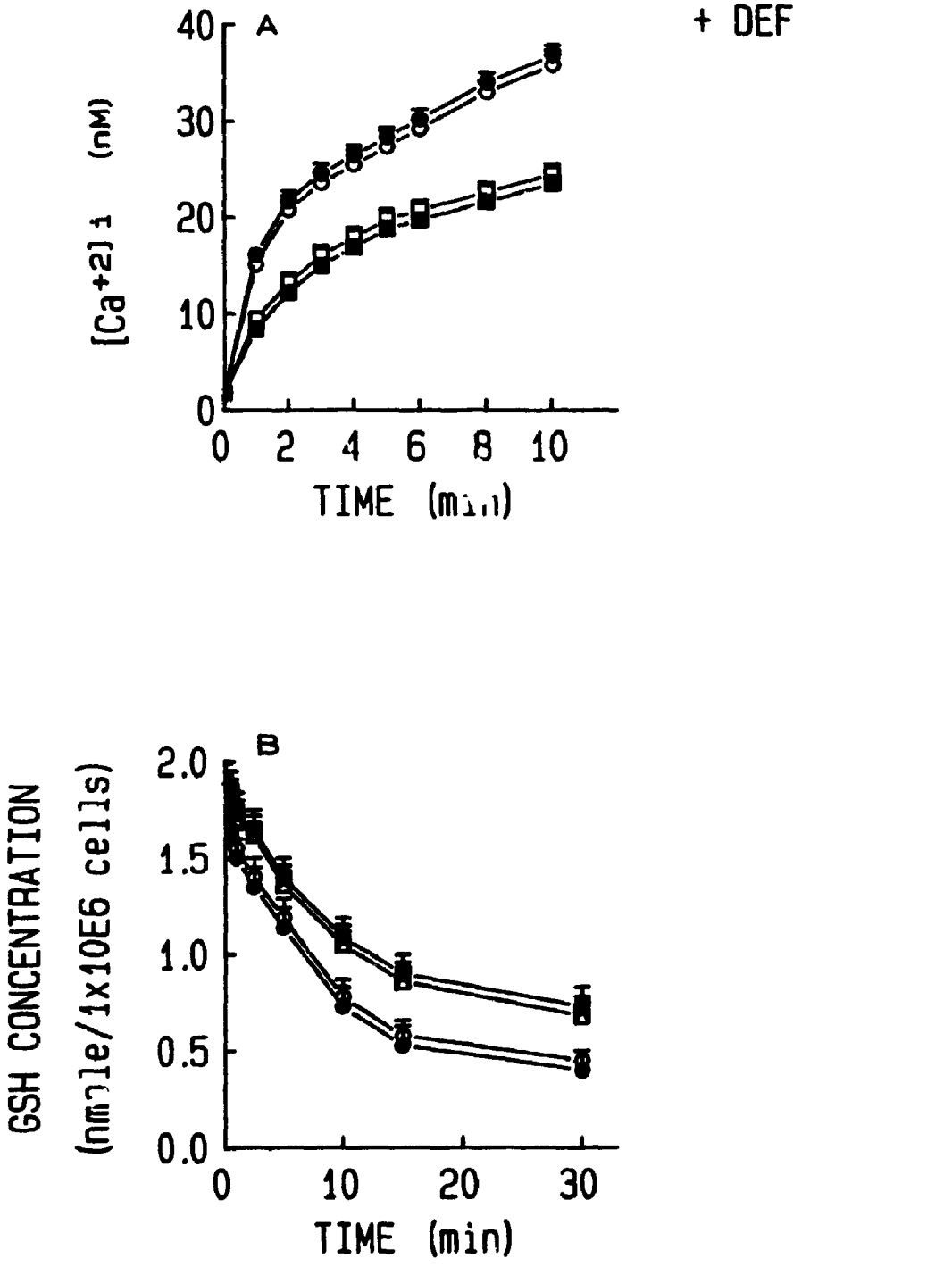
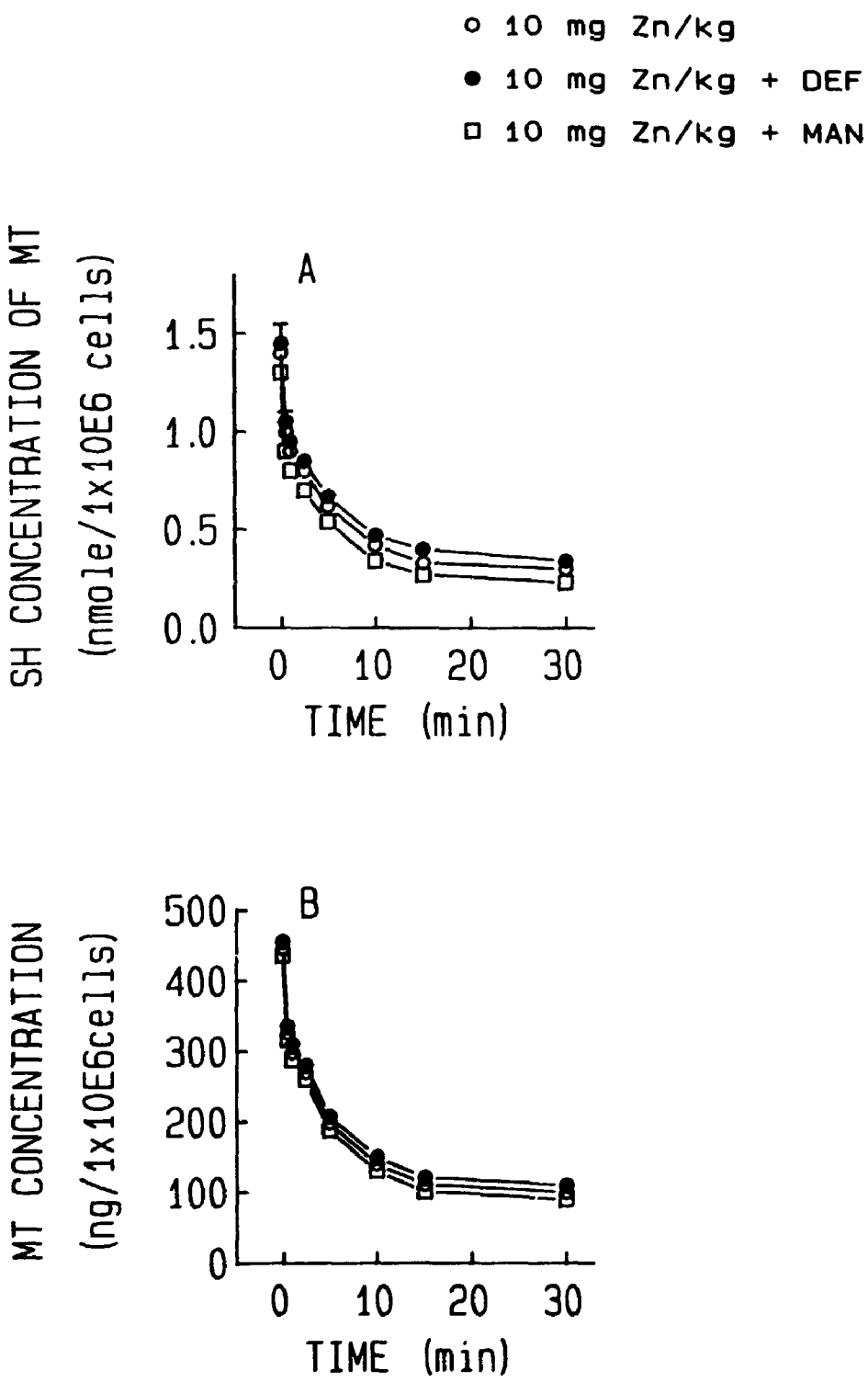

control
control + DEF
Zn-pretreated
Zn pretreated + DEF
A
[Ca+2]i (nM)
40
30
20
10
0
0 2 4 6 8 10
TIME (min)
B
GSH CONCENTRATION (nmole/1x10E6 cells)
2.0
1.5
1.0
0.5
0.0
0 10 20 30
TIME (min)

Figure 4.23. Effect of desferoxamine (DEF) or mannitol (MAN) on H_2O_2-induced changes in thiol (A) and metal binding capacity (B) of MT in Zn-pretreated Ehrlich cells. Ehrlich cells were preincubated with 500μM desferoxamine or 30 mM mannitol for 45 or 60 min, respectively, at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with 60μM H_2O_2.


10 mg Zn/kg
10 mg Zn/kg + DEF
10 mg Zn/kg + MAN
A
SH CONCENTRATION OF MT
(nmole/1x10E6 cells)
1.5
1.0
0.5
0.0
0
10
20
30
TIME (min)
B
MT CONCENTRATION
(ng/1x10E6cells)
500
400
300
200
100
0
0
10
20
30
TIME (min)

the protection against $[Ca^{+2}]i$ and cell blebbing.

To examine the protective mechanism(s) of Zn/Zn-MT in H_2O_2 cytotoxicity, the toxicity of H_2O_2 in control and Zn-pretreated cells was measured in the presence of either mannitol, a hydroxyl radical scavenger, or desferoxamine, a specific Fe (III) iron ion chelator. Desferoxamine (500 μM) or mannitol (30 mM) completely obliterated the H_2O_2-induced cell death and lipid peroxidation (fig. 4.19; fig. 4.21) in control and Zn-pretreated cells suggesting participation of hydroxyl radical via an Fe-catalyzed Fenton reaction. Desferoxamine or mannitol did not have any significant protection against decreases in GSH concentration and increases in $[Ca^{+2}]i$ (fig. 4.20; fig. 4.22) in control or Zn-pretreated cells. It is important to note that treatment of Zn-pretreated cells with either mannitol or desferoxamine failed to protect against H_2O_2 -induced oxidation of MT thiolate groups and loss of metal binding capacity of the protein (fig. 4.23).

4.4. DISCUSSION

The results of this study showed that Zn and Zn-MT in Ehrlich cells play an important role in modulating cellular response to H_2O_2-induced oxidative stress. Thus, Ehrlich cells with elevated Zn and Zn-MT levels were more resistant to H_2O_2-induced oxidative stress while Ehrlich cells with reduced Zn and Zn-MT levels were more susceptible to this treatment. These data are in agreement with findings of other studies showing the protective effect of Zn and/or Zn-MT in erythrocyte membranes, isolated microsomes and mitochondria, activated human neutrophils, cultured hepatocytes and other mammalian cells and hepatic tissues against oxidative stress induced by chemicals

such as CCl_4, xanthine/xanthine oxidase, certain antineoplastic drugs, t-butyl hydroperoxide, and H_2O_2 (Willson, 1988; Bray and Bettger, 1990; Hidalgo et al., 1988; Satoh et al., 1987; Cousins and Coppen, 1987; Ochi, 1988; Clarke and Lui, 1986).

Procedures used in the present study to manipulate the zinc status of Ehrlich cells provided a useful model to examine the functional role of Zn-MT in oxidative stress. With regards to the metal composition, MT of control, Zn-pretreated and Zn-deficient cells contained only Zn (fig. 4.5) with no detectable levels of Cu. In addition, the more than 10-fold difference in Zn-MT concentration existed between Zn-pretreated and Zn-deficient groups allowed testing of the influence of Zn-MT in H_2O_2-induced oxidative stress possible (Table 4.3; fig. 4.5). Moreover, important determinants of oxidative stress such as Fe, Cu, GSH and α-tocopherol concentrations as well as catalase activity were not affected by manipulation of cellular Zn status (table 4.2; fig. 4.3); this would allow one to discount the involvement of these parameters in explaining changes in H_2O_2-induced oxidative stress. These findings are consistent with those of other studies which showed a lack of responsiveness of α-tocopherol, GSH and Fe levels as well as catalase activity in liver and lung tissues of rats following Zn administration (Taylor et al., 1988; Bray and Bettger, 1990).

Changes of cellular Zn and Zn-MT levels in Ehrlich cells appear to affect the cellular response to H_2O_2 quantitatively rather than altering the qualitative characteristics of the response. Thus, the relative time-profiles of H_2O_2-induced changes in $[Ca^{+2}]i$, GSH oxidation, lipid peroxidation and cell death among control, Zn-retreated and Zn-deficient cells were similar. The protective effect of Zn-MT against H_2O_2 toxicity appears to be

due, at least in part, to the antioxidant properties of cysteinyl thiolates of MT. Oxidation of MT thiolate groups (fig. 4.14) provided evidence to support the involvement of MT in oxidative stress. The lack of effect of mannitol or desferoxamine (fig. 4.23) in protecting against H_2O_2-induced loss of thiolate concentration and metal binding capacity of the protein suggest the involvement of H_2O_2 as the major oxidant of MT. Evidence for interaction between MT and H_2O_2 resulting in decreases in the effective cellular concentration of H_2O_2, are reflected in the decreases in the oxidation of GSH, a substrate in the GSH-Px-mediated destruction of H_2O_2 (Pascoe and Reed, 1988). It was demonstrated in the present study that in Ehrlich cells with similar concentrations of MT and GSH (on the basis of SH content), the extent of H_2O_2-induced oxidation of these two sulphydryl molecules was of the same order of magnitude. This observation was consistent with that of Ochi (1988) who showed that elevation of Zn-MT in V99 cells was as efficient as GSH in protecting against tert-butyl hydroperoxide toxicity. The involvement of H_2O_2 as an oxidant of MT thiolate groups has also been demonstrated by Hartman et al., (1984) who showed that oxidation of copper-metallothionein by xanthine/xanthine oxidase is totally inhibited by catalase but unaffected by superoxide dismutase. Oxidation of Zn-MT thiolate groups has also been shown to occur during radiolysis (Thornalley and Vasak, 1985) or other forms of oxidative stress (Patierno et al., 1983; Thomas et al., 1986).

Results from in vitro studies have suggested that Zn ions released from MT following exposure to oxidants contribute significantly to the antioxidant function of MT (Thomas et al., 1986; Cousins and Coppen, 1987). The present study showed an apparent

relationship between H_2O_2-induced oxidation of MT thiolate groups, loss of metal binding capacity and changes in the subcellular and cytosolic distribution of Zn which is consistent with the H_2O_2-induced mobilization of MT-bound zinc (fig. 4.15). The mechanism(s) underlying the protective effect of Zn is not known, however, there is some in vitro evidence which suggests the transfer of Zn ions to membrane during the oxidation of MT thiolate groups may play a role in the protective function of MT against the damaging effects of lipid peroxidation (Thomas et al., 1986). We have also shown that elevation in non-MT-bound Zn (particulate and the high molecular weight cytosolic fractions) in control cells attained by preincubating with $ZnSO_4$ in vitro could also confer protection against H_2O_2 toxicity, although the effect was smaller (with respect to lipid peroxidation, GSH oxidation and cell death) than that observed in Zn-pretreated cells (ie. with MT induction). These data suggest the functioning of Zn ions as antioxidants which is consistent with report of other investigators (Bray and Bettger, 1990; Thomas et al., 1986; Willson, 1988). The protective mechanism of Zn-MT varies according to the parameters as discussed in the following.

Results of the present study showed that H_2O_2-induced increases in lipid peroxidation in control cells were mediated by OH radical mediated via an Fe-catalyzed Fenton reaction (fig. 4.19; fig. 4.21). Increase in lipid peroxidation of membrane lipids by a similar mechanism has been observed in cultured hepatocytes exposed to H_2O_2 (Rubin and Farber, 1984; Farber, 1990). It is well established that hydroxyl radicals can interact with unsaturated lipids of membranes to induce the process of lipid peroxidation (Halliwell and Gutteridge, 1984 a,b; Halliwell, 1987). It appears that the decreases in

lipid peroxidation in Zn-pretreated cells may be due to mechanism(s) involving the antioxidant properties of MT thiolate groups and perhaps Zn ions released from oxidized MT. As discussed in the above, MT can interact with H_2O_2 thus reducing the effective cellular concentration of H_2O_2. A decrease in cellular H_2O_2 would result in decreases in the generation of OH radical via the Fenton reaction. With respect to the antioxidant properties of Zn , a reasonable explanation may be that Zn ions mobilized to membranes, presumably following the oxidation of MT thiolate groups, can interfere with iron redoxing, thereby diminishing the conversion of H_2O_2 to OH radical (Bray and Bettger, 1990; Searle and Tomasi, 1982). Such an interference could take the form of competition for common binding sites or non-specific structural changes which alter iron binding. Results from in vitro study examining the role of Zn-MT in oxidative stress showed that the primary determinant of MT protection appears to be the Zn ions released subsequent to the oxidation of MT thiolate groups (Thomas et al., 1986).

It has been previously reported that oxidative stress elicited by menandione, tert-butyl hydroperoxide and H_2O_2 in isolated hepatocytes resulted in sustained increases in $[Ca^{+2}]i$ as assessed by either the activity of phosphorylase a or by the use of fluorescence indicators (Farber, 1990; Starke and Farber, 1986; Masaki et al., 1989). A very rapid and sustained increase in $[Ca^{+2}]i$ was also observed in the present study as measured by the fluorescent probe Indo-1; this effect was mediated by H_2O_2 directly and did not involve hydroxyl radicals since mannitol or desferoxamine failed to protect against H_2O_2-induced increases in $[Ca^{+2}]i$. This observation is consistent with those observed by Starke et al., (1986) who showed that calcium mobilization in hepatocytes is not the

consequence of the generation of toxic intermediates from H_2O_2. Although the mechanism(s) underlying the effect of H_2O_2 on calcium homeostasis is not known, it has been suggested that oxidation of protein SH groups, in particular those of Ca^{+2}-ATPase (Nicotera et al., 1985), which are important in the regulation of calcium homeostasis, may be responsible for the increases in $[Ca^{+2}]i$. The temporal relationship between oxidation of GSH and increases in $[Ca^{+2}]i$ observed in control and Zn-pretreated cells is consistent with this speculation, since oxidation of GSH is a good indicator of the oxidation of protein sulphydryl groups (McConkey and Orrenius, 1988; Orrenius, 1985; Bellomo and Orrenius, 1985). The protective effect against H_2O_2-induced increases in $[Ca^{+2}]i$ by Zn-pretreatment was not studied in detail; however, the Zn released from MT to the protective function of Zn-MT against H_2O_2-induced increases in $[Ca^{+2}]i$ is limited because preincubation of control cells with Zn (ie. no induction of MT) was more effective than Zn-pretreatment in protecting against H_2O_2 -induced increases in $[Ca^{+2}]i$. The mechanism underlying the protective effect of Zn is not known, but it may be related to its ability to mask specific membrane sulphydryl groups (Bray and Bettger, 1990) (ie Ca^{+2}-ATPase) which are known to be important in calcium homeostasis (Nicotera et al., 1985; Pascoe and Reed, 1988; Orrenius et al., 1988).

There are several mechanisms of oxidant-induced irreversible cell injury and disturbances in Ca^{+2} homeostasis has been proposed to be one of the most important one (Farber, 1990; Orrenius, 1988; Boobis et al., 1989). Under our experimental conditions, it appears that cell death observed in normal Ehrlich cells was mediated by hydroxyl radical-dependent peroxidation of membrane lipids and not by alterations in calcium

homeostasis, because procedures such as mannitol and desferoxamine treatment that blocked lipid peroxidation and cell death failed to modify the disturbances in Ca^{+2} homeostasis. This observation is consistent with the finding that cell death of hepatocytes exposed to H_2O_2 (Rubin and Farber, 1984) or tert-butyl hydroperoxide (Masaki et al., 1989) has been shown to occur by a mechanism involving lipid peroxidation and not by disturbances in calcium homeostasis. Since similar findings were observed in Zn-pretreated cells, it may be suggested that Zn/Zn-MT protected against H_2O_2-induced cell death by preventing lipid peroxidation.

The results of the present study showed that high Zn-MT levels can make Ehrlich cells more resistant to H_2O_2-induced oxidative injury. H_2O_2 treatment resulted in oxidation of MT thiolate groups and loss of its metal binding capacity with translocation of Zn released from oxidized MT to other cellular sites. Preincubation of Ehrlich cells with $ZnSO_4$ in vitro also conferred some degree of resistance to H_2O_2 toxicity, suggesting the inherent antioxidative property of Zn ions. These data suggested that Zn-MT can be considered as an antioxidant by virtue of its thiolate groups and its Zn ions that are released in the presence of oxidative stress.

CHAPTER FIVE

5.1 INTRODUCTION

Results presented in Chapter 4 showed that Ehrlich cells with high Zn-MT concentrations were resistant to H_2O_2-induced toxicity. The protective effect conferred by Zn-MT was due to the antioxidant properties of both cysteinyl thiolate groups of MT and Zn ions released concomitant to the oxidation of thiolate groups of the metalloprotein. The present study was carried out to investigate the role of Cu-MT in oxidative stress. Very little is known about the influence of Cu-MT in oxidative stress, however, Cu ions are known to have prooxidant properties. Copper, being a transition redox metal, it functions like iron in many respects. In fact, Cu(I) is a better catalyst than Fe(II) in the Fenton reaction: Fe(II) + H_2O_2 ---> OH + OH + Fe(III). It has been stated that if both Cu(I) and H_2O_2 are available in vivo, OH will form in biologically-significant amounts. In addition, Cu has been shown to exert its toxic effects by oxidizing cellular sulphydryl groups or by stimulating the chain reaction of lipid peroxidation resulting in cell damage (Aust, 1985; Halliwell and Gutterridge, 1984b; Hochstein et al., 1980). Therefore, if our hypothesis stated in the Introduction of Chapter 4 is correct, then Cu-MT would have prooxidant properties leading to enhancement in sensitivity of biological systems to oxidative stress.

5.2 MATERIALS AND METHODS

5.2.1 Animals.

Male Swiss albino CD-1 mice (approximately 30 g body weight) obtained from Charles River Breeding Laboratories Inc., Quebec were housed in groups of six to eight animals in polyethylene cages with free access to pelleted Purina laboratory chow and deionized water. The animals were kept at room temperature (22-24°C) and were exposed to alternate cycles of 12 h light and darkness.

5.2.2 Preparation of cells.

Ehrlich ascites tumour cells were grown in the peritoneal cavity of animals and transferred weekly to new hosts as described in section 4.2.2.

5.2.3 Tissue preparation.

Preparation of livers and Ehrlich cells for subcellular fractionation were carried out as described in section 4.2.3.

5.2.4 Treatment protocols to manipulate Cu and/or Cu-MT concentrations in host mice and Ehrlich cells.

It has been shown that normal Ehrlich cells contain very low levels of Cu with extremely very low binding of Cu to MT fraction (Kraker et al., 1985). To increase cellular Cu-MT concentration in Ehrlich cells control host mice were injected with a single dose of $CuSO_4$ (1 or 2 mg Cu/kg, ip) dissolved in 0.9% saline to hosts 5 days following inoculation of Ehrlich cells; Ehrlich cells were harvested 24 h following Cu administration as described previously; these cells were refered to as Cu-pretreated.

To increase the cellular non-MT-bound Cu concentration, Ehrlich cells isolated from control host mice were incubated with 0.6 ppm of $CuSO_4$ for 30 min at 37°C under air atmosphere and thereafter washed twice with PBS buffer to remove extracellular Cu ions. This procedure yielded Ehrlich cells with total Cu concentrations that were comparable to those of Cu-pretreated cells (1 mg Cu/kg); these cells were designated as Cu-preincubated cells.

In this study, Cu and Zn metabolism was also examined in hepatic tissues of host mice in order to ascertain the effectiveness of treatment protocols.

5.2.5 Experimental design.

To examine the influence of Cu-MT on cellular susceptibility to H_2O_2-induced toxicity, dose toxicity relationship was established in control and Cu-pretreated Ehrlich cells. For this, control cells and cells pretreated with 1 or 2 mg Cu/kg were inubated with different concentrations of H_2O_2 (7.5, 15, 30, 60, and 120 μM) at 37°C; cell viability was measured 45 min after addition of H_2O_2 and LC_{50} values were estimated. To characterize the H_2O_2-induced oxidative stress in control and Cu-pretreated cells, additional toxicity parameters ($[Ca^{+2}]i$, GSH, lipid peroxidation and cell blebbing) were monitored at different time intervals following exposure of cell preparations to 60 μM H_2O_2. Since Cu pretreatment of host mice is known to increase the binding of Cu to cellular components in addition to MT, toxicity studies were also carried out with Cu-preincubated cells to examine the influence of non-MT-bound Cu on H_2O_2-induced oxidative stress.

To investigate the involvement of Cu-MT in cellular response of oxidative stress, the effect of oxidant stress on Cu-MT was investigated. For this, the effects of H_2O_2 on the metal binding capacity, thiol content and metal content of MT of Cu-pretreated Ehrlich cells was examined. To examine whether or not thiolate groups of MT were prone to oxidation by H_2O_2, cytosols isolated from Ehrlich cells with and without prior H_2O_2 treatment were treated with the sulphydryl reductant 1,4-dithiothreitol (DTT) to examine possible regeneration of metal binding capacity of MT as measured by the Ag-heme saturation method.

To study the mechanism underlying the influence of Cu-MT on H_2O_2 toxicity. the following experiments were carried out with intact Ehrlich cells. To ascertain the role of Cu-MT in H_2O_2-induced oxidative stress, the effect of pretreatment with D-penicillamine, a Cu chelating agent, on H_2O_2-induced toxicity was examined in control and Cu-pretreated cells. To examine the mode of action of D-penicillamine, aliquots of cell suspensions were removed at 0, 5, 10 and 15 min following addition of H_2O_2 and the cytosolic distribution of Cu was analyzed by Sephadex G-75 chromatography to examine the possible chelation of Cu by D-penicilamine. In another study, the toxicity of H_2O_2 in control and Cu-pretreated cells was measured in the presence of either mannitol, a hydroxyl radical scavenger, or desferoxamine, a specific Fe(III) iron ion chelator to examine the role of Fe(II)-catalyzed Fenton reaction in the cytotoxicity of H_2O_2.

To examine the validity of Ehrlich cells as a model for studying the role of Cu-MT in oxidative stress, cellular parameters that are known to influence cellular response to

oxidative stress such as SOD and catalase activities as well as Cu, GSH, α-tocopherol and Fe concentrations were also measured in Ehrlich cells from all treatment groups.

5.2.6 Cytotoxicity study of H_2O_2 in Ehrlich cells.

Freshly isolated Ehrlich cells were used throughout this study. All incubations were performed in 10-ml Erlenmeyer flasks containing 1x10E7 cells/ml PBS buffer, pH 7.4, at 37°C under air atmosphere in a shaking water bath oscillating 80 cycles/min as described in section 4.2.5.

Pre-incubation of Ehrlich cells with 100 μM D-penicillamine, 30 mM mannitol and 500 μM desferoxamine were carried out at 37°C under air atmosphere for 30, 60 and 45 min, respectively. Cell suspensions were washed twice with ice-cold buffer and resuspended to original volume with the same buffer prior to challenge with H_2O_2.

5.2.7 Biochemical analyses and procedures.

5.2.7.1 Measurement of cell viability.

Cytotoxicity was measured by loss of cell viability as determined in section 4.2.7.1.

5.2.7.2 Measurement of cell blebbing.

The appearance of cell blebbing was observed under light microscopy as described in section 4.2.7.2.

5.2.7.3 Measurement of lipid peroxidation

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactants as described in section 4.2.7.3.

5.2.7.4 Measurement of $[Ca^{+2}]i$.

$[Ca^{+2}]i$ was measured fluorometrically using the Ca^{+2}-sensitive probe Indo-1 as described in section 4.2.7.4.

5.2.7.5 Measurement of superoxide dismutase activity.

Copper/zinc superoxide dismutase (SOD) in cytosols of Ehrlich cells was measured using the technique based on inhibition of pyrogallol (1,2,3-benzenetriol) autooxidation as described in section 4.2.7.5.

5.2.7.6 Measurement of catalase activity.

Catalase activity in sonicated homogenates was determined spectrophotometrically by following the disappearance of hydrogen peroxide at 240 nm as described in section 4.2.7.6.

5.2.7.7 Protein determination.

Protein determinations were estimated by the methods of Lowry et al. (1951), using crystalline bovine serum albumin (BSA) as the standard as described in section 3.2.4.5.

5.2.7.8 Measurement of reduced and total glutathione concentrations.

Reduced and total glutathione, more precisely non-protein sulphydryl, in Ehrlich cell cytosols was determined as described in section 4.2.7.8.

5.2.7.9 α-Tocopherol determination.

α-Tocopherol concentration in acetone extracts was determined as described in section 4.2.7.9.

5.2.7.10 Metallothionein determination.

Metallothionein concentrations from cytosols were estimated indirectly by measuring the metal binding capacity of the protein by the Ag-heme saturation method as described in section 4.2.7.10.

5.2.7.11 Measurement of thiol content of MT.

The thiol content of MT in heat-treated supernatants isolated from cytosols of incubation mixtures was determined as described in section 4.2.7.11.

5.2.7.12 Sephadex G-75 gel filtration study.

To study the chromatoghraphic separation profile of cytosolic Cu and Zn, an aliquot (2ml) of cytosol was chromatographed on a calibrated Sephadex G-75 column (1.5 cmx 55cm) as described in section 4.2.7.12.

5.2.7.13 Regeneration of metal binding sites of MT by 1,4 dithiothreitol (DDT).

To determine whether H_2O_2-induced loss of metal binding capacity of MT was due to oxidation of MT thiolates, heat-treated supernatant fractions of control and H_2O_2-treated samples were treated with the reducing agent DTT as described in section 4.2.7.13.

5.2.7.14 Metal analysis.

Estimation of total, cytosolic and particulate Cu, Zn and Fe concentrations of Ehrlich cells was performed following wet tissue digestion as described in section 4.2.7.14.

5.2.7.15 MT-I mRNA measurement.

Measurement of MT-I mRNA accumulation was performed as described in section 4.2.7.15.

5.2.11 Statistical analysis.

Statistical evaluation of data was performed by paired Students t test and two way analysis of variance (Gad and Weil, 1982). The level of significance was chosen at $p<0.05$.

5.3 RESULTS

i) Manipulation of Cu/Cu-MT status.

Administration of Cu (1 or 2 mg Cu/kg, ip) to host mice resulted in time-dependent

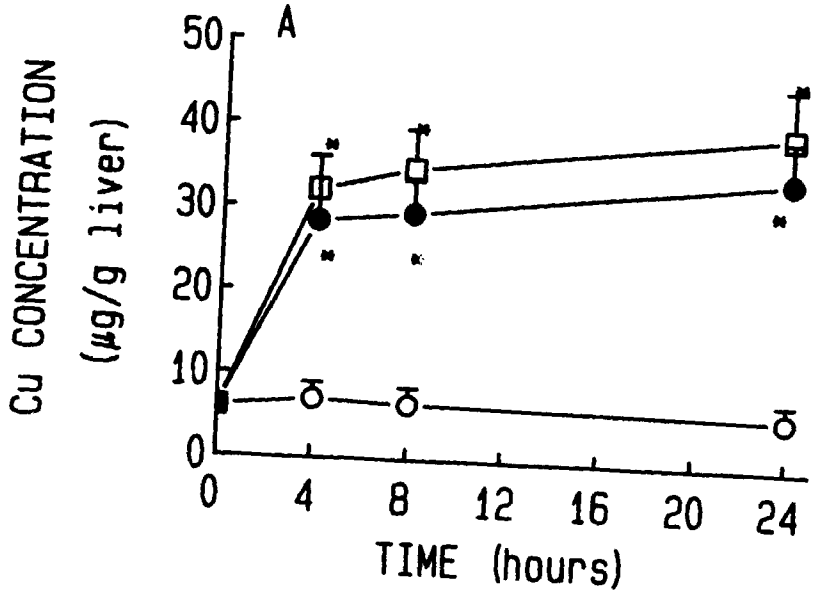
increases in total hepatic Cu and MT concentrations (fig. 5.1). Elevations in both Cu and MT were measured as early as 4 h after treatment and reached maximal levels 20 h later (fig. 5.1). Hepatic Cu was evenly distributed between the cytosolic and particulate fractions (fig. 5.2) and the cytosolic Cu was associated with both the HMW and MT fraction (Table 5.1). MT-bound Zn was significantly increased (approximately by 26%) following Cu admninistration (Table 5.1).

Pretreatment of host mice with $CuSO_4$ (1 or 2 mg Cu/kg) resulted in time- and dose-dependent increases in total cellular Cu and MT concentrations of Ehrlich cells which could be detected as early as 4 h after treatment and maximal levels were measured at 24 h (fig. 5.3). Increases in total Cu concentrations of Ehrlich cells at 24 h following treatment were mostly due to the elevation in cytosolic Cu which showed approximately 5-fold and 10-fold increases in the 1 and 2 mg Cu/kg groups, respectively (fig. 5.4). Furthermore, examination of the Sephadex G-75 elution profile of cytosols isolated from these cells showed that increases in MT-bound Cu was mostly responsible for the dose-dependent elevation in cytosolic Cu concentrations (fig. 5.5).

To examine whether or not the increases in MT concentrations in tissues or cells observed following administration of Cu were due to the induction of MT synthesis by a process involving increased transcription of MT gene, the accumulation of MT-mRNA levels in tissues was measured. Cu-pretreated Ehrlich cells showed accumulation of MT-mRNA by 4 h with maximal levels being attained at 8 h but declined to control levels 24 h following treatment (fig. 5.3). The levels of hepatic MT-mRNA were higher in host animals 6 days after innoculation of Ehrlich cells when compared to those of

Figure 5.1. Time-course of the effect of 1 mg Cu/kg- (●) or 2 mg Cu/kg-(□) pretreatment on cellular Cu (A) and MT (B) concentrations in hepatic tissues of host mice. Control host mice were injected with a single dose of $CuSO_4$ (1 or 2 mg/kg, ip) dissolved in 0.9% saline 5 days following inoculation of Ehrlich cells; host mice were killed 4, 8, or 24 h later. Values represent mean ± SEM (n = 6 animals).

* significantly different from corresponding control values, $p<0.05$.


A
Cu CONCENTRATION
(µg/g liver)
50
40
30
20
10
0
0
4
8
12
16
20
24
TIME (hours)

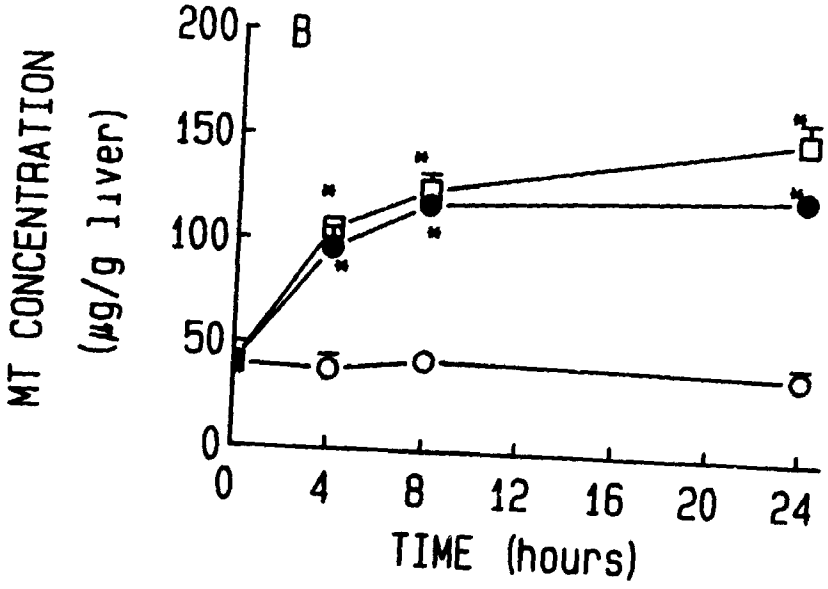

B
MT CONCENTRATION
(µg/g liver)
200
150
100
50
0
0
4
8
12
16
20
24
TIME (hours)

Figure 5.2. Hepatic total and cytosolic Cu (A) and Zn (B) concentrations as well as MT concentrations (C) in host mice. Normal mice (N) were treated with Ehrlich cells (C) and 5 days later were injected with a single dose of $CuSO_4$ (1 or 2 mg Cu/kg, ip) dissolved in 0.9% saline; host mice were killed 24 h after Cu-pretreatment. Values represent mean $\pm$ SEM (n = 6 animals).

a. significantly different from normal values, $p<0.05$.

b. significantly different from control values, $p<0.05$.

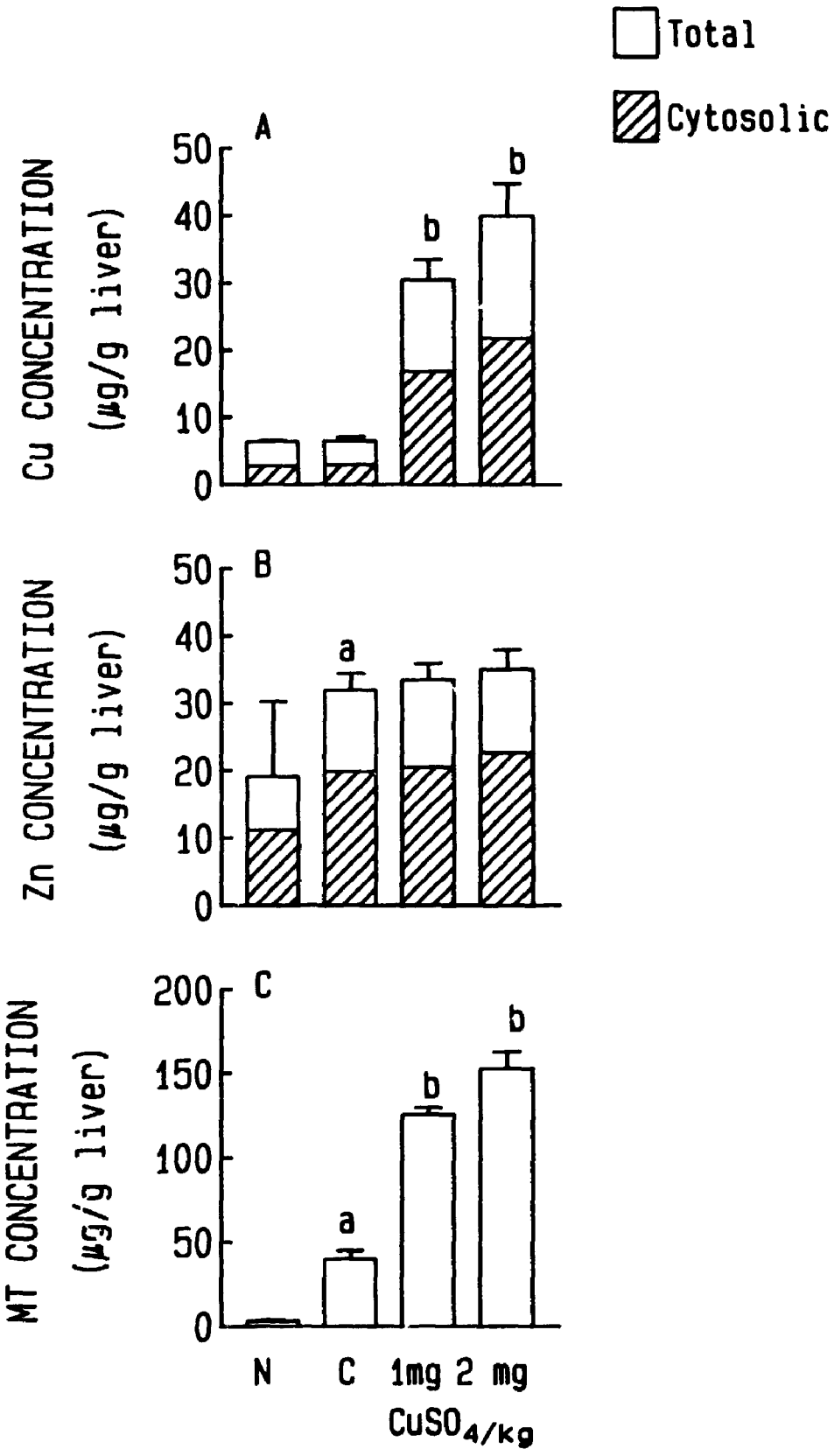

Total
Cytosolic
A
Cu CONCENTRATION
(μg/g liver)
50
40
30
20
10
0
b
b
B
Zn CONCENTRATION
(μg/g liver)
a
C
MT CONCENTRATION
(μg/g liver)
200
150
100
50
0
a
b
b
N
C
1mg
2 mg
CuSO4/kg

TABLE 5.1

Metal contents of cytosolic components of host mouse liver following Sephadex G-75 chromatographic separation[a]

Metal Concentration (μg/g liver)

	HMW[d]		MT[e]	
Treatment	Zn	Cu	Zn	Cu
Normal	10.50±1.70	2.70±0.35	ND	ND
Control host mice[b]	13.63±2.10	2.51±0.40	6.25±0.40	0.40±0.05
1 mgCu/kg[c]	13.70±1.95	9.20±1.40[f]	8.25±0.80[f]	7.40±0.90[f]
2 mgCu/kg[c]	14.80±1.45	10.20±1.45[f]	8.80±0.95[f]	12.15±1.60[f]

a. Values represent mean ± SEM from 3 animals.

b. Mice were killed 6 days post-inoculation with Ehrlich cells

c. Control host mice were injected with $CuSO_4$ 5-days post-inoculation with Ehrlich cells and killed 24 h later

d. High molecular weight component

e. Metallothionein component

f. Significantly different from corresponding control host value, $p < 0.05$.

ND. Not detected

Figure 5.3. Time-course of the effect of 1 mgCu/kg- (●) or 2 mg Cu/kg -(□) pretreatment on cellular Cu (A) and MT (B) concentrations as well as MT-I mRNA accumulation (C) in Ehrlich cells. Control host mice were injected with a single dose of $CuSO_4$ (1 or 2 mg/kg, ip) dissolved in 0.9% saline 5 days following inoculation of Ehrlich cells; host mice were killed 4, 8, or 24 h later. Values represent mean ± SEM (n = 6 animals).

a. significantly different from corresponding control values, $p<0.05$.

b. significantly different from corresponding Cu-pretreated (1 mg Cu/kg) values, $p<0.05$.

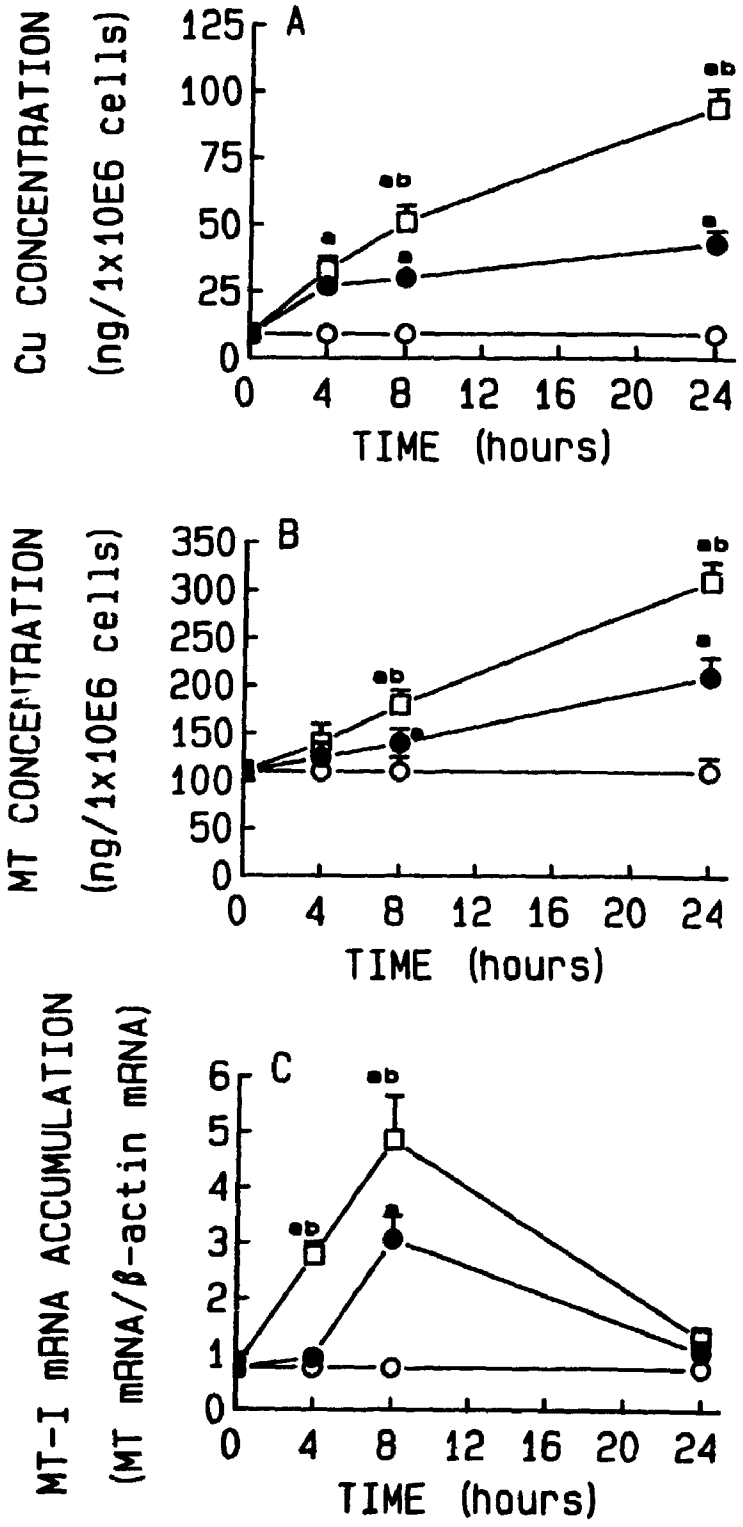

A
Cu CONCENTRATION
(ng/1x10E6 cells)
125
100
75
50
25
0
0 4 8 12 16 20 24
TIME (hours)
B
MT CONCENTRATION
(ng/1x10E6 cells)
350
300
250
200
150
100
50
0
0 4 8 12 16 20 24
TIME (hours)
C
MT-I mRNA ACCUMULATION
(MT mRNA/β-actin mRNA)
6
5
4
3
2
1
0
0 4 8 12 16 20 24
TIME (hours)

Figure 5.4. Total and cytosolic Cu (A), and Zn (B) concentrations as well as MT concentrations (C) of Ehrlich cells obtained 6 days post-inoculation from control, 1 mg Cu/kg- and 2 mg Cu /kg-pretreated host mice. Control host mice were injected with a single dose of $CuSO_4$ (1 or 2 mg /kg, ip) dissolved in 0.9% saline 5 days following inoculation of Ehrlich cells; host mice were killed 24 h later. Values represent mean ± SEM (n = 6 animals).

a. significantly different from corresponding control values, $p<0.05$.

b. significantly different from corresponding Cu-pretreated (1 mg Cu/kg) values, $p<0.05$.

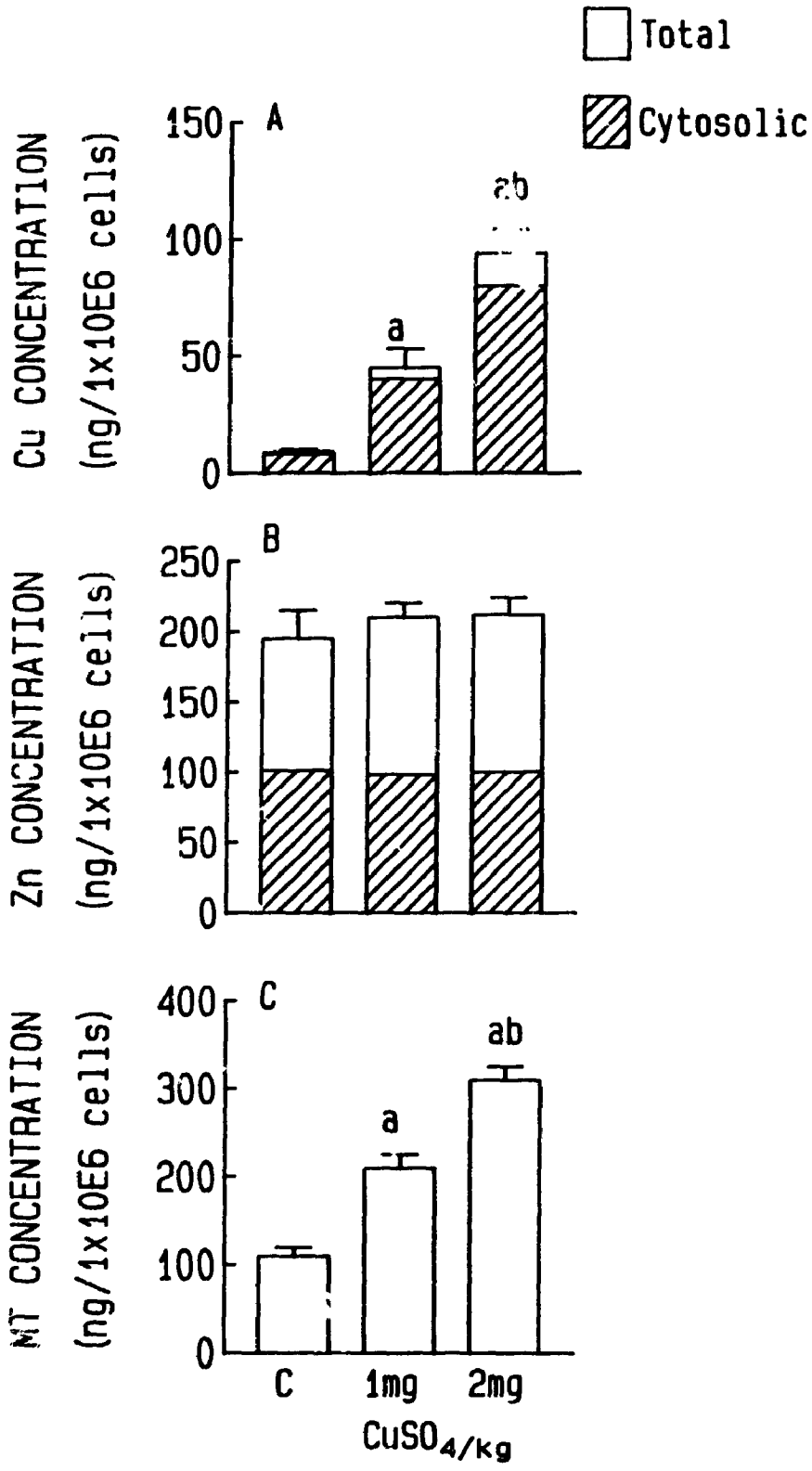

Total
Cytosolic
A
Cu CONCENTRATION (ng/1x10E6 cells)
150
100
50
0
a
ab
B
Zn CONCENTRATION (ng/1x10E6 cells)
250
200
150
100
50
0
C
MT CONCENTRATION (ng/1x10E6 cells)
400
300
200
100
0
a
ab
C
1mg
2mg
CuSO4/kg

Figure 5.5. Cytosolic distribution of Cu in Ehrlich cells isolated from control (————), 1 mg Cu/kg- (----) and 2 mg Cu/kg- (····) pretreated host mice. Cytosols were isolated from Ehrlich cell homogenates 6 days post-inoculation with Ehrlich cells and were chromatographed on a Sephadex G-75 column equilibrated with 10 mM Tris-HCl buffer, pH 8.6, at room temperature. Each cytosolic sample was analyzed chromatographically twice and the average values were used for the construction of the chromatogram.

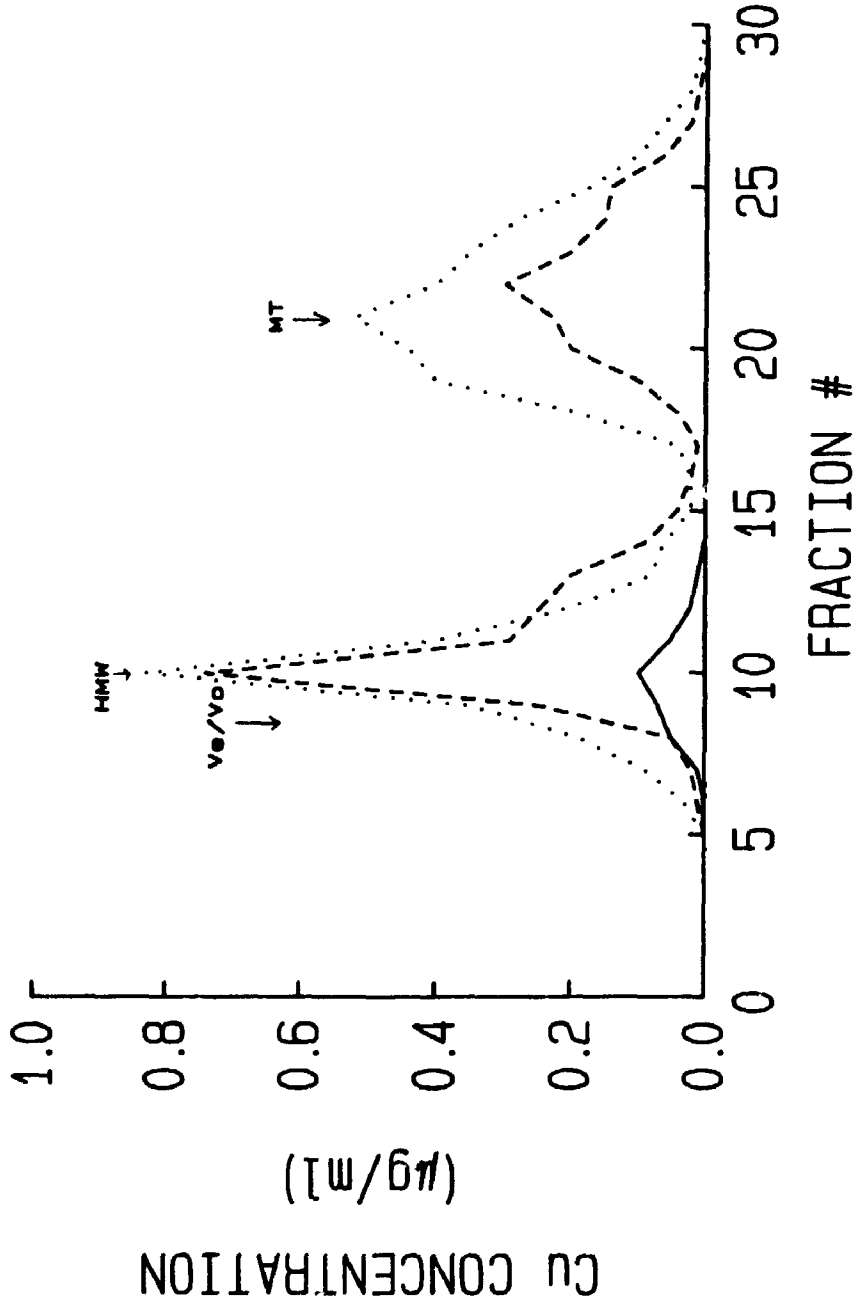

Cu CONCENTRATION
(μg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
FRACTION #
HMW
Ve/Vo
MT

Figure 5.6. MT-I mRNA accumulation in hepatic tissues following Cu pretreatment. Normal mice were treated with Ehrlich cells and 5 days later were injected with $CuSO_4$ (1 or 2 mg Cu/kg, ip) dissolved in 0.9% saline; mice were killed 4 h later. Varying amounts of total tissue RNA from liver of mice were immobilized on nylon filters and hybridized to radiolabeled MT-I and ß-actin cDNA probes. Values represent mean relative MT-I mRNA accumulation ± SEM as determined in triplicate measurement of single RNA samples.

a. significantly different from normal value, $p < 0.05$.

b. significantly different from value obtained from mice treated with Ehrlich cells, $p < 0.05$.

c. significantly different from Cu-pretreated (1 mg Cu/kg) value, $p < 0.05$.

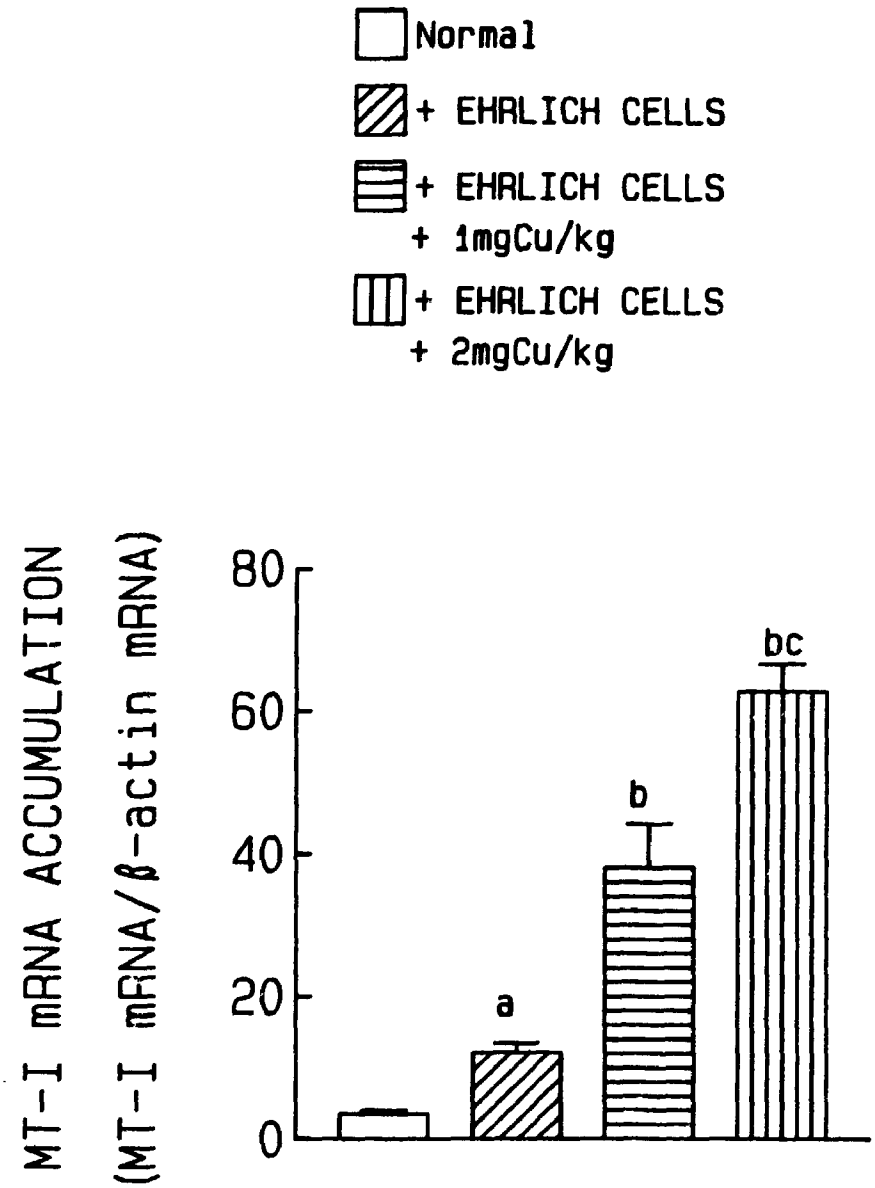

Normal
+ EHRLICH CELLS
+ EHRLICH CELLS
+ 1mgCu/kg
+ EHRLICH CELLS
+ 2mgCu/kg
MT-I mRNA ACCUMULATION
(MT-I mRNA/β-actin mRNA)
80
60
40
20
0
a
b
bc

TABLE 5.2

Effect of treatment[a] of host mice with Cu on Fe concentration and antioxidant activities of Ehrlich cells[b]

Treatment	Fe Concentration (ng/1x10E6 cells)	SOD (U/mg protein)	Catalase[c] (U/mg protein)	Total GSH (nmole/1x10E6 cells)	α-tocopherol (nmole/1x10E6 cells)
Control	110 ± 10	N.D	0.12 ± 0.01	1.96 ± 0.18	0.10 ± 0.02
1 mg Cu/kg	114 ±12	N.D	0.10 ± 0.03	1.95 ± 0.16	0.11 ± 0.01
2 mg Cu/kg	108 ± 8	N.D	0.12 ± 0.02	1.96 ± 0.10	0.10 ± 0.02

a. Control host mice were pretreated with $CuSO_4$ (1 or 2 mg Cu/kg, ip) 5 days post-inoculation with Ehrlich cells and killed 24 h later.

b. Values represent mean ± SEM (n=6-8 mice)

c. One unit of catalase activity is defined as the amount of catalase required to decompose 1μmole of H_2O_2 per minute at 25°C.

N.D Not detected

untreated animals (fig. 5.6). Moreover, marked elevations in MT-mRNA levels were detected 4 h following Cu treatment (fig. 5.6).

To further study the validity of the experimental model, some of the parameters that influence the response of Ehrlich cells to oxidative stress were also examined. As shown in table 5.2 and figure 5.4, concentrations of Fe, which is required for H_2O_2-induced irreversible cell injury (Starke and Farber, 1985), and the major cellular antioxidant systems such as GSH, Zn and α-tocopherol concentrations as well as catalase activity were not significantly affected following administration of Cu. Superoxide dismutase activity was not detected in any of the experimental groups examined (Table 5.2).

ii) Influence of Cu/Cu-MT status of Ehrlich cells on H_2O_2 toxicity.

Although MT-bound Cu was mostly responsible for the increases in total Cu concentrations, non-MT-bound Cu (cytosolic HMW and particulate fractions) was also elevated (Table 5.3). Therefore, this model alone would not be adequate to study the influence of Cu-MT on oxidative stress. To investigate whether or not non-MT-bound Cu would also affect oxidative stress-induced injury, control cells were preincubated with 0.6 ppm $CuSO_4$ in vitro for 30 min to obtain cellular Cu concentrations similar to cells pretreated with 1 mg Cu/kg, but without induction of MT synthesis. These Cu-preincubated cells contained similar cytosolic HMW-bound Cu concentrations, much higher concentrations in the particulate fraction and lower MT-bound Cu concentrations as compared to Cu-pretreated cells (Table 5.3).

The result of dose-toxicity relationship study gave estimate of LC_{50} values for the

control, 1 mgCu/kg- and 2 mg Cu/kg-pretreated cells to be 80 ± 9, 32 ± 8 and 12 ± 2 μM H_2O_2, respectively. The slopes of the log dose-toxicity lines among these three groups were not significantly different suggesting similar mechanisms of acute lethal injury (5.7). Experiments with Cu-preincubated cells revealed slight increases in sensitivity (LC_{50} 50± 5 μM H_2O_2) as compared to control cells, but the sensitivity to H_2O_2 in this group was significantly smaller than that observed in the Cu-pretreated cells (1 mg Cu/kg).

The characteristics of H_2O_2-induced oxidative stress in control cells and the influence of Cu-pretreatment and Cu-preincubation was also examined in this study. Exposure of control cells to H_2O_2 (60 μM) resulted in time-dependent decreases in cell viability (fig. 5.8), increases in lipid peroxidation (fig. 5.9), oxidation of GSH (fig. 5.10), increases in $[Ca^{+2}]i$ (fig. 5.11) as well as the appearance of bleb formation on cell surface(fig. 5.11). The magnitude of H_2O_2-induced changes in all of these parameters were significantly greater in Cu-pretreated cells as compared to control cells within the 60 minute exposure period. The extent of changes in these toxicity parameters between control and Cu-pretreated cells was highest for lipid peroxidation; this was followed in decreasing orders by $[Ca^{+2}]i$, cell blebbing and oxidation of GSH. Exposure of Cu-preincubated cells to 60 μM H_2O_2 resulted in significant increases in cell death (fig. 5.12) lipid peroxidation (fig. 5.12) and $[Ca^{+2}]$(fig. 5.13) with no significant changes in GSH oxidation (fig. 5.13) when compared to the corresponding response in control cells. It should be noted that the degrees of Cu-preincubation- related increases in H_2O_2-induced cell death and lipid peroxidation were lower when compared to those related to Cu-

Figure 5.7. Log dose-response of the killing of Ehrlich cells by H_2O_2. Control and Cu-pretreated Ehrlich cells were challenged with 7.5, 15, 30, 60, and 120 μM H_2O_2 for 60 min and viability was assessed by the trypan blue exclusion method. Viability was expressed as probit units by converting percents of response to normal equivalent deviations (N.E.D.). A N.E.D. is the response increment brought about by increasing or decreasing the log dose by one standard deviation. Each point represents the mean $\pm$ SEM of 5 observations.

a. significantly different from corresponding control value, $p < 0.05$.

b. significantly different from corresponding Cu-pretreated (1 mg Cu/kg) value, $p < 0.05$.

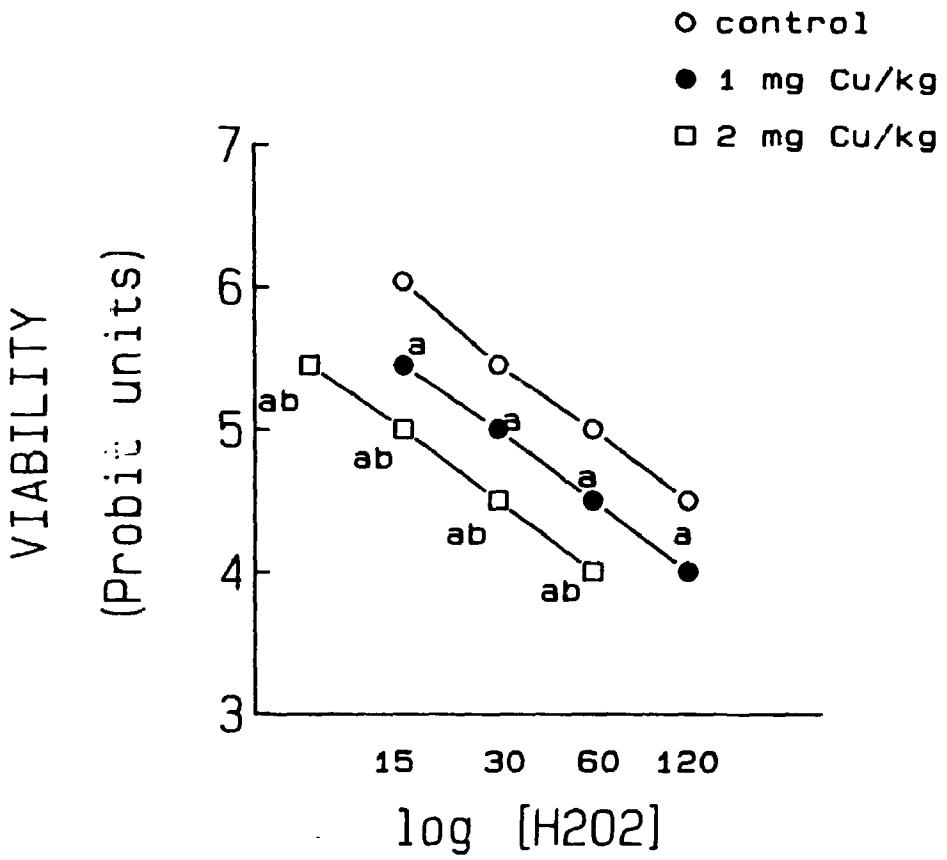

control
1 mg Cu/kg
2 mg Cu/kg
VIABILITY
(Probit units)
7
6
5
4
3
a
a
a
a
ab
ab
ab
ab
15
30
60
120
log [H202]

Figure 5.8. Changes in cell viability of Ehrlich cells isolated from control (○) and Cu-pretreated (1 mgCu/kg[●] and 2 mg Cu/kg [▵]) host mice following treatment of cells with H_2O_2 (60 μM). Cell viability was determined by the trypan blue exclusion method. Values represent mean ± SEM of 4 experiments. In the absence of H_2O_2, cell viability values were not significantly different from their respective control time zero value.

a. significantly different from corresponding control values, $p<0.05$.

b. significantly different from corresponding Cu-pretreated (1 mgCu/kg) value, $p<0.05$.

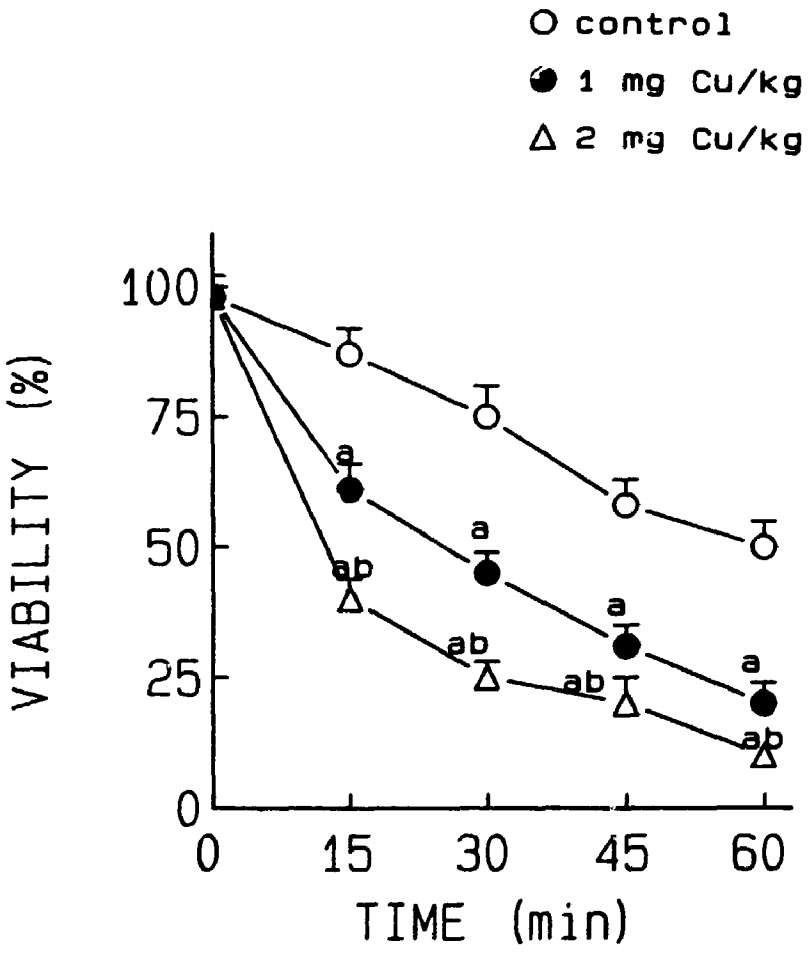

O control
1 mg Cu/kg
Δ 2 mg Cu/kg
VIABILITY (%)
100
75
50
25
0
0
15
30
45
60
TIME (min)
a
a
a
a
ab
ab
ab

Figure 5.9. Changes in lipid peroxidation of Ehrlich cells isolated from control (○) and Cu-pretreated (1mg Cu/kg[●] and 2 mg Cu/kg[□]) host mice following treatment of cells with H_2O_2 (60 μM). Formation of thiobarbituric acid reactants (abs at 535 nm) was taken as a measure of lipid peroxidation. Values represent mean ± SEM of 4 experiments.

a. significantly different from corresponding control values, $p < 0.05$.

b. significantly different from corresponding Cu-pretreated (1 mg Cu/kg) values, $p < 0.05$.

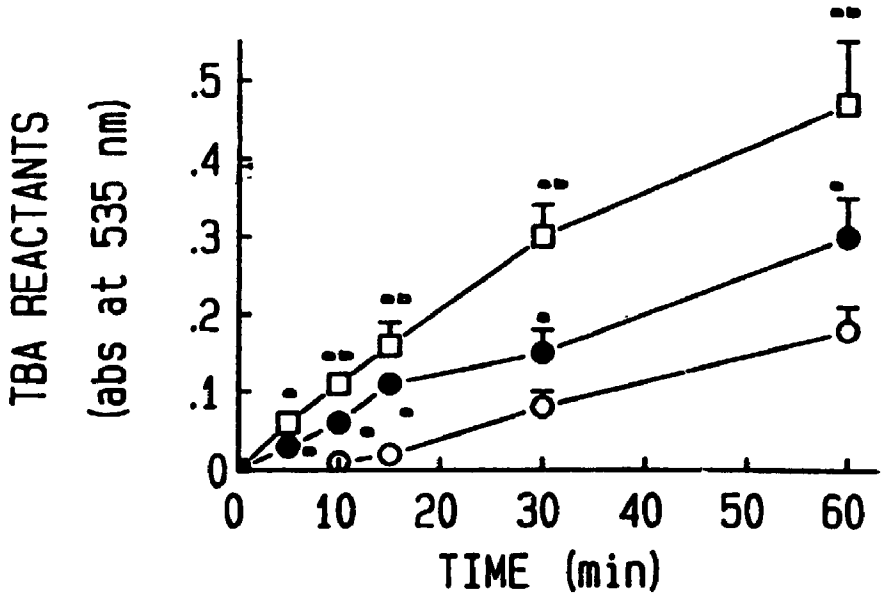

TBA REACTANTS
(abs at 535 nm)
.5
.4
.3
.2
.1
0
0
10
20
30
40
50
60
TIME (min)

Figure 5.10. Depletion of GSH in Ehrlich cells isolated from control (○) and Cu-pretreated (1 mg Cu/kg[●] and 2 mg Cu/kg[□]) host mice following treatment of cells with H_2O_2 (60 μM). Depletion of GSH was followed for 30 min (inset). Values represent mean ± SEM of 4 experiments.

a. significantly different from corresponding control values, $p < 0.05$.

b. significantly different from corresponding Cu-pretreated (1mgCu/kg) values, $p < .05$.

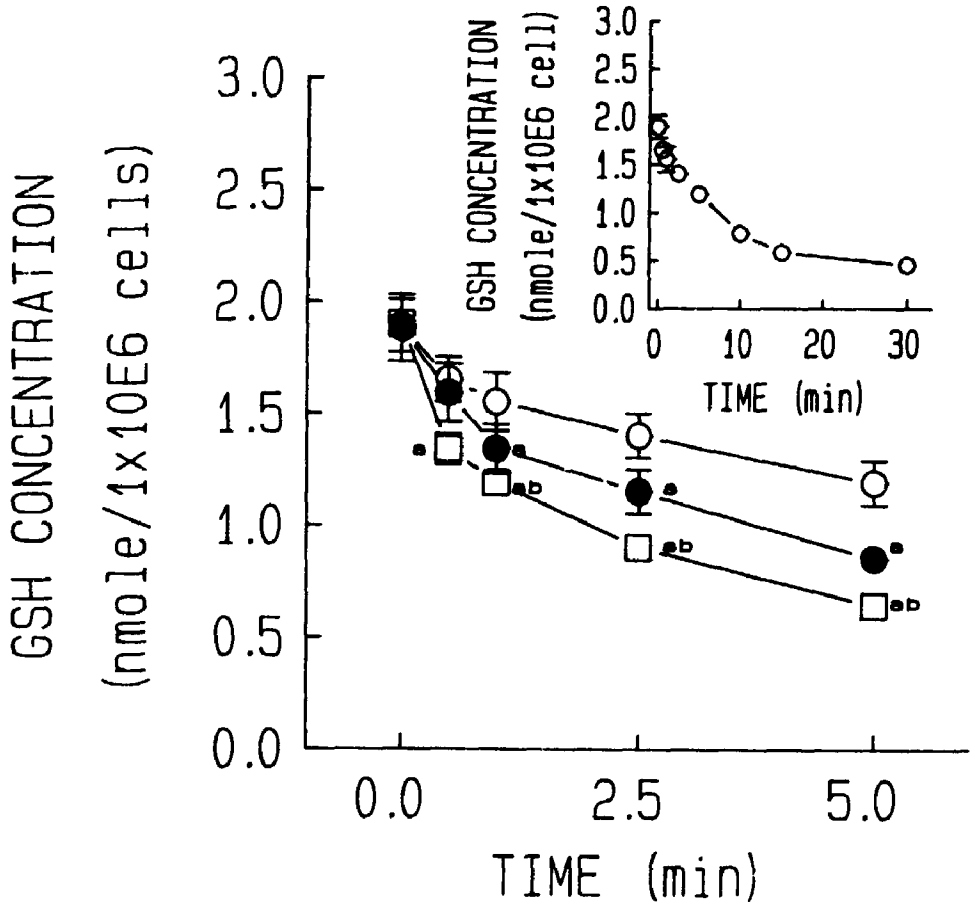
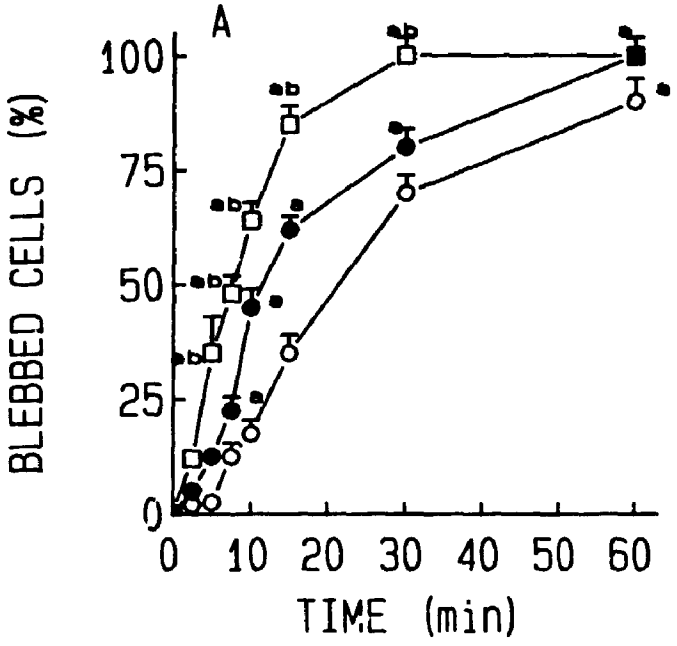

GSH CONCENTRATION
(nmole/1x10E6 cells)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
2.5
5.0
TIME (min)
GSH CONCENTRATION
(nmole/1x10E6 cell)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
TIME (min)

Figure 5.11. Changes in cell blebbing (A) and $[Ca^{+2}]i$ (B) in Ehrlich cells isolated from control (○) and Cu-pretreated (1 mg Cu/kg[◐] and 2 mg Cu/kg[□]) host mice following treatment of Ehrlich cells with H_2O_2 (60 μM). Cell blebbing was assessed subjectively by light microscopy. $[Ca^{+2}]i$ was measured fluorometrically using the Ca^{+2}-sensitive probe Indo-1. Values represent mean $\pm$ SEM of 4 experiments.

a. significantly different from corresponding control values, $p<0.05$.

b. significantly different from corresponding Cu-pretreated (1 mg Cu/kg) values, $p<0.05$.


A
BLEBBED CELLS (%)
100
75
50
25
0
0 10 20 30 40 50 60
TIME (min)

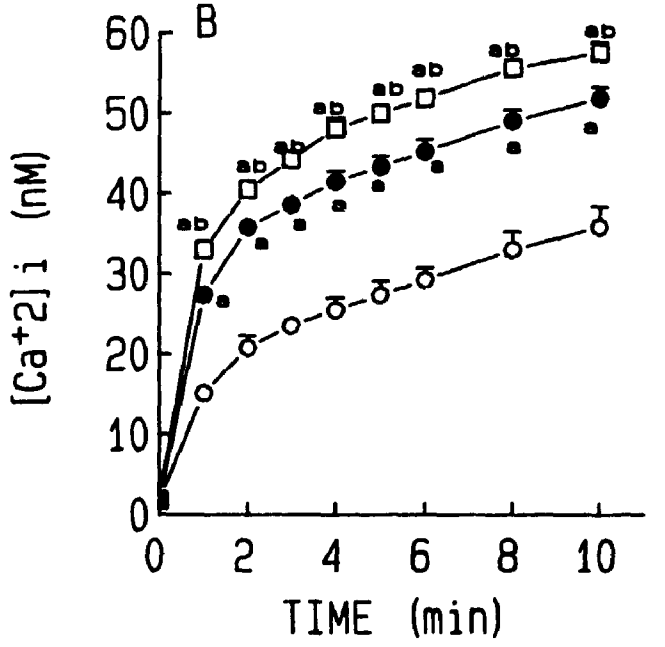
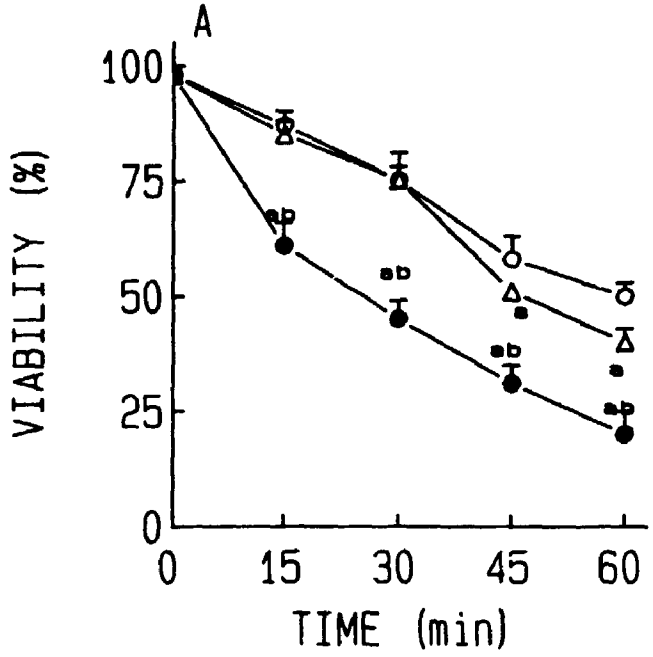

B
[Ca+2]i (nM)
60
50
40
30
20
10
0
0 2 4 6 8 10
TIME (min)

Figure 5.12. Changes in cell viability (A) and lipid peroxidation (B) in control (○), Cu-preincubated (△) and Cu-pretreated (1mg Cu/kg)(●) cells exposed to H_2O_2 (60 μM). Cu-pretreated cells were obtained from host mice as described in Fig. 5.4 To prepare Cu-preincubated Ehrlich cells were treated with 0.6 ppm $CuSO_4$ for 30 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2 Values represent mean ± SEM of 3 experiments.

a. significantly different from corresponding control values, $p<0.05$.

b. significantly different from corresponding Cu-preincubated values, $p<0.05$.


A
100
75
50
25
0
VIABILITY (%)
0
15
30
45
60
TIME (min)

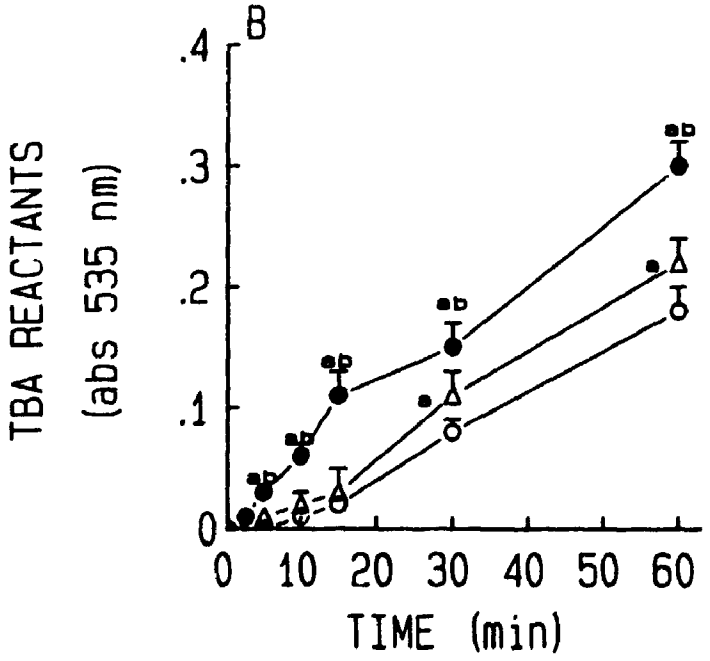
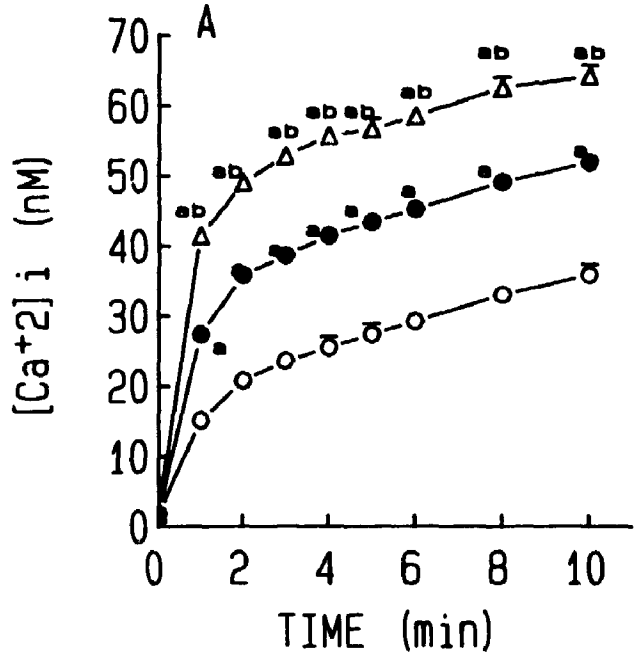

B
.4
.3
.2
.1
0
TBA REACTANTS
(abs 535 nm)
0
10
20
30
40
50
60
TIME (min)

Figure 5.13. Changes in $[Ca^{+2}]i$)(A) and GSH concentration (B) in control (O), Cu-preincubated (▵) and Cu-pretreated (1 mg Cu/kg)(●) cells exposed to H_2O_2 (60 μM). Cu-pretreated cells were obtained from host mice as described in figure 5.4. To prepare Cu-preincubated cells, Ehrlich cells were treated with 0.6 ppm $CuSO_4$ for 30 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2. Values represent mean ± SEM of 3 experiments.

a. significantly different from corresponding control values, $p<0.05$.

b. significantly different from Cu-pretreated (1 mgCu/kg) values, $p<0.05$.


A
70
60
50
40
30
20
10
0
[Ca+2]i (nM)
0
2
4
6
8
10
TIME (min)

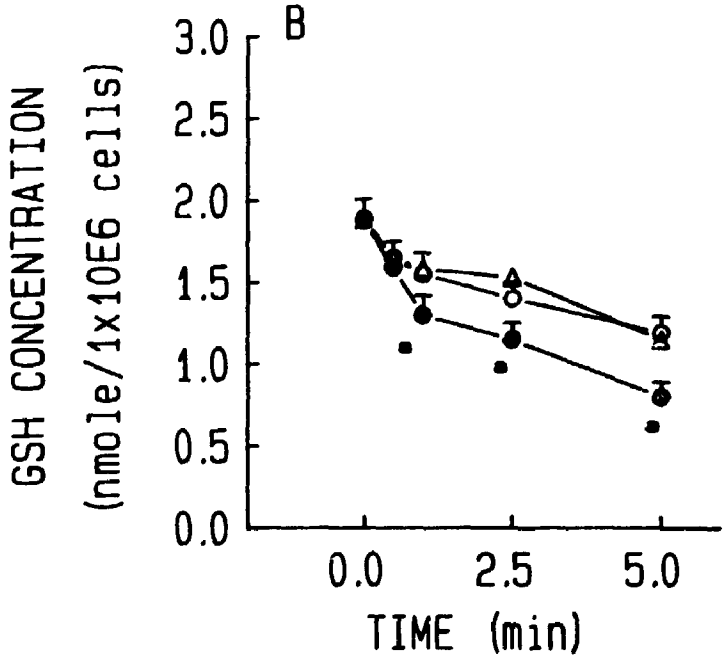

B
3.0
2.5
2.0
1.5
1.0
0.5
0.0
GSH CONCENTRATION
(nmole/1x10E6 cells)
0.0
2.5
5.0
TIME (min)

TABLE 5.3

Subcellular distribution of Cu in control, Cu-pretreated (1 mg Cu/kg) and Cu-preincubated (0.6 ppm Cu) cells[a]

	Cu concentration (ng/1x106 cells)			
		Cytosolic		
Treatment	Total	HMW	MT	Particulate
Control	9.00±1.05	7.08±0.85	ND	1.45±0.40
1mg Cu/kg[b]	45.50±3.10[d]	17.62±2.06[d]	18.0±2.10	10.00±2.10[d]
0.6ppm Cu[c]	40.58±2.04[d]	18.90±1.70[e]	0.80±0.10[e]	20.15±3.70[de]

a. Values represent mean±SEM from 4 experiments

b. Ehrlich cells were isolated from host mice 24 h after 1 mg Cu/kg $CuSO_4$ administration.

c. Ehrlich cells isolated from control host mice were treated with 0.6 ppm $CuSO_4$ for 30 min at 37°C; cells were washed twice with PBS buffer and resuspended to original volume with the same buffer.

d. significantly different from corresponding control values, $p<0.05$.

e. significantly different from corresponding Cu-pretreated values, $p<0.05$.

ND not detected

pretreatment (1 mg Cu/kg) while increases in H_2O_2-induced $[Ca^{+2}]i$ responses were higher in Cu-preincubated than Cu-pretreated cells (1 mg Cu/kg).

iii) Mechanism of enhancement of H_2O_2 sensitization by Cu-pretreatment.

To understand the mechanism underlying the role of Cu-MT in the sensitization of Ehrlich cells to H_2O_2, evidence of interaction between Cu-MT and H_2O_2 was examined. As shown in figures 5.14 and 5.15, exposure of Cu- pretreated Ehrlich cells to 60 μM H_2O_2 resulted in paralleled reduction of MT concentration (as measured by the Ag-heme saturation method, reflecting the treatment effect on metal binding capacity of MT)(fig. 5.14) and loss of MT sulphydryl groups (fig. 5.15). The H_2O_2-mediated loss in metal binding capacity was probably due to the oxidation of MT thiolate groups, since treatment with 1,4-dithiothreitol (DTT), a sulphydryl reductant, restored the metal binding capacity of MT samples to pre-H_2O_2 treatment values (fig. 5.16). Results presented in fig. 5.17, also showed that H_2O_2-induced oxidation and inactivation of metal binding sites of MT were accompanied by changes in the subcellular and cytosolic distribution of Cu. About 89% of total Cu in the Cu-pretreated cells (1 mg Cu/kg) was associated with the cytosol and this pool of Cu was distributed equally between the HMW and MT fraction (fig. 5.17; Table 5.4). Exposure of these Cu-pretreated cells to 60 μM H_2O_2 resulted in time-dependent decreases in MT-bound Cu measured within the 15 minute incubation period; this treatment effect was accompanied with concurrent increases in Cu concentrations in the HMW and particulate fraction(Table 5.4).

To ascertain the involvement of Cu and Cu-MT in H_2O_2-induced oxidative stress, the

effect of D-penicillamine, a Cu chelating agent, on H_2O_2-induced oxidative stress was examined. If the enhancement in H_2O_2-sensitivity observed in Cu-pretreated cells was solely due to chelatable Cu, then D-penicillamine should prevent the increases in toxic response beyond those observed in the control group. As shown in figure 5.18 and 5.19, this was the case with the increases in lipid peroxidation and cell death as well as oxidation of GSH. However, increases in $[Ca^{+2}]i$ were only partially sensitive to D-penicillamine blockade (fig. 5.19). It should be noted that D-penicillamine did not have any significant effects on H_2O_2-induced oxidative injury in control cells (data not shown) suggesting that the protective effect observed in Cu-pretreated cells was not due to its direct antioxidant properties. Moreover, the protective effect of D-penicillamine was not due to sequestration of Cu from HMW or particulate fractions because D-penicillamine did not have any significant protective effects on H_2O_2-induced oxidative injury in Cu-preincubated cells which contained elevated levels of Cu in these two fractions (data not shown). In addition, D-penicillamine did not alter the metal binding capacity or the Cu content of MT in Cu-pretreated cells in the absence of H_2O_2 exposure suggesting that the protective effect of D-penicillamine was not due to removal of Cu ions from MT during the preincubation period, ie. before H_2O_2 treatment. As shown in figure 5.17, D-penicillamine did not alter the H_2O_2-induced loss of metal binding capacity and Cu content of MT fraction of Cu-pretreated cells but it did reduce the apparent translocation of Cu to the HMW and particulate fractions; it is important to note that this releasable pool of Cu was recovered by D- penicilamine (fig. 5.17). These data suggest that that the protective effect of D-penicillamine may be due to sequestration of a Cu pool that

Figure 5.14. H_2O_2-induced reduction of MT concentration of Ehrlich cells isolated from control (A), 1 mgCu/kg (B) and 2 mg Cu/kg(C) pretreated host mice. Metallothionein concentration (or the metal binding capacity) was determined by the Ag-heme saturation method. To illustrate the effect of H_2O_2 on metal binding capacity of MT, the absolute changes within each group were compared. Values represent mean $\pm$ SEM of 4 experiments.

* Significantly different from corresponding time zero value, $p<0.05$.

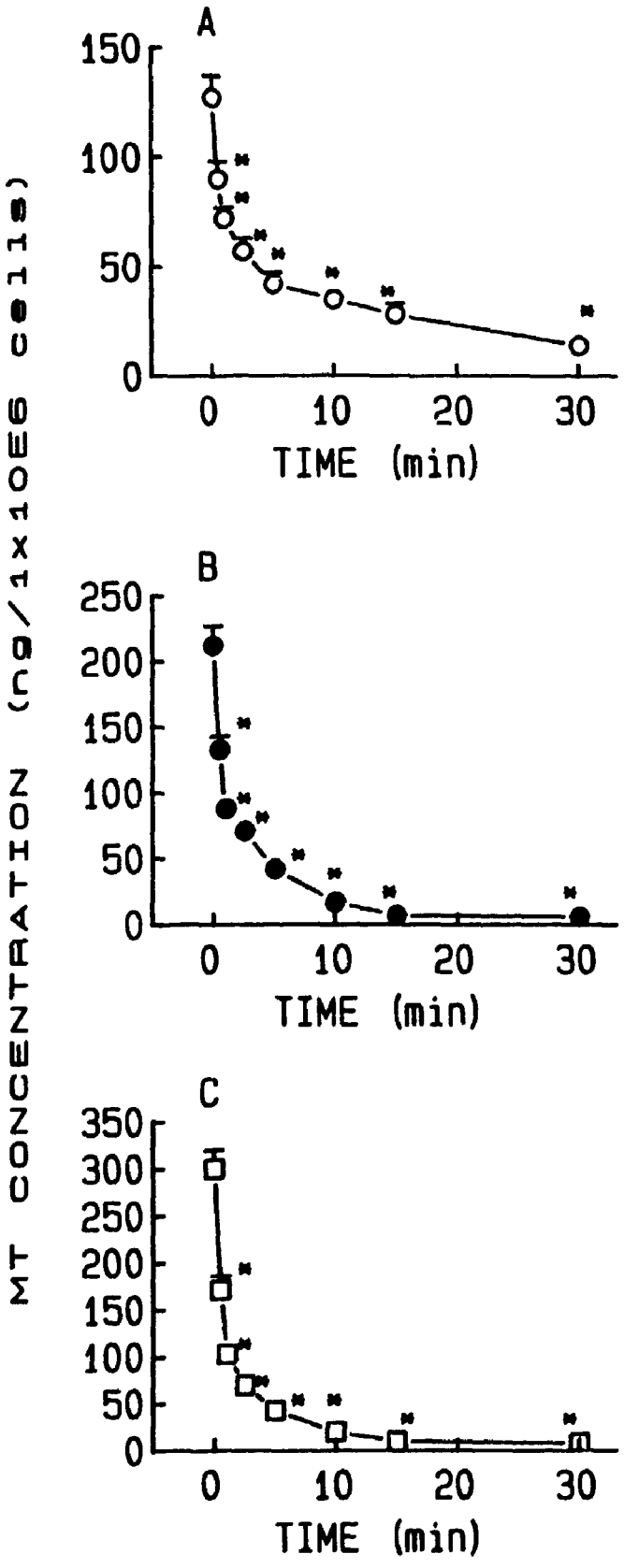

A
B
C
150
100
50
0
250
200
150
100
50
0
350
300
250
200
150
100
50
0
0
10
20
30
TIME (min)
MT CONCENTRATION (ng/1x10E6 cells)

Figure 5.15. H_2O_2-induced reduction of sulphydryl concentration of MT of Ehrlich cells isolated from control (A), 1 mg Cu/kg (B) and 2 mg Cu/kg (C) pretreated host mice. Thiol content of MT was measured following NbS_2 titration. To illustrate the effect of H_2O_2 on thiol content of MT, the absolute changes within each group were compared. Values represent mean $\pm$ SEM of 4 experiments.

* significantly different from corresponding time zero value, $p<0.05$.

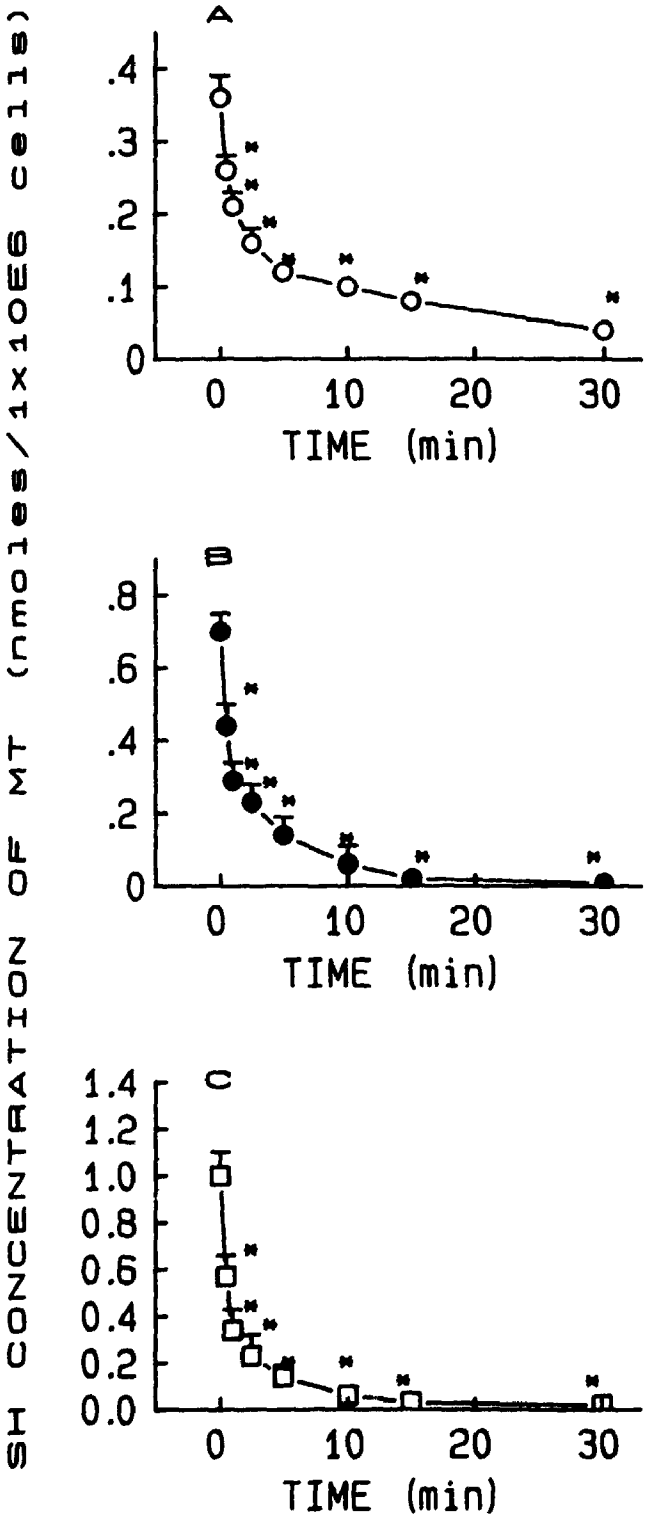

SH CONCENTRATION OF MT (nmoles/1x10E6 cells)
A
.4
.3
.2
.1
0
0
10
20
30
TIME (min)
B
.8
.6
.4
.2
0
0
10
20
30
TIME (min)
C
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
TIME (min)

Figure 5.16. In vitro 1,4-dithiothreitol (DTT)-dependent regeneration of metal binding capacity of MT in cytosols obtained from control (A), 1 mg Cu/kg (B) and 2 mg Cu/kg (C) pretreated Ehrlich cells. Heat-treated cytosolic supernatant fractions from control and H_2O_2 (60 μM)-treated incubations were treated with 5 mM 1,4-dithiothreitol (DTT) under nitrogen at room temperature for 30 min and the metal binding capacity was estimated by the Ag-heme saturation method. Values represent mean $\pm$ SEM of 3 experiments.

a. significantly different from corresponding time zero values, $p<0.05$.

b. significantly different from corresponding non-DTT-treated samples, $p<0.05$.

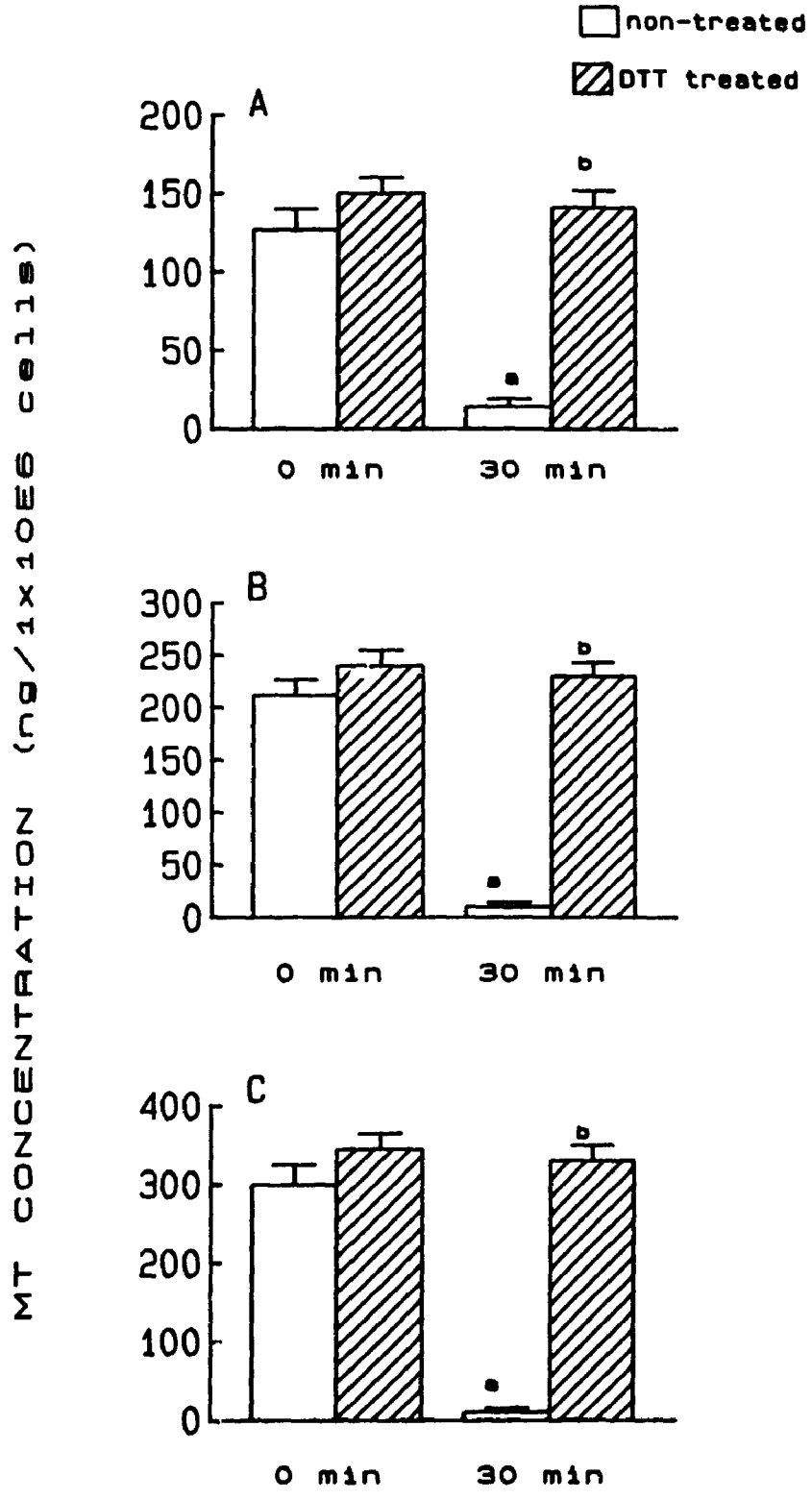

non-treated
DTT treated
MT CONCENTRATION (ng/1x10E6 cells)
A
200
150
100
50
0
a
b
0 min
30 min
B
300
250
200
150
100
50
0
a
b
0 min
30 min
C
400
300
200
100
0
a
b
0 min
30 min

Figure 5.17. Changes in distribution of Cu in cytosols of Ehrlich cells isolated from 1 mg Cu/kg-pretreated host mice (————) following treatment with H_2O_2 (60 μM) in the absence (----) or presence (····) of 100 μM D-penicillamine (PEN). For the D-penicillamine experiment, Ehrlich cells were preincubated with D-penicillamine for 30 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer, resuspended to original volume with the same buffer and challenged with H_2O_2 for 15 min. Cytosols isolated from Ehrlich cells were chromatographed on a Sephadex G-75 column equilibrated with 10 mM Tris-HCl buffer, pH 8.6, at room temperature. Two milliliter fractions were collected at an elution rate of 1 ml/min and measured for Cu by atomic absorption spectrophotometry.

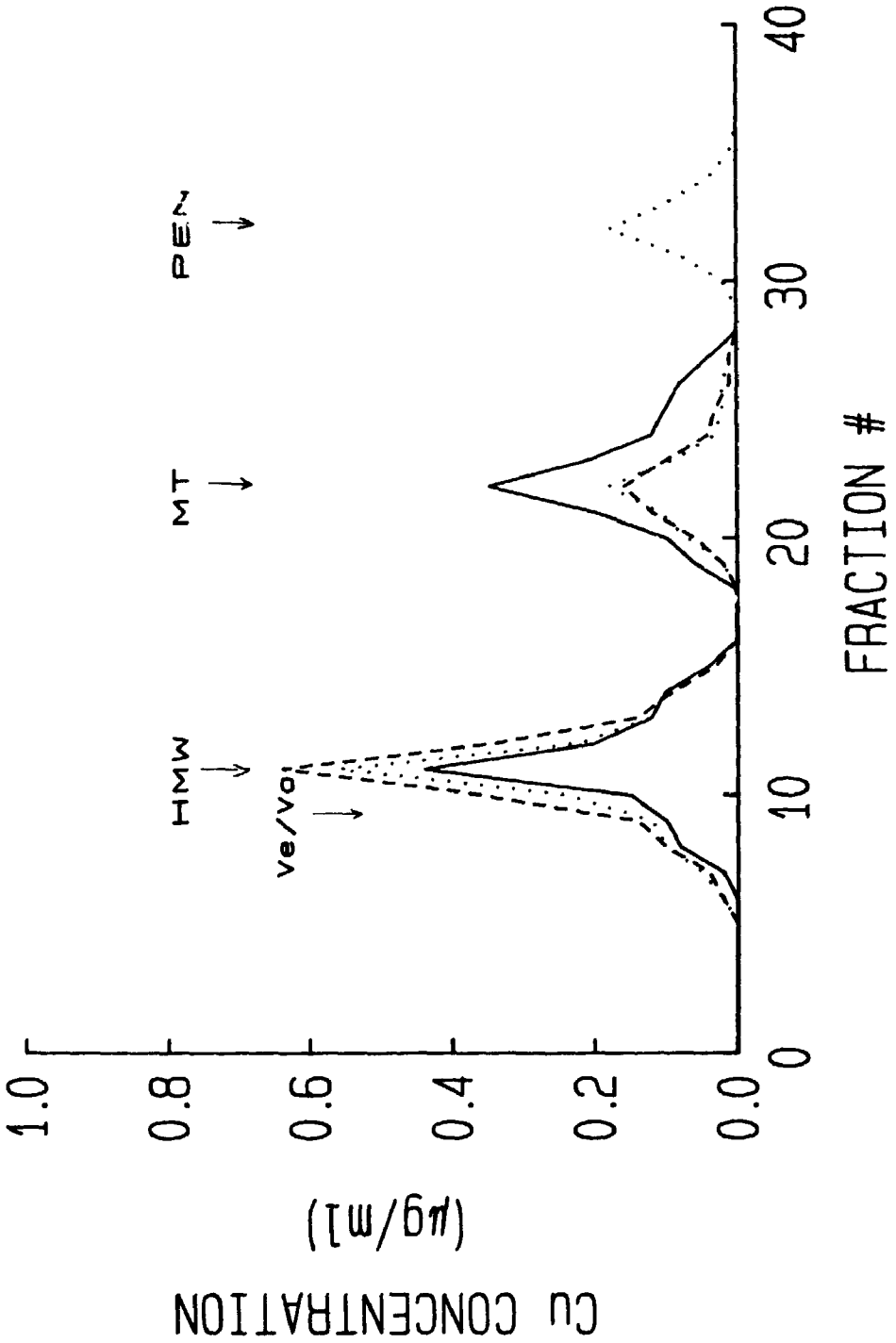

Cu CONCENTRATION
(μg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
FRACTION #
HMW
Ve/Vo
MT
PEN

TABLE 5.4

Effect of H_2O_2 on the subcellular distribution of Cu in Ehrlich cells isolated from host mice pretreated with 1 mg Cu/kg $CuSO_4$[a,b]

	Cu concentration (ng/1x10E6 cells)			
		Cytosolic		
Time of incubation (min)	Total	HMW	MT	Particulate
0	45.10±4.00	17.62±2.06	18.01±2.10	5.00±1.10
2.5	45.30±1.15	21.70±1.15[c]	9.30±1.60[c]	10.05±2.20[c]
5.0	45.35±3.00	22.80±1.80[c]	6.70±0.80[c]	12.50±1.85[c]
15.0	45.00±2.80	28.00±1.25[c]	0.70±0.07[c]	16.70±2.10[c]

a. Values represent mean ± SEM from 3 experiments

b. Ehrlich cells (1x10E7 cells/ml incubation) isolated from 1 mg Cu/kg Cu pretreated host mice were exposed to 60 μM H_2O_2 at 37°C. Aliquots of cell suspension were removed at appropriate times, washed once with ice-cold PBS buffer and resuspended to original volume with the same buffer. Homogenate, cytosol and particulate fractions were obtained following sonication of samples as described in Materials and Methods. To obtain HMW and MT fractions, an aliquot of cytosol was chromatographed on a Sephadex G-75 column.

c. significantly different from corresponding time zero values, $p < 0.05$.

Figure 5.18. Effect of D-penicillamine (PEN) on H_2O_2-induced changes of cell death (A) and lipid peroxidation (B) in Ehrlich cells isolated from Cu-pretreated (1 mg Cu/kg) host mice. Ehrlich cells were preincubated with 100 μM D-penicillamine for 30 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2. Values represent mean $\pm$ SEM of 3 experiments.

a significantly different from corresponding control values, $p < 0.05$.

b significantly different from corresponding non-PEN-treated Cu-pretreated calues, $p < 0.05$.

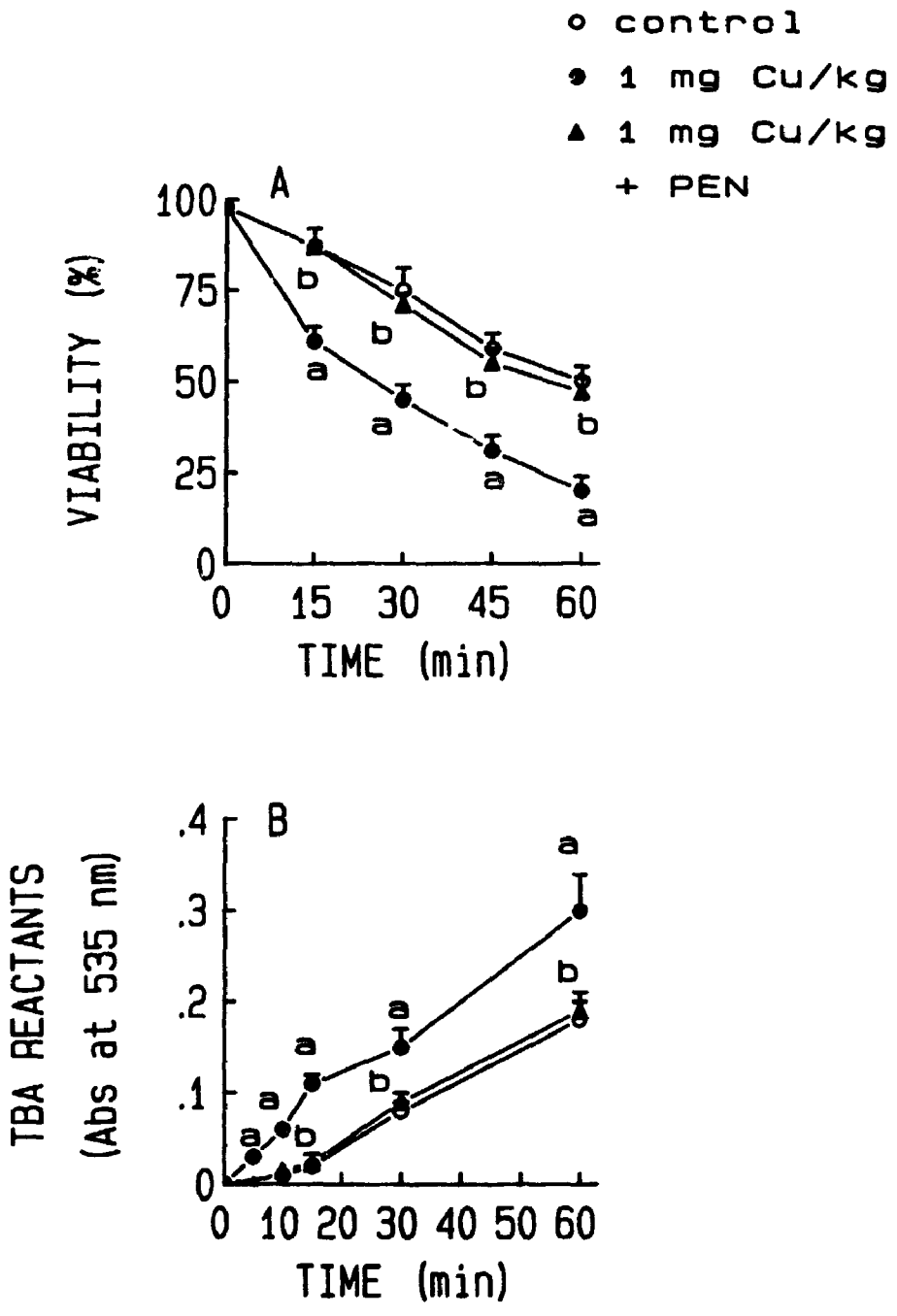

o control
• 1 mg Cu/kg
▲ 1 mg Cu/kg
+ PEN
A
VIABILITY (%)
100
75
50
25
0
0 15 30 45 60
TIME (min)
B
TBA REACTANTS
(Abs at 535 nm)
.4
.3
.2
.1
0
0 10 20 30 40 50 60
TIME (min)

Figure 5.19. Effect of D-penicillamine (PEN) on H_2O_2-induced changes of $[Ca^{+2}]i$ (A) and GSH concentration(B) in Ehrlich cells isolated from Cu-pretreated (1 mg Cu/kg) host mice. Ehrlich cells were preincubated with 100 μM D-penicillamine for 30 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2. Values represent mean $\pm$ SEM of 3 experiments.

a. significantly different from corresponding control values, $p<0.05$.

b. significantly different from corresponding non-PEN-treated Cu-pretreatred values, $p<0.05$.

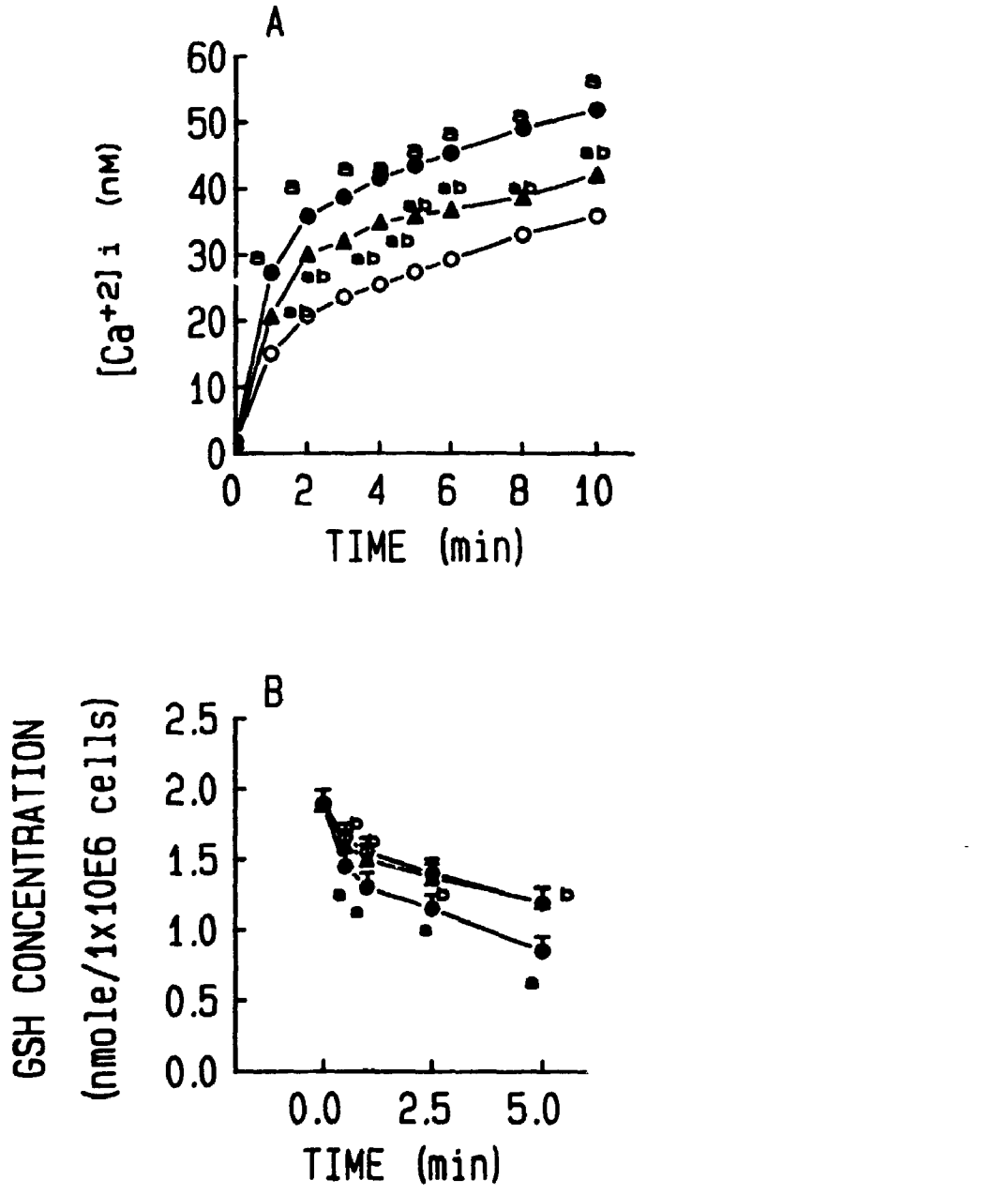

control
1 mg Cu/kg
1 mg Cu/kg + PEN
A
[Ca+2]i (nM)
60
50
40
30
20
10
0
0
2
4
6
8
10
TIME (min)
B
GSH CONCENTRATION
(nmole/1x10E6 cells)
2.5
2.0
1.5
1.0
0.5
0.0
0.0
2.5
5.0
TIME (min)

Figure 5.20. Effect of desferoxamine (DEF) in the absence or presence of D-penicillamine on H_2O_2-induced changes in cell viability (A) and lipid peroxidation (B) in Ehrlich cells isolated from control and Cu-pretreated (1 mg Cu/kg) host mice. Ehrlich cells were preincubated with 500 μM desferoxamine for 45 min at 37°C under air atmosphere in the presence or absence of 100 μM D-penicillamine. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged to H_2O_2 (60 μM). Values represent mean $\pm$ SEM of 3 experiments.

a. significantly different from corresponding non-desferoxamine treated vaslues, $p<0.05$.

b. significantly different from corresponding desferoxamine treated Cu-pretreated values, $p<0.05$.

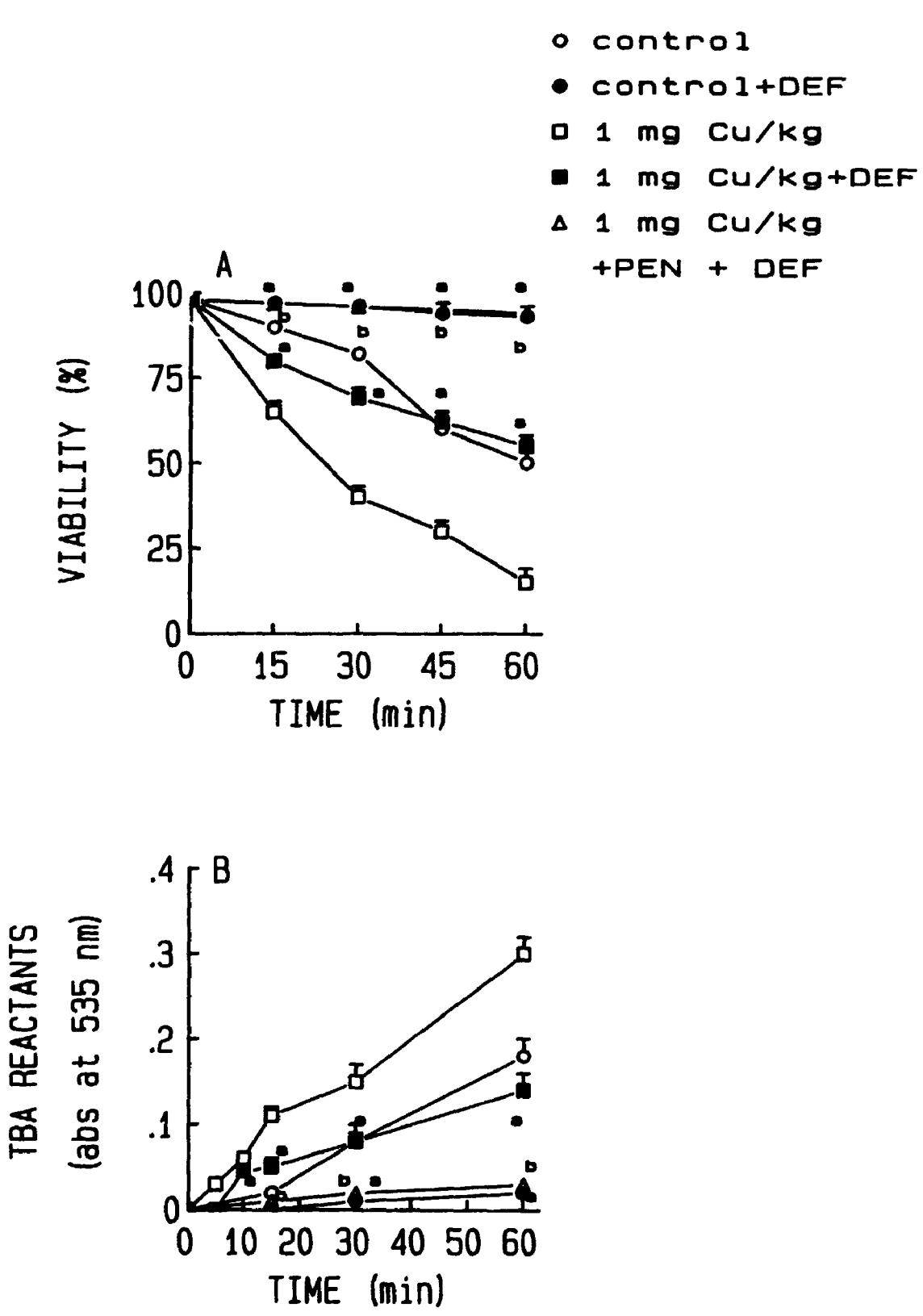

control
control+DEF
1 mg Cu/kg
1 mg Cu/kg+DEF
1 mg Cu/kg
+PEN + DEF
A
VIABILITY (%)
100
75
50
25
0
0
15
30
45
60
TIME (min)
B
TBA REACTANTS
(abs at 535 nm)
.4
.3
.2
.1
0
0 10 20 30 40 50 60
TIME (min)

Figure 5.21. Effects of desferoxamine (DEF) on H_2O_2-induced changes in $[Ca^{+2}]i$ (A) and GSH (B) concentrations in Ehrlich cells isolated from control and Cu-pretreated (1 mg Cu/kg) host mice. Ehrlich cells were preincubated with 500 μM desferoxamine for 45 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cell were the challenged with H_2O_2 (60 μM).

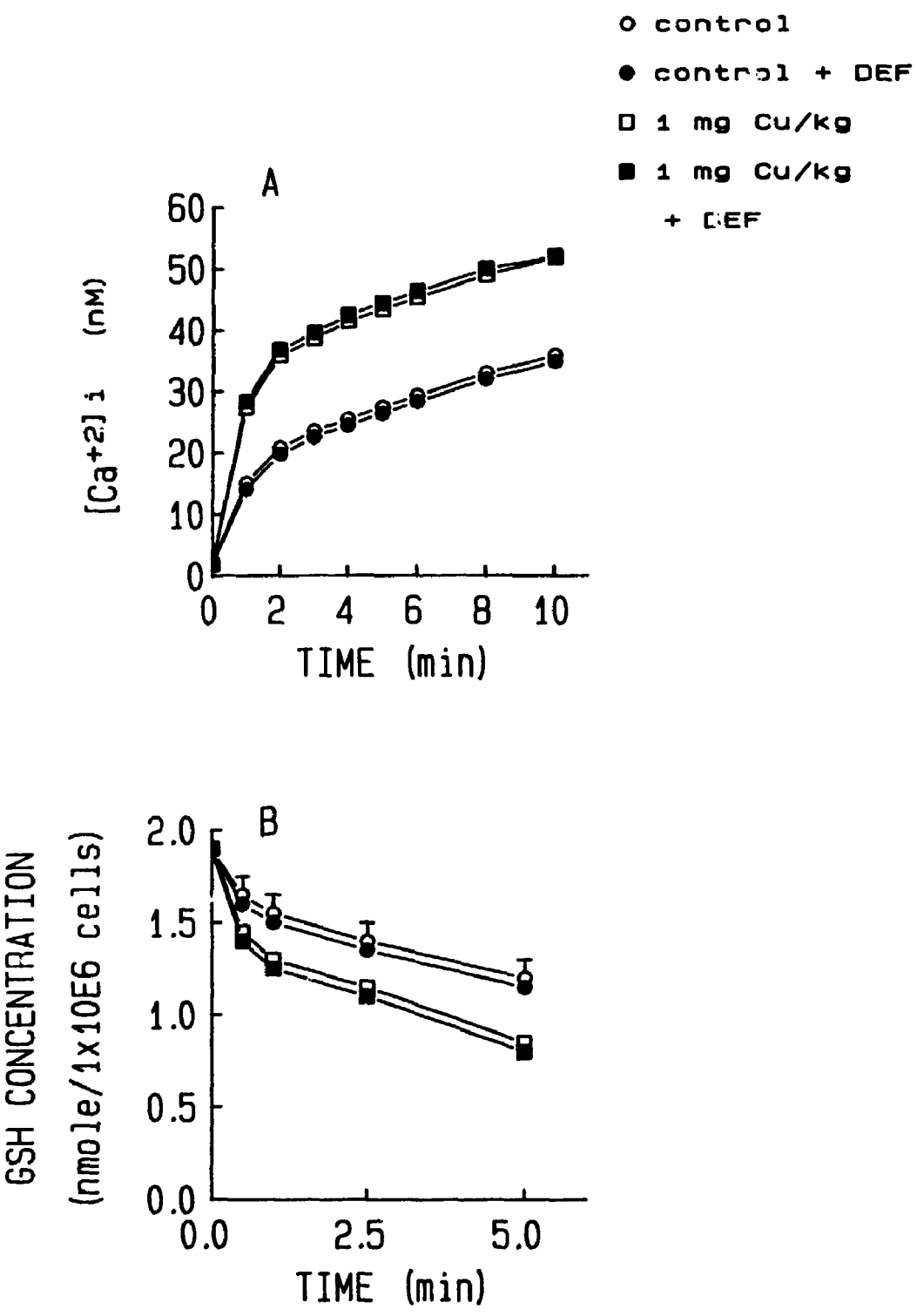

o control
● control + DEF
□ 1 mg Cu/kg
■ 1 mg Cu/kg + DEF
A
[Ca+2]i (nM)
60
50
40
30
20
10
0
0 2 4 6 8 10
TIME (min)
B
GSH CONCENTRATION (nmole/1x10E6 cells)
2.0
1.5
1.0
0.5
0.0
0.0 2.5 5.0
TIME (min)

Figure 5.22. Effect of mannitol (MAN) on H_2O_2-induced changes in cell viability (A) and lipid peroxidation (B) in Ehrlich cells isolated from control and Cu-pretreated (1 mg Cu/kg) host mice. Ehrlich cells were preincubated with 30 mM mannitol for 60 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2. Values represent mean ± SEM of 3 experiments.

* significantly different from corresponding non-mannitol treated values, $p < 0.05$.

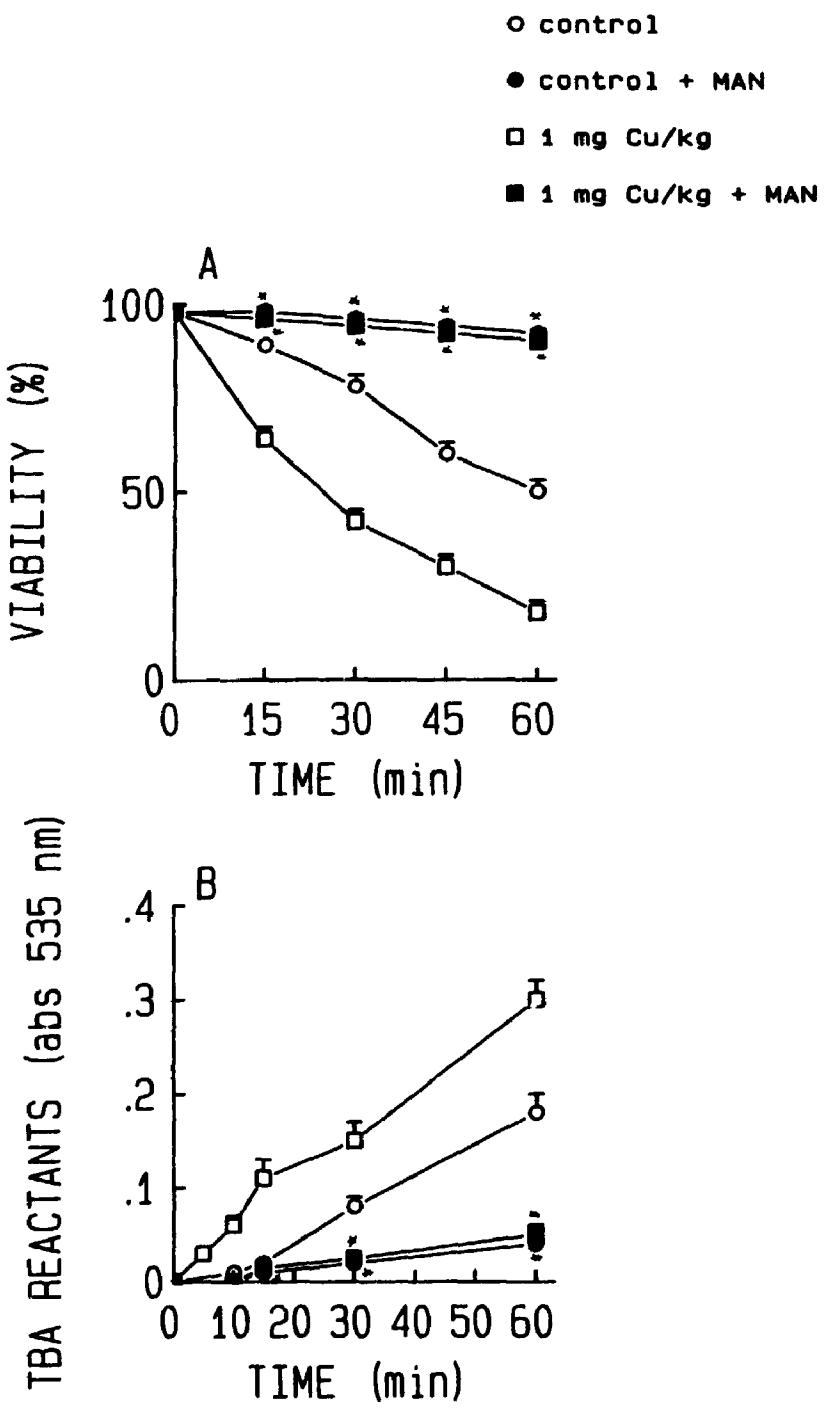
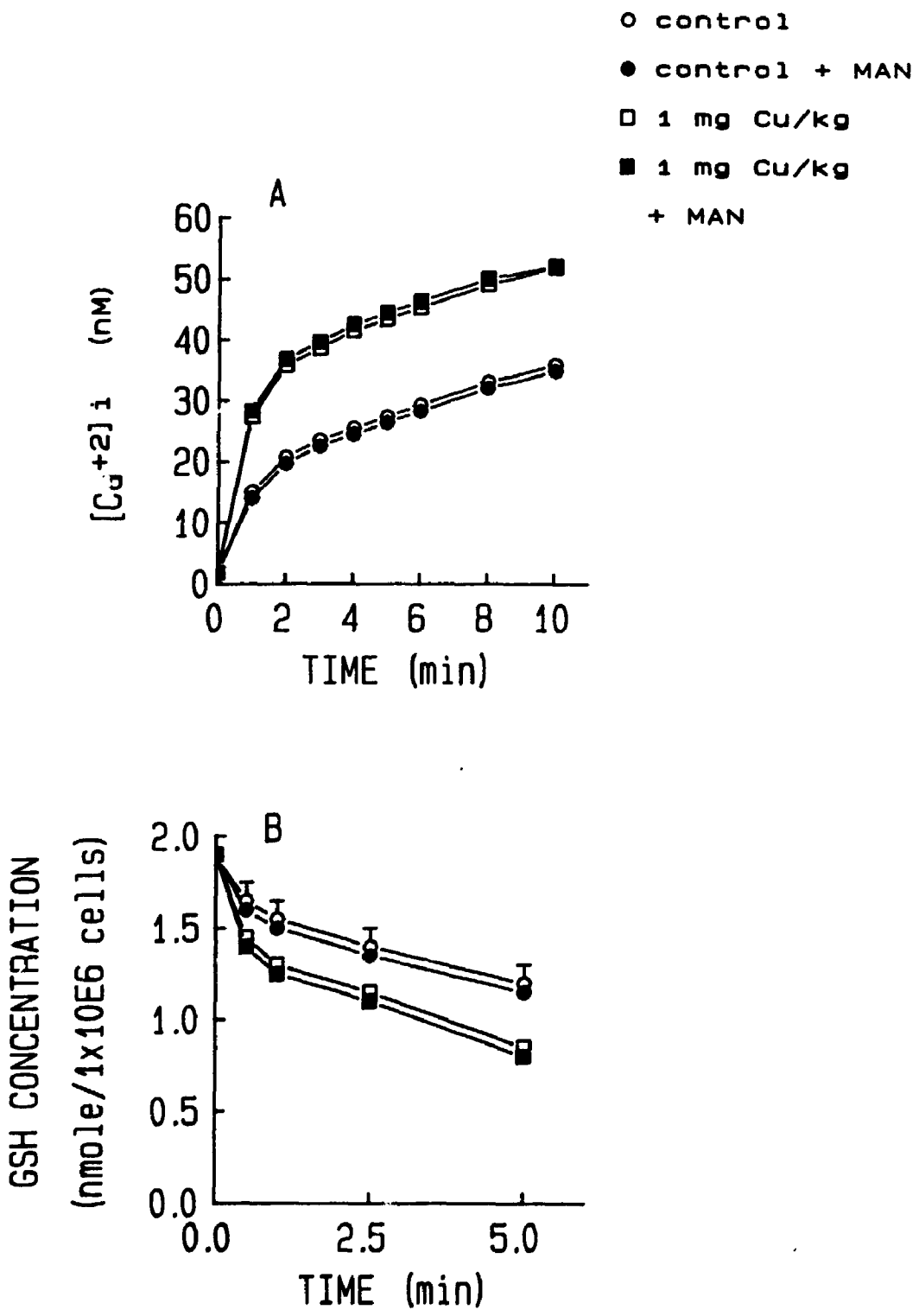

control
control + MAN
1 mg Cu/kg
1 mg Cu/kg + MAN
A
100
50
0
VIABILITY (%)
0 15 30 45 60
TIME (min)
B
.4
.3
.2
.1
0
TBA REACTANTS (abs 535 nm)
0 10 20 30 40 50 60
TIME (min)

Figure 5.23. Effect of mannitol (MAN) on H_2O_2-induced changes in $[Ca^{+2}]i$ (A) and GSH (B) concentrations in Ehrlich cells isolated from control and Cu-pretreated (1 mg Cu/kg) host mice. Ehrlich cells were preincubated with 30 mM mannitol for 60 min at 37°C under air atmosphere. Cell suspensions were washed twice with ice-cold PBS buffer and resuspended to original volume with the same buffer; cells were then challenged with H_2O_2 (60 μM). Values represent mean $\pm$ SEM of 3 experiments.


o control
● control + MAN
□ 1 mg Cu/kg
■ 1 mg Cu/kg + MAN
A
[Cu+2]i (nM)
60
50
40
30
20
10
0
0 2 4 6 8 10
TIME (min)
B
GSH CONCENTRATION (nmole/1x10E6 cells)
2.0
1.5
1.0
0.5
0.0
0.0 2.5 5.0
TIME (min)

was released from the oxidized MT.

To determine whether or not Cu-pretreatment increased toxicity by merely increasing the toxic response normally induced in control cells, additional studies were conducted with both control and Cu-pretreated (1 mg Cu/kg) cells to determine the mechanism(s) of toxicity involved. As shown in figures 5.20 and 5.22, desferoxamine (500 μM), a specific Fe(III)iron ion chelator or mannitol (30 mM), a hydroxyl radical scavenger, completely obliterated the H_2O_2-induced cell death and lipid peroxidation in control cells suggesting participation of OH radical generated via a Fe-catalyzed Fenton reaction. Similarily, H_2O_2-induced cell death and lipid peroxidation in Cu-pretreated cells was completely blocked by mannitol suggesting the involvement of OH radical (fig. 5.22). It should be noted that the presence of desferoxamine only reduced the H_2O_2 responses to the same levels observed following treatment of control cells: however, complete blockade of H_2O_2 toxicity was attained by a combination of D-penicillamine and desferoxamine (fig. 5.20). It should be noted that desferoxamine or mannitol had no significant protection against decreases in GSH concentration and increases in $[Ca^{+2}]i$ in neither control nor Cu-pretreated (fig. 5.21; fig. 5.23).

5.4 DISCUSSION

Most studies conducted to examine the role of MT in oxidative stress have focused on Zn-MT or Cd, Zn-MT (Thomas et al., 1986; Thornalley and Vasak, 1985; Cousins and Coppen, 1987) and these studies have revealed an antioxidant function of MT. The results of this study showed that increases in Cu status of Ehrlich cells, in particular the

elevation of Cu-MT concentration, enhanced susceptibility of Ehrlich cells to H_2O_2-induced oxidative injury. To the best of our knowledge, the prooxidant property of Cu-MT has only been reported in one other study in vitro showing the enhancement of xanthine/xanthine oxidase-induced microsomal lipid peroxidation by the presence of hepatic Zn, Cu-MT(II) (Arthur et al., 1987).

The Cu-pretreated Ehrlich cells used in the present study provided a useful model to examine the functional role of Cu-MT in oxidative stress. With regard to the metal composition, MT of Cu-pretreated cells contained predominantly Cu. In addition, the marked difference in MT concentration between groups made the testing of our hypothesis possible. Although the increases in toxic response observed in Cu-pretreated cells may be due to both Cu-MT and/or presence of additional Cu in other cellular components, the inclusion of a Cu-preincubated group in our experimental design would allow estimation of the possible inluence of non-MT-bound Cu associated with Cu-pretreatment protocol (Table 5.3). Indeed, comparison in H_2O_2 response between the two experimental groups suggest the predominant role of Cu-MT in increasing the sensitivity of cell death, lipid peroxidation and oxidation of GSH but not of $[Ca^{+2}]i$. Moreover, it is important that known determinants of oxidative stress such as Fe, Zn, GSH and α-tocopherol concentrations as well as catalase activity were not affected by Cu-pretreatment protocol discounting any Cu-related changes on cellular activational or protective mechanisms.

Elevation in Cu and Cu-MT in Ehrlich cells appear to exaggerate the cellular response to H_2O_2 quantitatively rather than altering the qualitative characteristics of the

response. Thus, the relative time-profiles of H_2O_2-induced changes in $[Ca^{+2}]i$, GSH oxidation, lipid peroxidation and cell death between control and Cu-pretreated cells were similar. Moreover, the parallel shift in the dose-toxicity relationship accompanying the reduction in LC_{50} values of H_2O_2 with Cu-pretreatments would also suggest no changes in mechanism(s) of acute lethal injury in Ehrlich cells with Cu-pretreatment.

The involvement of OH radical generated from H_2O_2 via an Fe-catalyzed Fenton reaction in the induction of lipid peroxidation in control cells has been discussed in Chapter 4. The increases in lipid peroxidation observed in Cu-pretreated cells, were also mediated by the hydroxyl radical; however, the formation of the ultimate oxidant was dependent on a Fe- and Cu-dependent mechanisms. Data from this study also showed that there was no interaction between the two mechanisms because the two effects were additive in nature (fig. 5.18 and 5.20). This is consistent with results reported in other study which showed that Cu-stimulated increases in lipid peroxidation did not involve mobilization of cellular Fe (Sandy et al., 1987). Results from previous study have also suggested that the enhancement of xanthine/xanthine oxidase-induced microsomal lipid peroxidation was due to the presence of Cu perhaps released from oxidized MT (Arthur et al., 1987).

It was reported in Chapter 4 that the rapid and sustained increases in $[Ca^{+2}]i$ observed in control cells challenged with H_2O_2 were mediated by H_2O_2 directly and did not involve hydroxyl radicals (fig. 21; fig. 23). In general, the H_2O_2-induced peroxidation of cellular membranes did not contribute to the alterations of $[Ca^{+2}]i$ in Cu-pretreated cells because blockade of lipid peroxidation by mannitol or desferoxamine

failed to modify the increase in $[Ca^{+2}]i$. Study conducted with Cu-pretreated cells in the presence of desferoxamine and mannitol also suggest that the increases in $[Ca^{+2}]i$ were due to the direct involvement of H_2O_2 and not the Cu-dependent formation of hydroxyl radical. The contribution of Cu released from MT in enhancing the H_2O_2-induced $[Ca^{+2}]i$ response was limited because D-penicillamine only provided partial protection against the elevations of $[Ca^{+2}]i$ suggesting that non-MT-bound Cu may be a more important determinant. This interpretation is consistent with the finding that Cu-preincubation, which resulted in increases in non-MT-bound Cu, was more effective than Cu-pretreatment in enhancing H_2O_2-induced $[Ca^{+2}]i$ response. The mechanism underlying the Cu effects in enhancing H_2O_2-induced increases in $[Ca^{+2}]i$ is not known; however, Cu has been shown to react with protein sulphydryls or increase susceptibility of sulphydryl groups to oxidants, thus resulting in enhanced depletion of intracellular thiols (Hochstein et al., 1980), an event known to be associated with disturbances in Ca^{+2} homeostasis (Orrenius, 1988; Pascoe and Reed, 1989).

The involvement of hydroxyl radical and lipid peroxidation in the mediation of H_2O_2-induced cell death in control Ehrlich cells has already been discussed in Chapter 4. The enhancement in H_2O_2-induced cell death in Cu-pretreated cells appear to be due to a hydroxyl radical mediated peroxidation of membrane lipids by a Cu-dependent mechanism as evidenced by the apparent effectiveness of metal chelators, D-penicillamine and desferoxamine as well as the antioxidant mannitol to protect against cell death and lipid peroxidation.

The effect of H_2O_2 on Cu-MT observed in Cu-pretreated cells provided evidence to

support the involvement of this metalloprotein in the enhancement of cellular susceptibility to oxidative stress by Cu-pretreatment. Data presented in figure 5.16, strongly suggest H_2O_2-induced oxidation of MT thiolates and loss of metal binding capacity and Cu content of MT. Oxidation of MT thiolates has also been demonstrated in yeast Cu-MT with H_2O_2 (Richter and Weiser, 1988; Hartman et al., 1984) and in hepatic pig Zn, Cu-MT(II) with xanthine/xanthine oxidase (Arthur et al., 1987).

Evidence indicating the importance of Cu ions lost from MT in the sensitizing effect of Cu-MT are reflected in the effectiveness of D-penicillamine, a Cu-chelating agent, in blocking the increases in lipid peroxidation, and decreases in cell viability and GSH concentration as well as the recovery of MT-bound Cu as penicillamine-Cu complexes(fig. 5.17). This interpretation is consistent with the observation that the levels of GSH, lipid peroxidation as well as cell death in response to H_2O_2 were not increased to any significant extent in Cu-preincubated cells. The mechanism by which Cu ions released from MT enhnaced H_2O_2 toxicity in Cu-pretreated cells has not been extensively investigated in this study; however, it is known that unchelatable form of Cu ions can interact with H_2O_2 in a Fenton reaction to generate the highly reactive hydroxyl radicals which interact with many cellular components to produce cell death, an directly oxidize cellular sulphydryls and propagate chemically-induced lipid peroxidation (Aust, 1985; Hochstein et al., 1980; Halliwell and Gutteridge, 1984b).

The results of this study revealed that increases in cellular Cu and Cu-MT concentrations increased the toxic responses to H_2O_2 toxicity. Oxidation of MT thiolate groups by H_2O_2 resulted in in decreases in metal binding capacity of MT, binding of Cu

to MT fraction and translocation of Cu to other cellular sites. D-penicillamine, a Cu chelating agent, suppressed the sensitization effect of Cu-pretreatment due to its ability to sequester Cu ions released from oxidized MT. These data suggest that Cu-MT has a prooxidative property and that Cu released from MT may be responsible for the prooxidative function of Cu-MT.

Results from Chapters four and five showed that the metal ions released from MT following oxidation of MT thiolate groups influence the sensitivity of Ehrlich cells to oxidative stress; Zn-MT protected against oxidative stress-induced toxicity due, at least in part, to the antioxidant properties of Zn ions while Cu-MT enhanced sensitivity to oxidative stress-induced toxicity due to the prooxidant properties of Cu ions. These data suggest that the metal composition of MT is a determinant of the function of MT in oxidative stress.

CHAPTER SIX

6.1 INTRODUCTION

The prooxidative property of Cu-MT as observed in Ehrlich cells (Chapter 5) has not been reported by other investigators. Therefore, additional studies were carried out to investigate the toxicological significance of the prooxidative property of Cu-MT in vivo. The developing guinea pig was used because hepatic Cu-MT concentrations have been reported to be very high in the 3-day old guinea pigs but decline rapidly to the low adult levels by day 7 of life (Lui, 1987; Srai et al., 1986). Accordingly, susceptibility of 3 day-old, 7-day-old and adult animals to Fe-induced oxidative stress in vivo was examined. Moreover, the responsiveness of 7-day-old animals pretreated with $CuSO_4$, an MT-inducing agent, to oxidative stress was also examined to better define the role of Cu and Cu-MT in the modulation of Fe-induced oxidative injury.

Ferric-nitrilotriacetate (FeNTA), being one of the more potent iron prooxidant compounds, was used in this study to induce oxidative stress (Goddard et al., 1986; Goddard and Sweeney, 1983). It, unlike other Fe complexes, produces a rapid and preferential deposition of iron in hepatocytes (Goddard and Sweeney, 1983; Bacon et al., 1983). FeNTA exerts its toxicity by stimulating the generation of reactive oxygen species in liver by undergoing NADPH-cytochrome-P-450 reductase-dependent cyclic oxidation and reduction reactions that require molecular oxygen and reducing equivalents (Goddard et al., 1986).

In vitro studies using incubations containing hepatic microsomes, NADPH and

cytosols from different age-groups were also carried out to examine the existence of possible correlation of hepatic Cu-MT in the different age groups and the magnitude of the observed oxidative stress-induced toxicity. D-penicillamine, a Cu-chelating agent, was also used to investigate the possible mechanisms underlying the role of MT-bound Cu in oxidative stress-induced toxicity.

6.2 MATERIALS AND METHODS

6.2.1 Animals and treatment.

Albino Hartley strain guinea pigs were obtained from Charles River Breeding Laboratories Inc., Quebec. All animals were housed in stainless-steel cages with free access to pelleted Purina laboratory guinea pig chow and distilled-deionized water. The animals were kept at room temperature (22-24°C) and were exposed to alternate cycles of 12 h light and darkness. Pregnant guinea pigs were caged separately and allowed to give birth normally and the litter size was adjusted to no more than three. The neonates were raised with their dams until they were used on days 3 and 7 after birth. Adult guinea pigs (500-550 grams body weight) were caged in groups of 4. Male guinea pigs were used throughout this study.

Guinea pigs were injected intraperitoneally with a single dose of $Fe^{+3}NTA$ (3.5 mg Fe/kg body weight) to induce oxidative stress. Injections were administered between 9-10 a.m. FeNTA solution was prepared shortly before use by the method of Goddard et al. (1986). For this, ferric nitrate and nitrilotriacetic acid were separately dissolved in distilled-deionized water and the solutions were mixed in a fourfold excess of NTA and

the pH was adjusted to 7.4 with 117 mM sodium bicarbonate. Control animals were treated with an equimolar concentration of NTA. To induce Cu-MT levels in the liver of 7-day-old guinea pigs, the animals were treated with a single intraperitoneal dose of cupric sulfate (0.5 mg Cu/kg body weight) 24 h earlier. Control animals were treated with an equivalent volume of 0.9% saline.

6.2.2 Experimental design.

IN VIVO STUDY: To examine the role of Cu-MT in FeNTA-induced oxidative stress, 3-day-old, 7-day-old and adult animals were treated with a single dose of FeNTA (3.5 mg FeNTA/kg, ip) and killed 0, 0.5, 1 or 2 h later. FeNTA-induced hepatotoxicity was assessed biochemically by measuring serum AST activity, hepatic reduced and oxidized GSH as well as microsomal lipid peroxidation levels. To further study the role of Cu-MT in FeNTA-induced oxidative stress, the 24 h Cu-pretreated 7-day-old guinea pigs were injected with FeNTA (3.5 mg FeNTA/kg body weight, ip) and examined 2 h later.

IN VITRO STUDY: To investigate the mechanism(s) underlying the age-related differences in FeNTA-induced hepatotoxicity, in vitro experiments were also carried out. Standard incubations were carried out in a 25 ml-Erlenmeyer flask with a final volume of 5.0 ml incubation mixture containing approximately 1.5 mg of washed hepatic microsomal protein, 10 mM NADPH, 50mM $MgCl_2$ and 30mM Tris-HCl, pH 7.4. The incubations were performed at 37°C with air as the gas phase in a metabolic shaker

oscillating at 100 cycles/min. Following a 3-min preincubation, the reactions were initiated by the addition of FeNTA to give a final concentration of 80 μM Fe in the incubation mixture. Aliquots of the incubation were removed at different times and the reactions were terminated by heat treatment in boiling water for 60 secs. The samples were centrifuged at 600 x g for 15 min in a Beckman TJ-6 centrifuge and the supernatant fractions were used for the measurement of lipid peroxidation, metal binding capacity, thiol content and Cu content of MT as well as reduced GSH levels when appropriate. Control incubations were conducted in the absence of FeNTA.

To examine whether or not the age-related differences observed in vivo were due to differences in the reactivity of hepatic microsomes, FeNTA-induced microsomal lipid peroxidation was measured in incubations containing microsomes isolated from animals of the 3 age groups.

To determine whether or not the enhancement in sensitivity to FeNTA-induced oxidative stress in 3-day-old guinea pigs was due to a "stimulating" factor(s) present in hepatic cytosols, FeNTA-induced peroxidation of microsomes isolated from liver of 3-day-old guinea pigs was measured in the presence of hepatic cytosols isolated from 3-day-old animals; cytosols isolated from livers of 7-day-old animals were used as negative controls. To investigate the heat-stability of the cytosolic factor(s) involved, hepatic cytosols isolated from 3-day-old or 7-day-old guinea pigs were heat-treated in boiling water for 1 min and centrifuged at 600 x g for 15 min and used instead of the normal cytosols.

To examine the nature of the interaction between FeNTA and Cu-MT, cytosols with

and without FeNTA treatment in vitro were treated with the sulphydryl reductant 1,4-dithiothreitol (DTT) and the metal binding capacity of MT was then measured by Ag-heme saturation method. The regeneration of metal binding sites would indicate Fe-induced oxidation of MT thiolates.

To examine the involvement of the release of Cu from MT in FeNTA-induced oxidative stress, standard incubations with or without 3-day-old cytosols were incubated in the presence of D-penicillamine, a Cu chelating agent. To examine whether the protective effect of D-penicillamine was due to sequestration of Cu released from MT subsequent to oxidation of MT thiolates, aliquots of the standard incubations were removed 30 minutes following addition of FeNTA, centrifuged at 105,000 x g for 60 min and chromatographed on a Sephadex G-75 column.

6.2.3 Tissue preparation.

Blood samples were collected from animals by cardiac puncture under light ether anesthesia. The collected blood was allowed to clot and was centrifuged; the serum was stored at -80°C and used within 48 h for enzyme analysis.

Livers were removed immediately after decapitation and washed in ice cold saline to remove excess blood. All subsequent steps were carried out at 0-4°C. Following washing, livers were quickly weighed and finely minced. Approximately 3 gram of liver sample was homogenized with a Potter-Elvehjem glass homogenizer and a loose-fitting Teflon pestle in a sufficient volume of 1.15% KCl-50 mM Tris-HCl buffer, pH 7.4, to

produce a 20% homogenate. The homogenate was centrifuged at 9000 x g for 10 min in a refrigerated Sorval RB-5 centrifuge. The post-mitochondrial supernatant was decanted and re-centrifuged at 105,000 x g for 60 min in a refrigerated Beckman L8-55 ultracentrifuge equipped with a Beckman 50.2Ti rotor to obtain the cytosolic and microsomal fractions. The microsomal fractions obtained from livers of control animals were resuspended in 150 mM Tris-HCl, buffer pH 7.4 and re-centrifuged at 105,000 x g for 60 min. The washed microsomal pellet was resuspended in 30 mM Tris-HCl buffer pH 7.4, and the microsomal suspension (approximately 10 mg/ml) was stored at 4°C and used within 2h for the in vitro study. For the measurement of lipid peroxidation in vivo, microsomal fractions were prepared as described previously except that the homogenizing medium contained 3 mM ethylenediamine-tetraacetic acid (EDTA).

6.2.4 Biochemical analyses and procedures.

6.2.4.1 Measurement of superoxide dismutase activity.

Copper/zinc superoxide dismutase (SOD) in hepatic cytosols was measured using the technique based on inhibition of pyrogallol (1,2,3-benzenetriol) autooxidation as described section 4.2.6.5.

6.2.4.2 Measurement of catalase activity.

Catalase activity in sonicated hepatic homogenates was determined spectrophotometrically by following the disappearance of hydrogen peroxide at 240 nm

as described in section 4.2.6.6.

6.2.4.3. Measurement of aspartate aminotransferase activity.

Activities of serum aspartate aminotransferase (AST) were determined by the method of Reitman and Frankel (1957), with a Sigma Diagnostic Kit No. 505. Enzyme activity was expressed as Sigma Frankel (SF) units per milliliter of serum.

6.2.4.4 Protein determination.

Protein determinations were estimated by the methods of Lowry et al. (1951), using crystalline bovine serum albumin (BSA) as the standard as described in section 3.2.4.5.

6.2.4.5 Determination of lipid peroxidation.

In vivo: Hepatic microsomes from treated and control animals was assayed for the presence of lipid-conjugated dienes and thiobarbituric acid reactants according to the method of Recknagel and Glende (1984) and Buege and Aust (1978), respectively. For the measurement of lipid diene conjugates, microsomal suspension (1.0 ml) ,was extracted with 5.0 ml of chloroform:methanol (2:1) and the extract was dried under a stream of nitrogen and the chloroform-free residue was then redissolved in cyclohexane (1.5 ml spectrophotometric grade) and absorbance at 243 nm was recorded against a cyclohexane blank. For the measurement of thiobarbituric acid reactants, microsomal fractions (0.5 ml) were added to 10% trichloroacetic acid and centrifuged at 600 x g for 3 min and the resulting supernatants were added to 1% thiobarbituric acid solution and

the mixture was incubated at 110°C for 10 min and the absorbance of the solution was measured 535 nm.

In vitro: In the in vitro study, TBA reacting material was taken as a measure of lipid peroxidation and was measured as described earlier. The change in absorbance at 535 nm was read against the value of the time zero sample.

6.2.4.6 Reduced and total glutathione concentration.

Reduced and total glutathione, more precisely non-protein sulphydryl, in hepatic cytosols was determined as described in section 4.2.6.8.

6.2.4.7. α-Tocopherol determination.

α-Tocopherol concentrations of hepatic tissues was determined as described in section 4.2.6.9.

6.2.4.8 Metallothionein determination.

Metallothionein concentrations from hepatic cytosols were estimated indirectly by measuring the metal binding capacity of the protein by the Ag-heme saturation method as described in section 4.2.6.10. Because of the close resemblance in amino acid composition between rat and guinea pig liver MT (Stillman et al., 1986), the concentration of MT in the samples was calculated by assuming that 18 g.atom of Ag is bound to each mole of thionein (Scheuhammer and Cherian, 1986) which has a molecular weight of 6050 as determined by amino acid analysis of rat hepatic MT (Onosaka and

Cherian, 1982).

6.2.4.9 Measurement of thiol content of MT.

The thiol content of MT of heat-treated supernatants isolated from cytosols in vivo or in vitro incubation mixtures were determined as described in section 4.2.6.11.

6.2.4.10 Sephadex G-75 gel filtration study.

To study the chromatographic separation profile of cytosolic Cu and Zn, an aliquot (2 ml) of hepatic cytosol was chromatographed at room temperature on a calibrated Sephadex G-75 column (1.5 cm x 55 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.6), containing 5 mM 1,4-dithiothreitol to prevent air oxidation of Cu-MT, and eluted with the same buffer at a rate of 1.0 ml/min. Fractions of 2 ml were collected and used directly for the quantitation of Cu and Zn by atomic absorption spectrophotometry.

6.2.4.11 Regeneration of metal binding sites of MT by 1,4 dithiothreitol (DTT).

To determine whether the FeNTA-induced metal binding loss of MT was due to oxidation of MT thiolates, heat-treated supernatant fractions of control and FeNTA-treated samples obtained from the in vivo experiments, were treated with the reducing agent DTT as described in section 4.2.7.13.

6.2.4.12 Metal analysis.

Estimation of total, cytosolic and microsomal Cu, Zn and Fe concentrations of

hepatic tissues was performed following wet tissue digestion as described in section 4.2.9.

6.2.5 Statistical analysis.

Results were analyzed by two-way analysis of variance (ANOVA) (Gad and Weil, 1982). The significance level was established at the $p<0.05$ level.

6.3 RESULTS

As shown in figure 6.1, serum AST and hepatic microsomal lipid peroxidation of control guinea pigs showed no age-related difference; however, their responses observed within the 2 h FeNTA treatment were age-related. The 3-day-old guinea pig showed a time-dependent increase in serum AST activity, which was not observed in 7-day-old or adult animals. Lipid peroxidation of microsomes as measured by the formation of diene conjugates (fig. 6.1B) and thiobarbituric acid reactants (fig. 6.1C) showed peak activities at 0.5 h after FeNTA administration and this treatment effect was higher in 3-day-old animals than in 7-day-old or adults animals. Data presented in figure 1 also showed that the hepatotoxic response in 7-day-old guinea pigs could be increased by 24 h Cu-pretreatment.

Levels of antioxidants (GSH and α-tocopherol) and antioxidative enzymes (SOD and CAT) in the liver of control guinea pigs did not show any significant differences among different age groups (Table 6.1). FeNTA treatment reduced hepatic GSH concentration and the reductions were of greater magnitude in the 3-day-old animals (Table 6.1). Also,

Figure 6.1. Effect of age- and Cu-pretreatment on iron nitrilotriacetate (FeNTA) (3.5 mg FeNTA/kg, ip)-induced changes in serum AST activity (A) and hepatic microsomal lipid peroxidation as measured by the formation of thiobarbituric acid reactants (B) and diene conjugates (C) in guinea pigs. Values represent mean ± SEM, (n = 4 animals). 7-day-old guinea pigs received a single dose of $CuSO_4$ (0.5 mg/kg, ip) 24 h prior to FeNTA (3.5 mg/kg, ip) treatment.

a. significantly different from corresponding time-matched NTA-treated control values, $p < 0.05$ (NTA treated time zero controls [not shown] were not significantly different from those of the corresponding non-treated values time-zero controls.

b. magnitude of FeNTA-induced changes in 3-day-old and Cu-pretreated 7-day-old guinea pigs differ significantly from that observed in 7-day-old and adult animals, $P < 0.05$.

3-day-old
7-day-old
adult
Cu-pretreated 7-day-old
A
SERUM AST ACTIVITY
(SF units/ml)
300
250
200
150
100
50
0
0
0.5
1.0
2.0
TIME (hours)
B
DIENE CONJUGATES
(Abs at 243 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C
TBA REACTANTS
(Abs at 535 nm)
.20
.15
.10
.05
0

TABLE 6.1
Antioxidative activities in the liver of developing guinea pigs
and their response to FeNTA treatment in vivo[a]

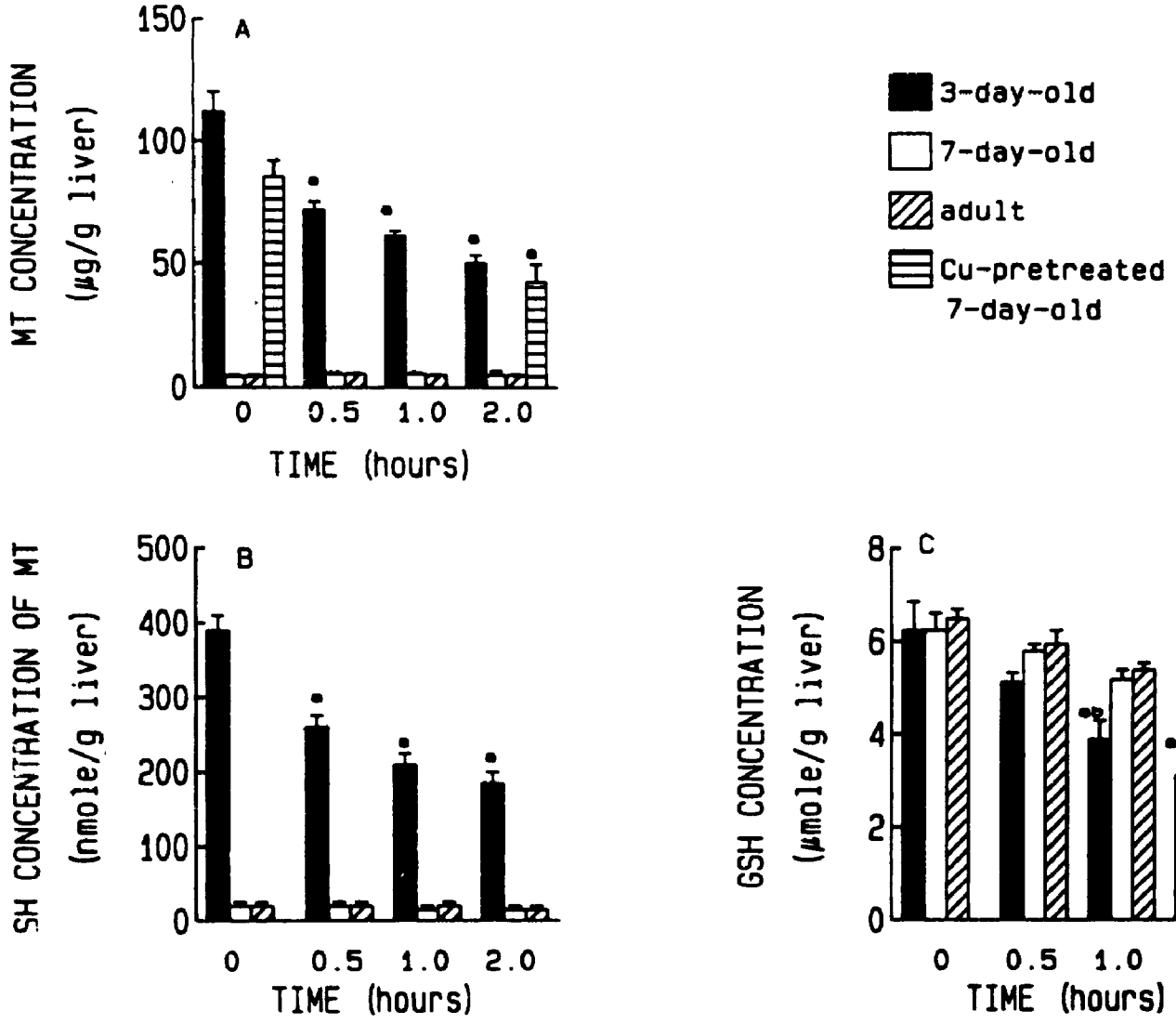
	NTA TREATED				FeNTA TREATED			
	3 day-old	7 day-old		adult	3 day-old	7 day-old		adult
		saline	Cu pretreated			saline	Cu pretreated	
SOD U/mg protein	15.0±2.0	14.6±1.7	13.8±2.1	38.7±1.2	-	-	-	-
Catalase U/mg protein	452±19	477±20	492±22	890±17	-	-	-	-
Total GSH μmole/g liver	6.4±0.1	6.4±0.2	6.2±0.1	6.7±0.3	6.3±0.3	6.2±0.4	6.1±0.3	6.67±0.5
Reduced GSH μmole/g liver	6.3±0.6	6.3±0.4	6.1±0.2	6.5±0.2	3.1±0.5[de]	5.00±0.3[d]	3.1±0.2[de]	5.1±0.3[d]
Oxidized GSH μmole/g liver	0.10±.01	0.13±0.03	0.15±.03	0.24±.05	3.2±0.3[de]	1.2±0.3[d]	2.9±0.2[de]	1.6±0.5[d]
α-tocopherol μg/g liver	8.6±0.3	8.0±0.5	8.2±0.2	8.64±0.3	-	-	-	-

a. Values represent mean ± SEM (n = 4 animals)
b. One unit of SOD activity is defined as the amount of SOD that inhibits the autooxidation of pyrogallol by 50%
c. One unit of catalase activity is defined as the amount of catalase required to decompose one μmole of H_2O_2/min at 25°C
d. Values of FeNTA-treated groups differ significantly from those of age-matched NTA-treated group, $p<0.05$
e. Magnitudes of FeNTA-induced changes in 3-day-old and Cu-pretreated 7-day-old guinea pigs differ significantly from those of corresponding 7-day-old and adult control guinea pigs, $p<0.05$.

Figure 6.2. Time-course of the effect of iron nitrilotriacetate (FeNTA) (3.5 mg/kg, ip) on MT concentration (A), sulphydryl concentration of MT (B) and reduced GSH concentrations (C) in 3 day-old, 7 day-old, adult and 7 day-Cu-pretreated guinea pigs. Seven day-old guinea pigs received a single dose of $CuSO_4$ (0.5 mg/kg, ip) 24 h prior to FeNTA (3.5 mg/kg,ip) treatment. Values represent mean $\pm$ SEM, (n = 4 animals).

a. significantly different from corresponding time-matched NTA-treated control values, $p<0.05$. (NTA-treated time zero controls [not shown] were not significantly different from the non-treated time-zero controls.

b. magnitude of FeNTA-induced changes in 3-day-old and Cu-pretreated-7-day-old guinea pigs differ significantly from that observed in 7-day-old and adult animals, $p<0.05$.


A
MT CONCENTRATION
(µg/g liver)
150
100
50
0
0
0.5
1.0
2.0
TIME (hours)
3-day-old
7-day-old
adult
Cu-pretreated
7-day-old
B
SH CONCENTRATION OF MT
(nmole/g liver)
500
400
300
200
100
0
0
0.5
1.0
2.0
TIME (hours)
C
GSH CONCENTRATION
(µmole/g liver)
8
6
4
2
0
0
0.5
1.0
2.0
TIME (hours)

Cu-pretreatment of 7-day-old guinea pigs enhanced the extend of treatment effect (Table 6.1).

To study the possible role of Cu-MT in the modulation of hepatic response to FeNTA-induced oxidative stress, the developmental profile of hepatic Cu-MT and the treatment-related oxidation of MT was examined. As shown in figure 6.2, MT concentration, as determined indirectly by the Ag-heme saturation method, and its thiol content in hepatic cytosols were 27-fold higher in the 3-day-old animals than in the 7-day-old and adult animals. These parameters were decreased in a time-dependent manner reaching 48% to 52% reductions by 2 h following FeNTA treatment in 3-day-old guinea pigs, while no such effects were observed in 7-day-old and adult animals. Hepatic MT concentration of 7-day-old animals was increased by 20-fold 26 h after Cu administration and this was decreased by 50% 2 h following FeNTA treatment (fig. 6.2). To determine whether the Fe-induced loss of metal binding capacity of MT was due to oxidation of the protein thiolate groups, hepatic cytosols were treated with a reducing agent in vitro. Incubation of samples isolated from 3-day-old and Cu-pretreated 7-day-old control animals with DTT resulted in slight but significant increases in the Ag-binding capacity of MT, presumably due to the restoration of the thiolate groups that were oxidized during isolation procedures (fig. 6.3). Moreover, such treatment restored the metal-binding capacity of MT that was lost following FeNTA treatment in vivo, thus indicating an oxidative rather than a non-specific destructive effect of FeNTA on MT and also ruling out the possibility of the release of the metalloprotein from damaged hepatocytes (fig. 6.3). It should be noted that the MT sulphydryl concentration of hepatic cytosols of 3-

Figure 6.3. In vitro DTT-dependent regeneration of metal binding capacity of MT of hepatic cytosols obtained from 3-day-old (A) and Cu-pretreated 7-day-old (B) guinea pigs 2h following FeNTA (3.5 mg /kg, ip) treatment. Heat-treated cytosolic fractions from control and FeNTA-treated animals were treated with 5 mM DTT under nitrogen at room temperature for 30 min and the metal binding capacity was estimated by the Ag-heme saturation method. Values represent mean $\pm$ SEM, (n = 4 animals).

a. significantly different from corresponding NTA-treated value, $p < 0.05$

b. significantly different from corresponding non-DTT-treated samples, $p < 0.05$.

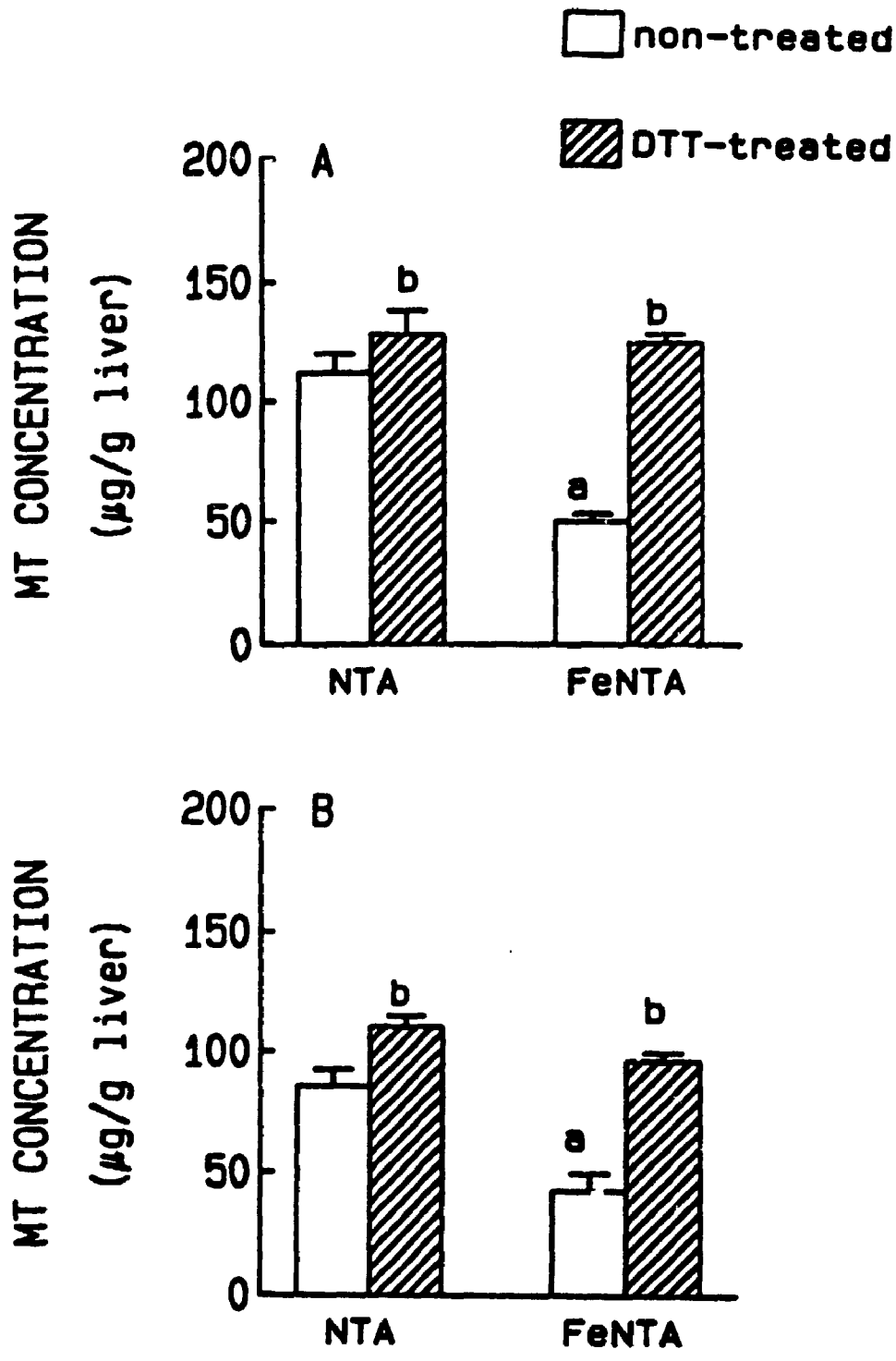

non-treated
DTT-treated
A
MT CONCENTRATION (µg/g liver)
200
150
100
50
0
b
b
a
NTA
FeNTA
B
MT CONCENTRATION (µg/g liver)
200
150
100
50
0
b
b
a
NTA
FeNTA

TABLE 6.2
Total, cytosolic and microsomal metal concentrations of guinea pig livers
2-hours after FeNTA (3.5 mg/kg,ip) administration[a]

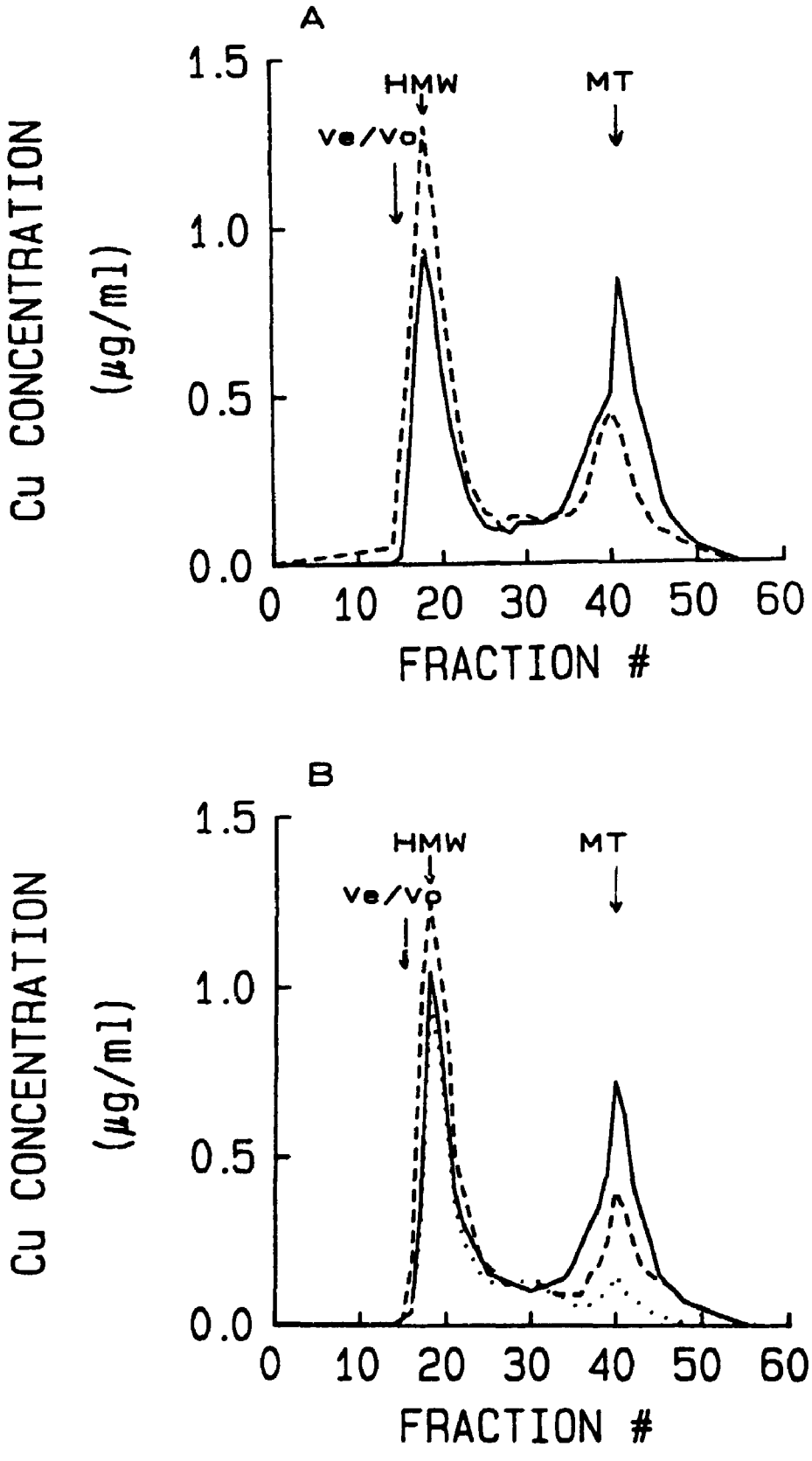
GROUP	NTA TREATED				FeNTA TREATED			
	3 day-old	7 day-old		Adult	3 day-old	7 day old		adult
μg/g liver		Saline	Cu-pretr.[b]			Saline	Cu-pretr.	
COPPER								
Total	115.4±10.5[c]	18.9±2.0	62.4±3.4[c]	11.6±2.3	118.6±9.6	19.4±1.8	70.4±5.2	16.7±4.9
Cytosolic	41.5±3.1[c]	10.5±1.3	30.1±2.8[c]	7.8±1.1	41.5±2.5	12.9±1.0	40.1±2.7	7.5±1.1
Microsomal	17.8±3.2[c]	4.9±0.8	4.9±0.8[c]	3.6±0.5	18.0±2.3	5.0±0.7	5.1±0.1	3.3±0.5
IRON								
Total	105.9±10.0[c]	57.3±5.3	57.4±2.8	52.1±4.7	140.4±9.9[d]	87.5±3.4[d]	99.9±3.7[d]	124.0±8.5[d]
Cytosolic	32.0±3.1	35.0±3.4	33.0±2.1	34.3±3.1	59.0±13.9[d]	56.0±2.8[d]	60.0±1.7[d]	69.4±6.1[d]
Microsomal	50.1±3.7[c]	15.7±2.5	16.0±1.7	12.2±2.1	85.5±3.7[d]	22.9±2.5[d]	24.8±1.9[d]	28.0±3.5[d]
ZINC								
Total	23.6±1.1	25.6±3.7	47.1±2.9[c]	24.4±2.5	20.0±2.2	26.0±2.8	42.0±3.4	27.4±3.4
Cytosolic	10.7±0.9	11.5±1.6	20.9±1.1[c]	10.5±1.1	10.9±1.0	11.9±2.1	18.5±1.3	11.5±1.1
Microsomal	9.08±2.1[c]	6.5±1.5	6.7±0.8	1.8±0.2	7.6±1.5	6.3±1.1	6.8±1.0	1.2±0.4

a. Values represent mean ± SEM (n = 4 animals)
b. 7-day-old guinea pigs received a single dose of $CuSO_4$ (0.5 mg/kg, ip) 24h prior to FeNTA treatment
c. Significantly different from corresponding 7 day-old and adult control calues, $p < 0.05$.
d. Values of FeNTA-treated group differ significantly from age-matched NTA-treated group, $p < 0.05$.

day-old guinea pig were 6% of tissue GSH concentration; however, the fractional loss (%) of sulphydryl groups within the two hours of treatment was greater with MT (fig. 6.2).

Total Cu concentrations of hepatic tissues were 6- and 10-fold higher in 3-day-old animals as compared to 7-day-old and adult animals, respectively (Table 6.2). Cytosolic Cu concentrations, which accounted for approximately 36 - 67% of total, were approximately 4-fold higher in 3-day-old animals as compared to the other two age groups. Age-related difference also existed in microsomal Cu concentration but the magnitude of the difference was much smaller as compared to that of cytosolic Cu (Table 6.2). Copper pretreatment in 7-day-old guinea pigs increased both total and cytosolic Cu concentrations of hepatic tissues by approximately 3-fold without altering those of the microsomal fraction. As shown in table 6.2, these hepatic Cu parameters of the 4 experimental groups were not significantly affected by FeNTA administration. However, FeNTA did alter the distribution of Cu in the hepatic cytosol of 3-day-old and Cu-pretreated 7-day-old animals. As shown in figure 6.4, Sephadex G-75 gel filtration study of hepatic cytosols revealed that Cu was associated with both the HMW (51%) and MT (46%) fractions of 3-day-old guinea pigs while Cu binding was restricted only to the HMW fraction in both 7-day-old and adult animals. Copper pretreatment of 7-day-old animals resulted in significant increases in cytosolic Cu with most of it being bound to the MT fraction (52%) (fig. 6.4B). Two hours following FeNTA treatment, it was observed that the binding of Cu to MT fraction was reduced by 46% in both 3-day-old and Cu pretreated 7-day-old animals, with a concurrent increment in the Cu content of

Figure 6.4. Distribution of Cu in hepatic cytosols of 3-day-old (A) and Cu-pretreated 7-day-old (B) guinea pigs 2h following FeNTA (3.5 mg FeNTA/kg, ip)(------) or NTA (———) injection. Normal 7-day-old guinea pigs (·····) received a single dose of $CuSO_4$ (0.5 mg Cu/kg,ip) 24 h prior to FeNTA treatment. Hepatic cytosols were isolated from homogenates pooled from 4 animals and were then chromatographed on a Sephadex G-75 column equilibrated with 10 mM Tris-HCl buffer, pH 8.6 at room temperature. Two milliliter fractions were collected at an elution rate of 1 ml/min and measured for Cu by atomic absorption spectrophotometry. Each cytosolic sample was analyzed chromatographically twice and the average values were used for the construction of the chromatogram.


A
1.5
1.0
0.5
0.0
HMW
MT
Ve/Vo
Cu CONCENTRATION
(µg/ml)
0
10
20
30
40
50
60
FRACTION #
B
1.5
1.0
0.5
0.0
HMW
MT
Ve/Vo
Cu CONCENTRATION
(µg/ml)
0
10
20
30
40
50
60
FRACTION #

the HMW fraction (fig. 6.4). This treatment had no apparent effects on the distribution of cytosolic Cu in the 7-day-old and adult animals (data not shown).

Although Zn and Zn-MT are known to have antioxidant properties (Thomas et al., 1986; Bray and Bettger, 1990), they did not contribute to the observed age-related difference in FeNTA -induced toxicity since there were no age-dependent differences in total, cytosolic or microsomal Zn concentrations (Table 6.2). However, pretreatment of 7-day-old guinea pigs with $CuSO_4$ resulted in approximately 2-fold increases in the total and cytosolic Zn concentrations of the liver. Examination of the gel filtration profile of hepatic cytosols isolated from control animals receiving no FeNTA treatment revealed that no measurable amounts of Zn was associated with the MT fraction in the 3 age groups, although small amounts of MT-bound Zn were present in Cu-pretreated 7-day-old animals (data not shown). Therefore results of the gel filtration study indicate that hepatic MT of 3-day-old guinea pigs existed basically in the form of Cu-MT, which was depleted by day 7 of age.

The data pertaining to the possible role of hepatic Fe deposition in FeNTA toxicity is shown in table 6.2. In the control groups, total and microsomal Fe concentrations of 3-day-old guinea pigs were higher than those measured in 7-day-old and adult animals, while cytosolic Fe concentrations were similar in all groups. The amounts of Fe (approximately 35 $\pm$ 3 μg Fe/g) deposited in livers of 3-day-old, 7-day-old and Cu-pretreated 7-day-old animals after FeNTA treatment were similar with the majority of this Fe being recovered from the cytosolic fraction (Table 6.2).

To elucidate the mechanism(s) underlying the age- and Cu-pretreatment-related

TABLE 6.3
Influence of Hepatic Cytosols Isolated From 3-Day-Old or 7-Day-Old Guinea Pigs on FeNTA-Induced Microsomal Lipid Peroxidation In Vitro[a]

Incubation Condition[b]			TBA Reactants (Abs 535 nm)[c] Time (min)		
Microsomes	Cytosol	Basal(-) FeNTA(+)	15	30	60
3-day-old	-	—	0.015 ± 0.005	0.040 ± 0.010	0.060 ± 0.010
	-	+	0.320 ± 0.020[d]	0.580 ± 0.030[d]	0.790 ± 0.050[d]
7-day-old	-	—	0.020 ± 0.008	0.040 ± 0.010	0.050 ± 0.015
	-	+	0.345 ± 0.035[d]	0.610 ± 0.045[d]	0.805 ± 0.040[d]
Adult	-	—	0.025 ± 0.005	0.035 ± 0.005	0.060 ± 0.010
	-	+	0.310 ± 0.020[d]	0.590 ± 0.030[d]	0.815 ± 0.025[d]
3-day old	3-day-old	—	0.025 ± 0.005	0.055 ± 0.010	0.080 ± 0.020
		+	0.580 ± 0.030[d,e]	1.100 ± 0.080[d,e]	1.390 ± 0.120[d,e]
3-day old	7-day-old	—	0.025 ± 0.005	0.055 ± 0.010	0.065 ± 0.015
		+	0.400 ± 0.050[d]	0.620 ± 0.040[d]	0.805 ± 0.075[d]

a Values represent mean ± SEM, (n = 3 experiments).

b Incubations were carried out at 37°C under air atmosphere in a final volume of 5.0 ml incubation mixture containing approximately 1.5 mg washed microsomal protein and NADPH. Reactions were initiated by the addition of 80 μM FeNTA.

c After 15, 30 or 60 min of incubation, 0.5 ml of incubation mixture was added to 1.0 ml of 10% TCA and TBA reactants were measured at 535nm

d Value of FeNTA-treated sample differs significantly from its corresponding basal value, $p < 0.05$.

e Magnitude of FeNTA-induced changes in presence of cytosols isolated from livers of 3-day-old guinea pigs differs significantly from those observed in the present or absence of cytosols isolated from 7-day-old guinea pigs, $p < 0.05$.

differences in FeNTA toxicity, the following in vitro experiments were carried out. Firstly, incubation of 80 μM FeNTA with microsomes isolated from 3-day-old, 7-day-old or adult animals resulted in similar increases in microsomal lipid peroxidation as measured by the formation of thiobarbituric acid reactants, thus showing lack of age-related differences in the reactivity of hepatic microsomes to FeNTA-induced toxicity (Table 6.3). Secondly, the role of cytosolic factor(s) in the modulation of Fe toxicity was studied by measuring the effect of hepatic cytosols isolated from 3-day-old and 7-day-old animals on Fe-induced microsomal lipid peroxidation in vitro. As shown in table 6.3, the presence of cytosol isolated from 3-day-old guinea pig livers increased the peroxidation of microsomal lipids isolated from 3-day-old animals by 74% as compared to those in the absence of cytosol, while 7-day-old cytosol had no apparent effects at all (table 6.3). Result of this study suggest the presence of a stimulatory cytosolic factor(s) in 3-day old animals, which disappeared between day 3 and 7 postpartum and that age-related decreases in sensitivity to FeNTA-induced oxidative stress was not due to emergence of a protective mechanism during development.

Since MT is known to be relatively heat stable, hepatic cytosols of 3-day-old guinea pigs were heated for 1 minute and the supernatant was tested for its ability to stimulate FeNTA-induced lipid peroxidation following removal of the aggregated protein(s). As shown in figure 6.5A, the stimulatory effect of cytosol from 3-day-old guinea pigs was not reduced by prior heat treatment; this was consistent with the heat-stability property of Cu-MT. To ascertain the specificity of the heat treatment procedures on hepatic cytosols, other parameters were also examined. Heat-treatment of cytosols resulted in

TABLE 6.4

Effect of Heat-treatment on Copper, Iron, MT and GSH Concentration of Cytosols Isolated from Livers of 3-Day-Old and 7-Day-Old Guinea Pigs[a]

	Non-heat-treated cytosols		Heat-treated cytsols[b]	
μg/ml cytosol	3-day-old	7-day-old	3-day-old	7-day-old
Cu				
Total	11.52 ± 1.71	4.20 ± 0.80	6.45 ± 0.30[f]	0.93 ± 0.11[f]
HMW-bound[c]	5.90 ± 0.40	4.10 ± 0.90	0.60 ± 0.05[f]	0.82 ± 0.12[f]
MT-bound[c]	5.30 ± 0.40	ND	5.10 ± 0.65	ND
Fe				
Total	8.90 ± 0.90	9.75 ± 1.10	0.80 ± 0.09[f]	0.71 ± 0.06[f]
HMW-bound[c]	7.60 ± 0.82	7.80 ± 0.45	0.67 ± 0.03[f]	0.61 ± 0.14[f]
MT-bound	ND[g]	ND	ND	ND
MT[d]	26.70 ± 2.15	2.95 ± 0.30	24.75 ± 1.70	2.51 ± 0.30
GSH[e]	533.50 ± 15.80	522.41 ± 10.15	500.90 ± 13.65	485.54 ± 18.66

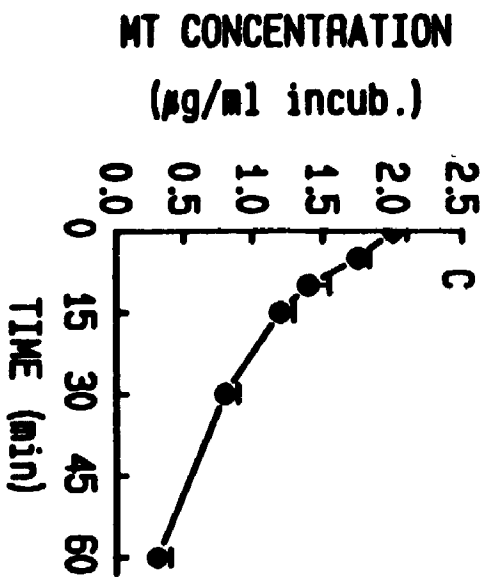
a Values represent mean ± SEM (n = 3 experiments).
b Cytosols from control guinea pigs were heat-treated for 1 min in boiling water, centrifuged at 600Xg for 15 min and the resulting supernatants were used to measure metals, MT and GSH concentrations.
c Measured by atomic absorption spectrophotometry following Sephadex G-75 chromatographic analysis of supernatants.
d Estimated by the Ag-heme saturation method.
e Estimated by titration with NbS_2.
f Significantly different from corresponding non-heat-treated sample value, $p<0.05$.
g ND - non-detectable.

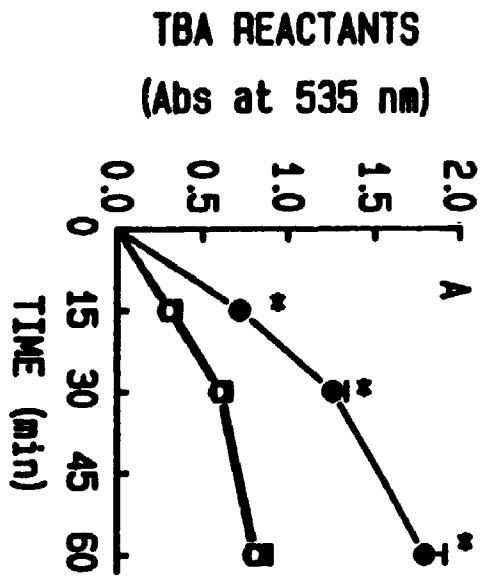
significant decreases in cytosolic Cu, Zn and Fe concentrations with no significant changes in the metal binding capacity of MT (Table 6.4). Sephadex G-75 filtration study revealed that heat-treatment did not reduce the binding of Cu to the MT fraction to any significant extent, but the binding of Zn, Cu and Fe to the HMW fraction was almost completely obliterated; this procedure did not, however, alter the reduced GSH levels (Table 6.4). It should be noted that heat-treatment of 7-day-old cytosol did not increase FeNTA-induced microsomal lipid peroxidation suggesting that the heat labile cytosolic cellular defence mechanisms such as SOD, GSH-Px and CAT, did not play an important role under these in vitro experimental conditions.

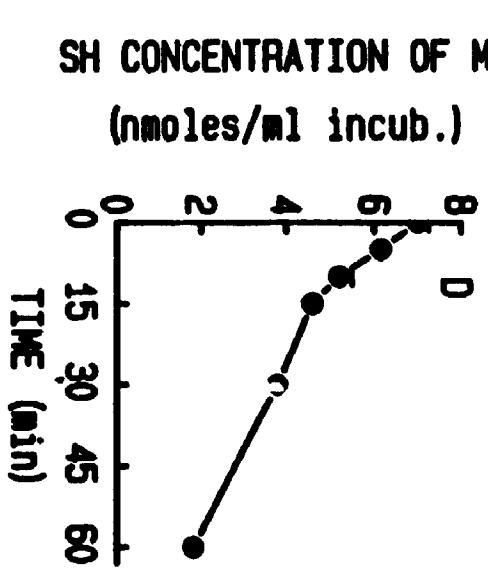
To further examine the involvement of Cu-MT in FeNTA-induced oxidative stress, the effect of FeNTA on cytosolic Cu-MT in vitro was also examined. As shown infigure 6.5C, FeNTA decreased the metal binding capacity of MT (as determined by the Ag-heme saturation method) in a time-dependent manner, which was accompanied by a parallel reduction in the thiol content of the metalloprotein (fig. 6.5B); a significant positive linear correlation (correlation coefficient, r=.987) was observed between these two parameters. In addition, the gel filtration profile (figure 6.6), showed that FeNTA treatment also reduced the binding of Cu ions to MT with a concurrent increment in the Cu content of the HMW fraction. To determine the role of the metal ions of Cu-MT in enhancing FeNTA-induced toxicity, the effect of D-penicillamine,a chelating agent for removing free Cu ions, on FeNTA-induced microsomal lipid peroxidation in vitro was examined. As shown in table 6.5, 15 μM D-penicillamine completely blocked the stimulatory effect of control cytosols of 3-day-old animals on microsomal lipid

Figure 6.5. Interaction between heat-treated cytosol from 3-day-old or 7-day-old guinea pig liver and FeNTA-induced oxidant stress. Changes in microsomal lipid peroxidation (A), GSH concentration (B), metal binding capacity of MT (C) and sulphydryl concentration of MT (D). The standard incubation was carried out at 37°C under air atmosphere in a final volume of 5.0 ml incubation mixture containing approximately 1.5 mg washed microsomal protein and NADPH and the reactions were initiated by the addition of 80 μM FeNTA. Aliquots were removed at 0, 15, 30 and 60 min of incubation period and were terminated by heat treatment in boiling water for 1.0 min. In vitro incubations were carried out in the absence of cytosols (○) or in the presence of heat-treated hepatic cytosols isolated from 3-day-old (●) or 7-day-old guinea pigs (□). Heat-treatment procedure in hepatic cytosols removed heat-labile proteinaceous components but not Cu-MT. Values represent mean ± SEM, (n = 5 experiments).

* magnitude of FeNTA-induced changes in incubations containing 3-day-old-heat-treated cytosols differ significantly from corresponding incubations containing 7-day-old heat treated cytosol or no cytosol, $p<0.05$.


MT CONCENTRATION
(µg/ml incub.)
0.0
0.5
1.0
1.5
2.0
2.5
C
TIME (min)
0
15
30
45
60


TBA REACTANTS
(Abs at 535 nm)
0.0
0.5
1.0
1.5
2.0
A
TIME (min)
0
15
30
45
60


SH CONCENTRATION OF MT
(nmoles/ml incub.)
0
2
4
6
8
D
TIME (min)
0
15
30
45
60

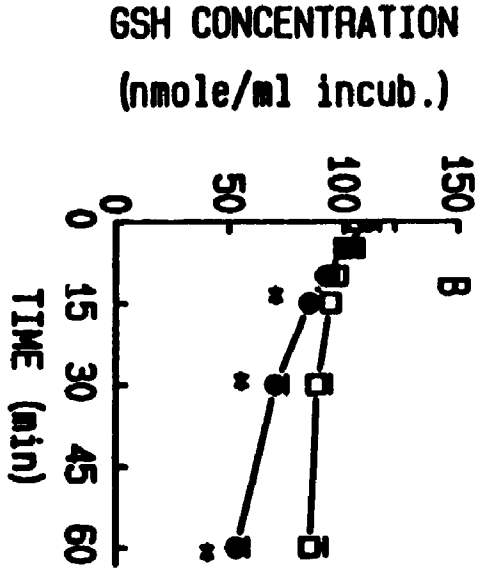

GSH CONCENTRATION
(nmole/ml incub.)
0
50
100
150
B
TIME (min)
0
15
30
45
60

Figure 6.6. Changes in distribution of Cu in hepatic cytosols of 3-day-old guinea pigs following incubation with FeNTA. The stardard incubation was carried out at 37°C under air atmosphere in a final volume of 5.0 ml incubation mixture containing approximately 1.5 mg washed microsomal protein and NADPH (———); the reactions were initiated by addition of 80 μM FeNTA (---) in the presence of 15 μM D-penicillamine (···) and incubations were carried out for 30 min. Aliquots of were pooled and chromatographed on a Sephadex G-75 column as described in figure 6.4 in triplicates.

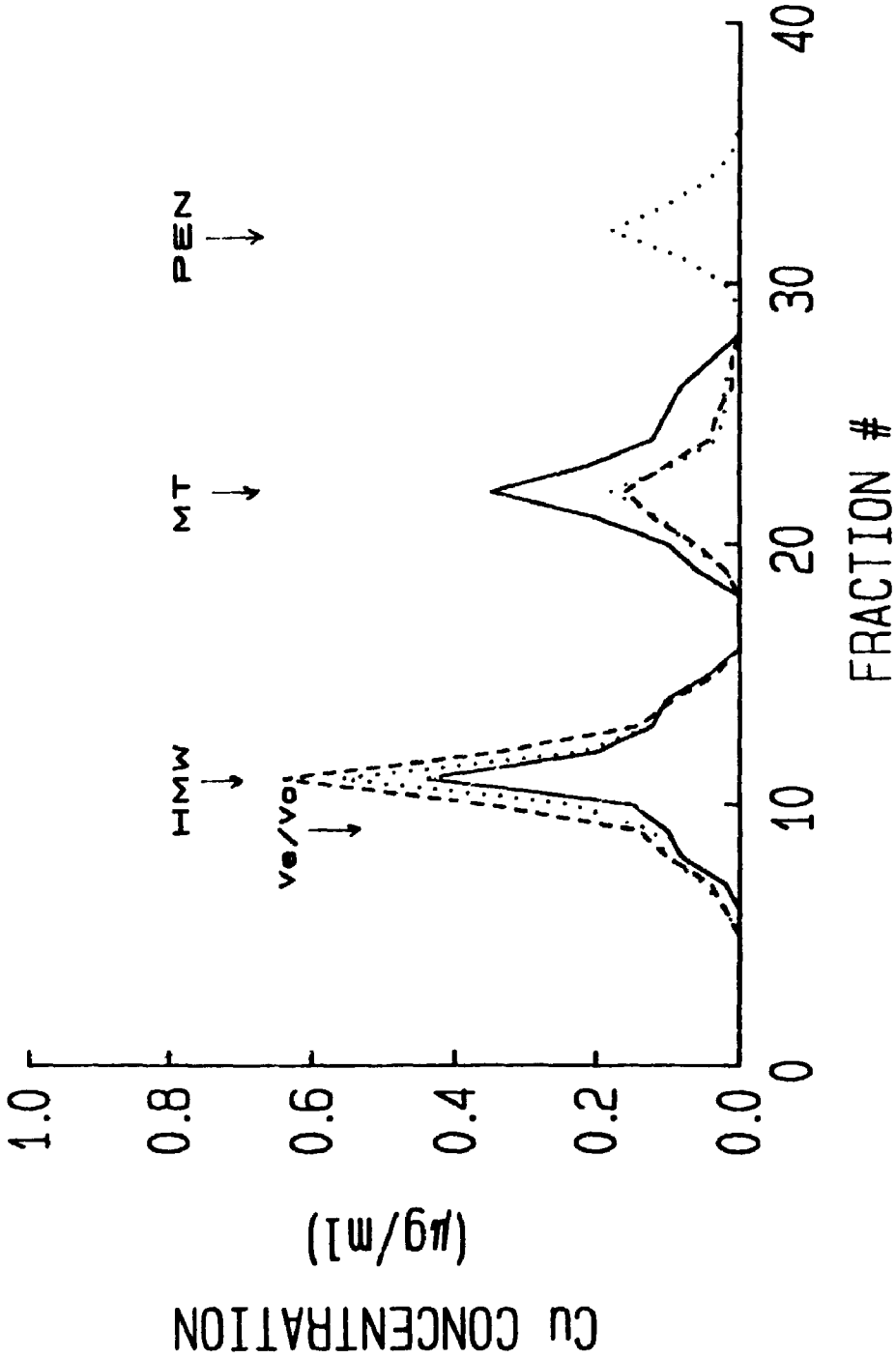

PEN
MT
HMW
Ve/Vo
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
FRACTION #
Cu CONCENTRATION
(μg/ml)

TABLE 6.5

Effect of D-penicillamine on FeNTA-induced change in microsomal lipid peroxidation and MT concentrations in incubations[a] containing no cytosol or control cytosols isolated from 3 day-old or 7 day-old guinea pigs[b]

	TBA REACTANTS (Abs 535 nm) Time (min)			MT CONCENTRATION[d] (μg MT/ml incubation) Time (min)			
Cytosol	0	30	60	0	15	30	60
NONE	0.023±0.005	0.045±0 005	0.065±0.005	-	-	-	-
+PEN	0.025±0.005	0.040±0.005	0.070±0.010	-	-	-	-
+FeNTA	0.320±0.020[e]	0.590±0.030[e]	0.790±0.045[e]	-	-	-	-
+PEN+FeNTA	0..350±0.020[e]	0.590±0.015[e]	0.795±0.065[e]	-	-	-	-
3 DAY-OLD	0.030±0.005	0.050±0.005	0.070±0.010	2.15±0.10	2.00±0.15	1.96±0.11	1.90±0.20
+PEN	0.025±0.005	0.045±0.005	0.078±0.005	2.05±0.15	1.95±0.10	1.93±0.08	1.87±0.15
+FeNTA	0.580±0.030[e]	1.100±0.090[e]	1.370±0.075[e]	2.15±0.10	1.20±0.15[e]	0.80±0.05[e]	0.45±0.06[e]
+PEN+FeNTA	0.330±0.025[ef]	0.620±0.055[ef]	0.810±0.035[ef]	2.10±0.20	1.25±0.10[e]	0.87±0.13[e]	0.54±0.09[e]
7 DAY-OLD	0.025±0.005	0.040±0.005	0.065±0.010	0.20±0.01	0.18±0.02	0.18±0.02	0.17±0.01
+PEN	0.020±0.005	0.045±0.005	0.075±0.005	0.21±0.02	0.19±0.01	0.18±0.02	0.17±0.01
+FeNTA	0.380±0.035[e]	0.620±0.050[e]	0.805±0.040[e]	0.21±0.01	0.08±0.01[e]	0.05±0.01[e]	0.03±0.01[e]
+PEN+FeNTA	0.350±0.020[e]	0.590±0.015[e]	0.795±0.065[e]	0.20±0.02	0.07±0.01[e]	0.04±0.01[e]	0.02±0.00[e]

a. The reaction was initiated by the addition of 80 μM FeNTA (where appropriate) to standard incubation mixture containing hepatic microsomes isolated from 3 day-old control guinea pigs.

b. Values represent mean±SEM from 5 experiments.

c. After 15, 30 and 60 min of incubation, 0.5 ml of incubation mixture was added to 1.0 ml of 10% TCA and TBA reactants were measured at 535 nm.

d. Estimated by the Ag-heme saturation method.

e. Value of FeNTA-treated sample differs significantly from its corresponding basal value, $p < 0.05$

f. Value of FeNTA-treated sample in the presence of penicillamine differs significantly from its corresponding FeNTA-treated value, $p < 0.05$

peroxidation. It should be noted that D-penicillamine did not have any significant effects on FeNTA-induced microsomal lipid peroxidation in the absence of cytosols or in the presence of cytosols isolated from liver of 7-day-old guinea pigs, suggesting that the observed protective effect was not likely due to its direct antioxidant properties. In addition, D-penicillamine did not alter the metal binding capacity of MT (Table 6.5) or the Cu content of the MT fraction (fig. 6.6) of hepatic cytosols of 3-day-old control animals in the absence of FeNTA exposure, suggesting that the protective effect of penicillamine was not due to removal of Cu ions from MT during the preincubation period (ie. before FeNTA treatment). As shown in table 6.5, 15 μM D-penicillamine did not alter the FeNTA-induced-loss of metal binding capacity of MT and Cu content from the MT fraction, but it did reduce the redistribution of Cu from MT to the HMW fraction suggesting that the protective effect of D-penicillamine was due to the sequestration of Cu released from MT subsequent to the oxidation of the protein thiolates.

6.4 DISCUSSION

Most studies conducted to examine the role of MT in chemical toxicity have focused on MT containing Zn and/or Cd (Clarke and Lui, 1986; Thomas et al., 1986; Cousins and Coppen, 1987) and Zn-MT is generally recognized to possess antioxidant property (Thomas et al., 1986; Thornalley and Vasak, 1985; Cousins and Coppen, 1987). However, results of the present study have shown that the presence of MT in the form of Cu-MT in hepatic tissues may actually be associated with heightened sensitivity to

FeNTA-induced hepatic toxicity indicating the importance of the metal complement of MT as a determinant in its toxicological function.

Decreases in GSH/GSSG ratio and increases in microsomal lipid peroxidation are generally recognized as sensitive indices of oxidative stress (Jenkinson et al., 1988; Girroti, 1985; Mitchell et al., 1984;). The observed changes in these parameters following FeNTA treatment would suggest that the treatment-related elevations in serum transaminase (AST) activity was related to oxidative injury. These findings were consistent with the reported toxicological properties of Fe compounds (Goddard et al., 1986; Bacon et al., 1983; Ganote and Nahara, 1973; Golberg et al., 1962) and more importantly the results suggest that age- and Cu-pretreatment related differences in Fe-toxicity were due to factors associated with the modulation of oxidative stress.

The developing guinea pigs used in the present study provided an appropriate model to examine the functional role of Cu-MT in oxidative stress. With regards to the metal content, hepatic MT from 3-day-old guinea pigs contained high levels of Cu with no measurable amounts of Zn allowing unambiquous interpretation of data. Moreover, the magnitude of the difference in hepatic MT and MT-bound Cu between 3-day-old and 7-day-old animals provided an excellent comparison between the 2 age groups to ascertain the role of Cu-MT in oxidative stress. Also, it is important that known determinants of oxidative stress have been shown in Table 6.1 and 6.2 of this study and other other studies not to vary between 3-day- old and 7-day-old animals; these include: i) Fe deposition in hepatic tissues following FeNTA treatment; ii) microsomal NADPH-cytochrome P-450 reductase activity (Mitchell, 1983) and iii) cellular defense system

such as superoxide dismutase, catalase, GSH , GSH-peroxidase (Rickett and Kelly, 1990) and α-tocopherol. These findings are further substantiated by the results of our in vitro studies showing: i) similar reactivity of hepatic microsomes isolated from 3-day-old, 7-day-old and adult animals to FeNTA-induced lipid peroxidation and ii) failure of hepatic cytosols isolated from 7-day-old animals to provide protection against FeNTA-induced lipid peroxidation in hepatic microsomes of 3-day-old and 7-day-old and adult animals. These data clearly indicate that the observed age-related decrease in sensitivity between day 3 and 7 postpartum was not due to development of a protective mechanism.

Data from the in vitro study suggest the presence of a "sensitizing" factor(s) in the liver of 3-day-old animals and its disappearance by day 7 postpartum, which corresponds very well to the ontogenic profile of hepatic Cu and Cu-MT (Lui, 1987; Srai et al., 1986). Moreover, elevation of hepatic Cu-MT in 7-day-old guinea pig by Cu-pretreatment and the associated increase in responsiveness to FeNTA toxicity in vivo also provide support of such a relationship between tissue Cu-MT and tissue sensitivity to oxidative stress. In addition, it was observed in our recent study (Chapter 5) that treatment of host mice with $CuSO_4$ resulted in the induction of Cu-MT in Ehrlich ascites tumor cells and enhanced their susceptibility to H_2O_2-induced oxidative stress in vitro. Furthermore, Cu-pretreatment has been associated with enhancement of other biological systems to prooxidants. Thus, pretreatment of mice with $CuSO_4$ resulted in increases in lethal and cardiac toxicity to adriamycin (Satoh et al., 1987) and decreases in life-span of mice exposed to paraquat (Kohen and Chevion, 1985). Addition of $CuSO_4$ to cell suspensions containing E. coli or hepatocytes enhanced the cytotoxicity of paraquat

(Kohen and Chevion, 1985) or diquat (Sandy et al., 1987) respectively, agents known to undergo redox cycling and induce oxidative stress (Kappus, 1987; Sandy et al., 1987). Although in these studies the role of MT has not been examined, the findings are supportive of our present observation. It should be noted that although our in vitro data strongly indicated the involvement of Cu-MT, the participation of non-MT bound Cu in vivo cannot be ruled out completely at this time.

The effect of Fe-induced oxidative stress on Cu-MT provides another line of evidence to support its involvement in oxidative stress. Data presented in figure 6.3 strongly suggest the Fe-mediated oxidation of MT thiolates and loss of metal binding capacity. Oxidation of MT thiolates has also been demonstrated in yeast Cu-MT with H_2O_2 (Richter and Weser, 1988; Hartman et al., 1984) or in incubation of hepatic pig Zn,Cu-MT(II) with xanthine/xanthine oxidase (Arthur et al., 1987). It appears that there is considerable evidence indicating interaction between FeNTA and Cu-MT both in vivo and in vitro.

The precise role of Cu-MT in oxidative stress is not known. Our hypothesis, which was based on previous findings with Zn-MT, is concerned with the participation of Cu ions in oxidative stress upon their release from MT following oxidant-dependent inactivation of binding sites. Evidence for the Fe-induced oxidation of Cu-MT in 3-day-old and Cu-pretreated 7-day-old animals and the related loss of Cu from MT have already been discussed in the above; moreover, in vitro studies provide additional data indicating the importance of Cu ions released from MT in the sensitizing effect of Cu-MT. Firstly, the effectiveness of the copper chelating agent, D-penicillamine, in

blocking the increases in Fe-induced microsomal lipid peroxidation produced by hepatic cytosols from 3-day-old animals, suggests that Cu ions lost from MT were involved in the enhancement of the sensitivity to FeNTA-induced oxidative stress. Secondly, Fe-induced changes in cytosolic Cu distribution also offer additional supports. As shown in figure 6.6, FeNTA treatment in vivo resulted in increases in Cu binding to HMW component with concurrent decreases in the Cu content of MT; this pool of Cu was most likely originated from MT as shown clearly in vitro studies in which other major sources of Cu were excluded from the incubation mixture. Although the high molecular weight Cu-binding component has not been characterized in this study, other reports have shown that the major binding component in this fraction is ferritin. Ferritin is generally considered as an Fe storage protein but more recent studies have shown that it also provides high capacity-low affinity binding for various metal ions, including Cu ions and has been suggested to act as a first line of defence against metal toxicity before the emergence of more specific detoxication mechanisms (eg. induction of MT synthesis) (Joshi, 1988). Therefore, the observed deposition of Cu to HMW component, probably ferritin, during FeNTA-induced oxidative stress in 3-day-old animals in vivo and in vitro may suggest the functioning of such a cellular defence mechanism. This interpetation was also consistent with the observed increases in the stimulating potency of cytosols isolated from 3-day-old animals after heat treatment, which is known to remove this ferritin-based Cu-detoxication mechanism. But more importantly, the translocation of Cu to the HMW component during oxidative stress would reflect a transient increase in the concentration of intracellular free Cu ion which may participate in the i) generation

of reactive oxygen species via a Fenton reaction (Aust, 1985; Halliwell and Gutteridge, 1984b), ii) propagation of membrane lipid peroxidation (Hochstein et al., 1980) and iii) oxidation of cellular sulphydryl groups (Hochstein et al., 1980) and thus contributing to the heightened FeNTA-induced oxidative injury observed in 3-day-old guinea pig.

Among MTs with different metal compositions, Cu-MT is known to be most susceptible to oxidation (Minkel et al., 1980; Geller and Winge, 1982); however, direct comparisons with the oxidation of other sulphydryl compounds, especially GSH in vivo have not been reported. Results of the present study showed that the fractional (%) loss or oxidation of Cu-MT thiolate groups was greater than that of GSH in the liver of 3-day-old guinea pig within the first hour following FeNTA treatment, despite the existence of much higher (15-fold) GSH than MT-thiolate concentration in the liver. Although the actual turnover rate of GSH in treated animals has not been determined, our findings suggest high reactivity of Cu-MT towards Fe-induced cellular oxidants. Furthermore, these data suggest that GSH-Px, the major cytosolic antioxidant system (Toyoda et al., 1989), may not be adequate to prevent oxidation or activation of Cu-MT during oxidative stress; this also points out the toxicological significance of Cu-MT in oxidant stress. However, it should be noted that the relative abundance of various reactive oxygen species generated in the FeNTA model system could have favoured the oxidation of Cu-MT. In this respect, it is interesting to note that our preliminary data from in vitro experiments carried out in the presence of SOD, CAT, mannitol or desferoxamine, suggest that FeNTA-dependent oxidation of Cu-MT involved primarily superoxide anion, while hydrogen peroxide is known to be a major oxidant for the GSH-Px system.

Therefore, it remains to determine whether similar relative reactivity exists between Cu-MT and GSH with other types of prooxidants which may be an important determinant of the toxicological significance of tissue Cu-MT.

It is apparent from the results of the present study that elevations of tissue Cu-MT concentration may result in increases in susceptibility to oxidative stress. This raises concerns of whether conditions or biological systems known to be associated with high hepatic levels of Cu or Cu-MT would be at risk to oxidant stress: this includes Wilson's disease, cholestatic syndromes and primary biliary cirrhosis, Indian childhood cirrhosis (Sternlieb 1980), active chronic hepatitis, as well as livers of human fetuses and newborn infants (Bloomer and Lee, 1978; Sass-Kortsak, 1965; Bruckman and Zondek, 1939). The prooxidative property of Cu-MT observed in the present study was apparently contradictory to the antioxidative function ascribed to Zn-MT (Thomas et al., 1986). This would make extrapolation of animal ontogeny data to humans difficult. While the liver of developing mammals shows marked elevations in MT concentration, the metal composition and content of MT are highly species specific. Perinatal liver of most rodent species, such as rat, mouse and rabbit, contains Zn-MT (Bakka and Webb, 1981; Mason et al., 1980) and hepatic MT of perinatal guinea pig is basicaly a Cu-metalloprotein (Lui, 1987); however, MT of human fetal livers contains high levels of both Cu and Zn (Riordan and Richards, 1980). Therefore, additional experiments using appropriate models are needed to determine the role of hepatic MT in oxidative stress during human development.

CHAPTER SEVEN

SUMMARY

This research was concerned with the role of metallothionein (MT) in chemical toxicity mediated by reactive intermediates generated from xenobiotic and oxygen metabolism. Metallothionein is a ubiquitous, low molecular weight, cysteine-rich (30% of total amino acids), metal binding protein. Since sulphydryl compounds are known to act as nucleophiles or reductants in biological systems, it was hypothesized that because of its high cysteinyl thiolate concentration, MT would be reactive towards intermediates generated from xenobiotic and oxygen metabolism. It is also known that all cysteinyl thiolate groups of MT participate in the binding of metals (Zn and Cu) and their inactivation by reactive intermediates generated from xenobiotic or oxygen metabolism results in release of metal ions from the metalloprotein. Moreover, zinc and copper ions, by themselves, are known to have direct influence in oxidant stress. Therefore, it was hypothesized that the metal released from MT concomitant with the oxidant-induced inactivation of metal binding sites would also contribute to the modulating role of MT in oxidant toxicity.

To investigate the role of cysteinyl thiolate groups of MT as scavengers of chemically-reactive intermediates from xenobiotic metabolism, the interaction between purified Zn, Cd-MT and CCl_4 was investigated in vitro. Carbon tetrachloride was used as the model compound because it is believed to exert its toxicity by covalent binding of its metabolites to proteins or lipids or via induction of oxidative stress. Incubation of purified Cd, Zn-MT with CCl_4 in the presence of hepatic microsomes and NADPH

resulted in a time-dependent depletion of MT thiols with the concurrent reduction in the metal-binding sites of the protein. Moreover, this reaction also resulted in the release of Zn and Cd from MT. Results from experiments conducted to determine whether or not the CCl_4-induced decrease in MT-thiol content was due to the scavenging of CCl_4 metabolite(s) showed that the trichloromethyl radical, chloroform and phosgene as well as the products of CCl_4-induced microsomal lipid peroxidation were not directly involved. Although covalent binding of $^{14}CCl_4$ to MT was detected following incubation in the presence of microsomal bioactivation system, it did not account for the CCl_4-induced loss of MT thiol groups for the following reasons: i) prior oxidation of sulphydryl groups of MT by hydrogen peroxide did not alter the binding; and ii) anaerobiosis did not alter the extent of covalent binding but obliterated the inhibitory effect of CCl_4 on MT thiol content. Measurement of the thiol content of CCl_4-treated MT after treatment with 1,4 dithiothreitol, a sulphydryl reducing agent, revealed that all the thiol groups that were lost subsequent to CCl_4 treatment could be regenerated. These data suggest that CCl_4-linked oxidation of MT, rather than the covalent binding of $^{14}CCl_4$ metabolite(s), may be responsible for the CCl_4-induced loss of metal binding sites of MT, indicating an antioxidative property of MT thiolate groups.

To determine the role of metal complement of MT in chemical toxicity, the influence of Zn-MT and Cu-MT on oxidative stress was compared, since Zn and Cu ions by themselves, are known to have antioxidant and prooxidant properties, respectively. To examine the role of Zn-MT in oxidative stress, Ehrlich cells with three different Zn-MT concentrations were exposed to H_2O_2. Zn-MT concentration of control Ehrlich ascites

tumour cells was increased 4-fold 24 h after pretreatment of host mice with 10 mg $ZnSO_4$ / kg, ip and was decreased by 50% following dietary Zn restriction in the host. In vitro toxicity testing revealed that Ehrlich cells with elevated Zn and Zn-MT levels were more resistant to H_2O_2 toxicity (loss of cell viability, increase in lipid peroxidation and increase in $[Ca^{+2}]i$) while Ehrlich cells with reduced Zn and Zn-MT levels were more susceptible to this treatment. H_2O_2-treatment resulted in oxidation of MT thiolate groups and loss of its metal binding capacity, with concurrent reduction in the binding of Zn to MT. Preincubation of Ehrlich cells with $ZnSO_4$ in vitro, which increased total Zn content without altering MT concentration, also conferred some degree of resistance to H_2O_2 toxicity, suggesting the inherent antioxidative property of Zn ions.

To examine the role of Cu-MT in oxidative stress, Ehrlich cells with two different concentrations of Cu-MT were exposed to H_2O_2. Treatment of hosts with a single dose of $CuSO_4$ (1 or 2 mg/kg, ip) 24h prior to cell collection resulted in dose-dependent increases in Cu and Cu-MT concentration in Ehrlich cells. This effect dramatically increased the toxic responses to H_2O_2 that appeared to be directly related to Cu and Cu-MT concentrations. H_2O_2-treatment resulted in oxidation of MT thiolates, reduction in binding of Cu to MT fraction and translocation of Cu to other cellular sites with the majority of this Cu being recovered in the HMW component of the cytosolic fraction.

To investigate the mechanism(s) by which Cu-pretreatment enhanced sensitivity to H_2O_2 toxicity, additional in vitro experiments were conducted. Pretreatment of Ehrlich cells with D-penicillamine, a Cu chelating agent, suppressed the sensitization effect of Cu-pretreatment on H_2O_2 toxicity and prevented the translocation of Cu ions to cytosolic

high molecular weight fractions and particulate fractions. These data suggested that the protective effect of D-penicillamine was due to sequestration of Cu released from MT subsequent to the oxidation of protein thiolates. To determine whether or not Cu-pretreatment increased H_2O_2 toxicity by merely increasing the toxic response normally induced in control cells, additional studies were conducted with control and Cu-pretreated Ehrlich cells. Desferoxamine or mannitol completely obliterated the H_2O_2-induced cell death and lipid peroxidation in control cells suggesting the involvement of OH radical generated via a Fe-catalyzed Fenton reaction. In Cu-pretreated cells, H_2O_2-induced cell death and lipid peroxidation were partially protected by desferoxamine but completely obliterated by mannitol or a combination of desferoxamine and D-penicillamine. These data suggested that the increases in H_2O_2-induced toxicity in Cu-pretreated cells was mediated by a OH radical generated from a Cu-catalyzed Fenton reaction. The results of this study showed that Cu-MT enhances sensitivity to oxidant stress due to the prooxidative properties of Cu released from MT concomitant to inactivation of MT thiolates.

Since the prooxidative property of Cu-MT has not been reported by other investigators, additional studies were carried out to investigate the toxicological significance of Cu-MT. Neonatal guinea pigs were used as the experimental model because hepatic Cu and Cu-MT concentrations were high in the 3-day-old guinea pigs but declined to low adult levels by 7-days of life. Comparison of the hepatotoxic responses to iron-nitrilotriacetate (FeNTA) (3.5 mg Fe/kg,ip), a prooxidant, within 2h of treatment in the three age groups revealed heightened sensitivity in the 3-day-old guinea pig as

evidenced by elevation in serum aspartate aminotransferase activity and lipid peroxidation and decreases in GSH/GSSG ratio. Furthermore, pretreatment of 7-day-old animals with the MT inducing agent $CuSO_4$ (0.5mg/kg, ip) 24h prior to administration of FeNTA resulted in increases in hepatic Cu and Cu-MT levels by 7-fold and 6-fold, respectively, as well as increases in the hepatotoxic responses. Metal binding capacity, thiol content and Cu content of hepatic MT of 3-day-old and Cu-pretreated 7-day-old animals were reduced by FeNTA treatment; these reductions were restored in vitro by the sulphydryl reductant 1,4-dithiothreitol. Results of in vitro studies showed the stimulation of FeNTA-induced microsomal lipid peroxidation by hepatic cytosols isolated from 3-day-old but not those of 7-day-old animals. The stimulatory effect of cytosol isolated from 3-day-old animals was not modified by heat-treatment and this further supports the involvement of the heat stable MT. The role of Cu ions lost from MT subsequent to oxidation of MT thiolates was shown by the effectiveness of D-penicillamine, a Cu chelating agent, in antagonizing the FeNTA-induced microsomal lipid peroxidation. The results of this study suggested that Cu-MT may have a prooxidative property in vivo and tissues with high Cu-MT levels may be particularly susceptible to oxidative stress.

The results of this research showed that the cysteinyl thiolate groups of MT possess antioxidant property, acting as a reductant of H_2O_2 and products of CCl_4 metabolism. More importantly, MT can act either as an antioxidant or a prooxidant. Thus, Zn-MT and Cu-MT can be considered as antioxidative and prooxidative agents, respectively. Results of this research also indicated that the metal ions that are bound to MT are not biologically inactive; this is especially true for Cu. The identification of Cu-MT as a

risk factor in oxidative stress is of significance because MT is traditionally considered as a detoxication mechanism or storage site for metals. To most investigators, the deposition of excessive amounts of Cu in the form of Cu-MT in tissues is not associated with risk.

APPENDIX I

CHEMICALS

NADPH and 1,4-dithiothreitol (DTT) were purchased from Boehringer Manheim (Montreal, Quebec. Canada). Chelex-100 (50 - 100 mesh) was purchased from BioRad (Mississauga, Ontario, Canada). Cadmium chloride, ferric nitrate, zinc sulphate and cupric sulphate were purchased from Fisher Scientific Co. (Toronto, Ontario, Canada). Radioactive $^{14}CCl_4$ was purchased from NEN Reasearch Products, Dupont (Boston, MA, USA). Sephadex G-75 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Pyrogallol, catalase, glutathione (reduced form), 2-thiobarbituric acid, 5,5-dithiobis-(2-nitrobenzoic acid)(NbS_2), D-penicillamine, desferoxamine and mannitol were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Fisher Scientific Co. (Toronto, Ont., Canada).

Normal and zinc-deficient diets (in pellet form) were obtained from Ziegler, Bros. Inc.,(Gardens, PA, USA). The normal diet contained 40 ppm Zn, 12 ppm Cu and 65 ppm Fe whereas the Zn deficient diet contained 1 ppm Zn with normal levels of Cu and Fe.

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