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# Extracellular Calcium Effects on Cell Viability and Thiol Homeostasis

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**Studies of chemically induced cell injury and death, which have used as model systems freshly isolated rat hepatocytes and hepatocytes in culture, are discussed. An important model uses the omission of $Ca^{2+}$ from the medium during rat hepatocyte incubations. $Ca^{2+}$ omission induces an intense oxidative stress within hepatocytes incubated in a 95% $O_2$ + 5% $CO_2$ atmosphere. The relationship of calcium homeostasis to the parameters of oxidative stress is important to understanding the progression from reversible to irreversible injury. In the $Ca^{2+}$ omission model, the vitamin E (Vit. E) content of hepatocytes is important for the prevention of cell injury. Recent studies with rat hepatocytes show that ruthenium red (RR) and $La^{3+}$, which block $Ca^{2+}$ translocation through the mitochondrial uniport, can prevent malondialdehyde (MDA) formation, reduced glutathione (GSH), and protein-SH loss, Vit. E loss, and LDH leakage induced by $Ca^{2+}$ omission from the incubation medium. $Ca^{2+}$ omission promoted a marked loss of mitochondrial transmembrane potential ($\Delta\varphi$) that was prevented by RR, EGTA, Vit. E, and desferrioxamine. The absence of extracellular $Ca^{2+}$ may cause mitochondrial $Ca^{2+}$ cycling that contributes to the observed oxidative stress, resultant loss of cell viability, and protein thiol homeostasis. Chemical agents including a glutathione-depleting agent, ethacrynic acid, and a redox cycling agent, adriamycin, increase the loss of cell viability caused by a $Ca^{2+}$-free medium, but they have some additional effects on cellular processes. The demise of cell viability by the agent is also preventable by Vit. E supplementation. $Ca^{2+}$ has a role in cell injury that appears to uniquely involve mitochondrial homeostasis.**

## Introduction

The loss of cell viability due to injury can be thought of as a sequence of events characterized by reversible but potentially debilitating changes in cell function and morphology (*1*). After an extensive insult, the stages of cell injury pass a point of irreversible damage where recovery is impossible. This irreversible reaction to an injurious insult is defined by cell death (*1*) and is characterized in general by a loss of membrane integrity, collapse of cell morphology, and loss of cell viability. The role of extracellular calcium in toxicological effects of chemicals is presented.

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## Proposed Role of Extracellular Calcium

Only recently have investigations into this point of irreversibility due to cell injury concentrated on cellular aspects of $Ca^{2+}$, even though pathologists decades ago observed that there was a correlation between calcification and tissue necrosis. Furthermore, most early mechanistic studies were based on a proposed role for $Ca^{2+}$ in the process of cell death (*2-5*), as demonstrated by Trump et al. (*1,6*) who have showed that increases in levels of cytosolic $Ca^{2+}$ in excitable tissue following initial cell injury were primary to cell death.

An appreciation of the potential role of a loss of $Ca^{2+}$ homeostasis in irreversible cell injury can be developed from an understanding of the intricate processes required to maintain normal physiological $Ca^{2+}$ levels. Intracellular $Ca^{2+}$ is maintained at a steep concentration gradient from extracellular concentrations in the millimolar range down to the 0.1 to 0.4 μM range for free $Ca^{2+}$ in the cell cytosol (*7,8*). This gradient, coupled with a large electrochemical gradient, serves as a driving force to transport $Ca^{2+}$ into the cell. Although total cellular calcium ranges from 3 to 30 nmole/mg cell protein, intracellular ionized $Ca^{2+}$

concentrations are maintained at 0.1 μM or less by several mechanisms (*9*). Intracellular homeostasis of calcium has been recently reviewed (*10*). In addition to $Ca^{2+}$ exchange systems, numerous $Ca^{2+}$-binding proteins, such as calmodulin, sequester intracellular $Ca^{2+}$ to a limited extent (*11,12*). Thus, a complex homeostatic system has evolved to maintain low cytosolic $Ca^{2+}$ by compartmentation in the intracellular matrix and by extrusion to the extracellular milieu. The regulatory role of $Ca^{2+}$ in normal cell processes suggests that a prolonged disturbance in the normally low cytosolic $Ca^{2+}$ levels would have detrimental effects on the cell.

As mentioned, an early finding of an association of $Ca^{2+}$ accumulation within necrotic tissue stimulated investigations into the relationship between cell death and the observed increase in tissue calcium. It was believed that this accumulation was causally related to cell death, and a hypothesis was advanced that it represented an influx of extracellular $Ca^{2+}$ from interstitial spaces into injured cells (*5*). 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. Farber et al. (*13*) termed this extracellular calcium-dependent mechanism the final common pathway to cell death.

This hypothesis, which was reviewed (*14,15*), used monolayers of primary cultures of rat hepatocytes as a model of cytotoxicity. Cultured hepatocytes were incubated with an air atmosphere with or without added $Ca^{2+}$ in the medium (3.6 mM) and exposed for 1 to 6 hr to a wide variety of agents, including such diverse chemical structures as ethylmethanesulfonate (EMS), the $Ca^{2+}$ ionophore A23187, and asbestos. In all cases cell death, as measured by uptake of trypan blue, was greater in the presence of extracellular $Ca^{2+}$ (*13*). Influx of extracellular $Ca^{2+}$, driven by its concentration and electrochemical gradients, was suggested to be the trigger for the final processes leading to cell death.

The initial reports of this phenomenon were followed by equally convincing demonstrations that extracellular $Ca^{2+}$ in the presence of toxic agents either had no significant effects on cell death in both freshly isolated rat hepatocyte suspensions (*16*) and cultured rat hepatocyte monolayers (*17*) or, more surprisingly, actually resulted in diminished cell death of isolated hepatocytes, when compared with cells incubated without the addition of $Ca^{2+}$ (*18*). Orrenius and colleagues (*18*) observed opposite results using the isolated hepatocyte model, as those reported by Farber and coworkers (*13*), using the cultured hepatocyte model. Importantly, our laboratory noted that in the absence of toxic chemicals, extracellular $Ca^{2+}$ afforded greater viability to isolated hepatocytes than was found with those exposed to low $Ca^{2+}$ (*19*). Although these studies refuted the claims that cell death was dependent on the presence of extracellular $Ca^{2+}$, the question remained as to why these two experimental systems produced opposing results.

## The Protective Effect of Vitamin E during Calcium Omission

In the aforementioned studies, freshly isolated cells were incubated in buffered salt solution (usually a Krebs-Henseleit buffer), whereas cultured hepatocyte monolayers were incubated in a medium containing not only salts, but amino acids, vitamins, and, occasionally, fetal calf serum. This serum itself contained an endogenous mixture of salts, vitamins, and proteins. Since the major criterion of irreversible cell injury is damage to the cell plasma membrane allowing the leakage of intracellular macromolecules, we examined the relationship of vitamin E, as a major membrane protective factor, to the outcome of toxic chemical insults to freshly isolated rat hepatocytees. The results of this study (*20*) indicated that the differences in $Ca^{2+}$-dependent toxicities observed in the previous studies were related to the presence or absence of the antioxidant vitamin E (as esters of α-tocopherol), in the incubation medium. For example, when isolated hepatocytes were incubated in the absence of extracellular $Ca^{2+}$, the addition of vitamin E (as α-tocopherol succinate) to the incubation medium provided maximal protection against toxicity. This model simulated the cultured hepatocyte model that contained relatively comparable amounts of vitamin E (as α-tocopherol phosphate and α-tocopherol) in the incubation medium (*21*) and showed the least toxicity in the absence of $Ca^{2+}$. On the other hand, without the addition of α-tocopherol succinate to the isolated hepatocyte medium, the absence of extracellular $Ca^{2+}$ resulted in maximal toxicity, as previously observed in the studies with isolated cells incubated in Krebs-Henseleit buffer alone (*18*). The results of these three studies (*13,18,20*) have been compared elsewhere in more detail (*22*).

Acting in concert with the glutathione redox system, vitamin E serves an important role as a lipophilic antioxidant. A requirement for vitamin E is necessitated by the cellular production of active oxygen species such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($^{\cdot}OH$), which can be generated by the metabolism of certain xenobiotics, principally redox active compounds, or via leakage of electrons from mitochondrial (*23*) and microsomal (*24*) transport systems. Although $^{\cdot}OH$ is the most reactive species (*25*), all of these oxyradicals are capable of oxidative attack on membrane lipids and the vitamin E series to control the membrane disruption resulting from lipid peroxidation. However, vitamin E has received only limited attention for its role in protecting cells or whole animals against injury from toxic chemicals. Some controversial results have been reported about its effectiveness as a protective mechanism against chemically

induced toxicity *in vivo,* suggesting that the protective effects of vitamin E may include cellular macromolecules other than lipids.

## Relationship between Calcium and Cellular Glutathione

Mammalian cells have evolved protective mechanisms to minimize injurious events that might result from toxic chemicals and normal oxidative byproducts of cellular metabolism. A major endogenous protective system is the glutathione redox system. Glutathione is present in high concentrations (generally in the millimolar range) in most mammalian cells as reduced glutathione (GSH), with minor fractions being oxidized glutathione (GSSG), mixed disulfides of GSH and other cellular thiols, and minor amounts of other thioethers (*26*). GSH acts both as a nucleophilic scavenger of numerous compounds and their metabolites, via enzymatic and chemical mechanisms. It converts electrophilic centers to thioether bonds and is a cofactor in the GSH peroxidase-mediated destruction of $H_2O_2$. GSH depletion to about 20 to 30% of total glutathione levels can readily impair the cell's defense against the toxic actions of such compounds and against the oxidative damage from $H_2O_2$ and may lead to the rapid demise of the cell and to cell death (*27,28*).

The finding that altered cell $Ca^{2+}$ content affects viability of cells differently, depending on the constituent makeup of the extracellular environment, was exploited to examine the relationship of GSH and the glutathione redox system during the course of events leading to chemically induced death. In the isolated hepatocyte model system, there are several means by which the GSH level and associated enzyme systems can be modulated.

The concentrations of amino acids, vitamins, and minerals in the incubation medium can be readily controlled experimentally, allowing an evaluation of their effects on endogenous cytoprotective systems such as the GSH redox system. GSH can be depleted directly by conjugation with electrophiles and indirectly by the addition of inhibitors of GSH biosynthesis and regeneration (*29,30*). GSH is synthesized *in vivo* from γ-glutamylcysteine and glycine via GSH synthetase (EC 6.3.2.3), with the sulfur functionality of cysteine supplied with methionine via the cystathionine pathway (31). GSH synthesis can be diminished by buthionine sulfoximine (BSO) inhibition of γ-glutamylcysteine synthetase (EC 6.3.2.2), the cytosolic rate-limiting enzyme. GSH is maintained in a redox couple with GSSG within the cell and is regenerated by GSH reductase (EC 1.6.4.2), a cytosolic NADPH-dependent enzyme. Inhibition of this enzyme, and hence GSH regeneration, with 1,3-bis(2-chlorethyl)-1-nitrosourea (BCNU) also depletes intracellular GSH (*32*). Alternatively, GSH content in isolated hepatocytes can be augmented by the addition of its sulfur-containing precursors, such as cysteine (or *N*-acetyl cysteine) or methionine, to maximize biosynthesis (*33*).

To conduct these studies, isolated hepatocytes were incubated in Fischer's medium made to contain < 0.08 mM $Ca^{2+}$ to 3.5 mM $Ca^{2+}$ for up to 5 hr at 37°C. Viable cells were separated from nonviable cells by centrifugation through a layer of dibutyl phthalate oil, leaving the nonviable cells in the upper layer (*34*). All biochemical analyses were conducted with viable cells only, overcoming the problem of contributions from dead cells. With this experimental protocol, calcium-depleted hepatocytes undergo a physiologically induced oxidative type of injury. This injury is characterized by a 58% decrease in intracellular GSH, with 70% of this accounted for by the efflux of GSH and oxidation to GSSG (*35*). Using BSO to inhibit GSH biosynthesis, the remaining 30% of the loss of intracellular GSH was found to be accounted for, mainly by a reduction in its biosynthesis resulting from a decrease of its precursor pools. This was accompanied by precursor efflux, especially those supplying the cysteine sulfur, to the extracellular medium (*35*).

Lowered hepatocellular $Ca^{2+}$ content causes the loss to the medium of not only sulfur-containing amino acids, but also glutamate and aspartate. The depletion of GSH is followed by leakage of intracellular $K^+$ (*19,35*), a sensitive indicator of reversible cell injury (*36*); however, no major leakage of lactate dehydrogenase (LDH) is observed. More importantly, these losses of low molecular weight constituents are indicative of the minor extent of leakage of the plasma membrane of the cells, without loss of substantial membrane integrity or cell viability during the 5-hr incubation period. Plasma membrane alteration is also noted by the formation of morphological surface protrusions known as blebs, as visualized under scanning electron microscopy (*37*), and also by the diffusion of malondialdehyde to the incubation medium as a byproduct of peroxidation of membrane fatty acids (*35*). Since the extent of injury described by these parameters is reversible (*1,38*), it thereby constitutes early stages in the pathway to cell death.

The mechanism whereby lowered cell $Ca^{2+}$ leads to these biochemical and morphological changes is apparently due to the redistribution of intracellular $Ca^{2+}$ stores. Omission of extracellular $Ca^{2+}$ eradicates the usually high $Ca^{2+}$ concentration gradient across the plasma membrane, leading to an initial loss of cytosolic $Ca^{2+}$ as it is continually displaced from the cell (*38*). This lowering of cytosolic $Ca^{2+}$ is proposed to trigger the release of $Ca^{2+}$ stored in internal membrane sites into the cytosol, thereby raising cytosolic $Ca^{2+}$ above physiological levels, in spite of lowered total cell calcium. This rise in cytosolic $Ca^{2+}$ has been proposed to stimulate calcium-dependent membrane-bound phospholipases (*39–41*) and to activate certain calcium-dependent nonlysosomal proteases (*42*). The activation of these enzymes would be expected to alter

lipids and proteins of the mitochondrial and plasma membranes, and in particular, the cytoskeletal structure, resulting in the loss of mitochondrial function and leaky plasma membranes. During massive oxidative stress, this would lead to cell death.

In a series of reports by Orrenius et al. (*43,44*), it was demonstrated that during menadione-induced $H_2O_2$ production in isolated hepatocytes, the depletion of intracellular GSH and modification of key protein thiols (ProSH) result in the perturbation of the normal flux and storage of intracellular $Ca^{2+}$ at internal organelle sites, particularly the mitochondrial and endoplasmic reticular membranes. This results in the release of free $Ca^{2+}$ into the cell cytosol. The thiol-containing plasma membrane $Ca^{2+}$ 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 $Ca^{2+}$. This reasoning has been extended to earlier studies on the role of extracellular $Ca^{2+}$ in cell death (*18,20,45*), whereby an intracellular disturbance in calcium homeostasis, rather than an influx of extracellular $Ca^{2+}$, is considered to have been responsible for the events leading to cell death (*44*), as described above. Accumulating evidence thus implicates the disturbance of intracellular calcium homeostasis as a major event that triggers membrane degenerative processes within chemically intoxicated cells and has been proposed to be a common mechanism in the pathway to chemically induced cell death (*41,46,47*). However, substantial evidence has been presented to equally claim that increased cytosolic $[Ca^{2+}]$ plays no major role in cytotoxicity during exposure of hepatocytes to $H_2O_2$ or $CCl_4$ (*48,49*).

## Role of Mitochondria

In the calcium-omission model, the internal site of $Ca^{2+}$ storage that is immediately affected by altered cytosolic $Ca^{2+}$ content remains uncertain. Mitochondria are capable of taking up large amounts of $Ca^{2+}$ (*50*), driven by the proton translocation-generated membrane potential, which is sensitive to ruthenium red (*51*). This has led to the general belief that the mitochondrion can be a major regulator of cytosolic $[Ca^{2+}]$. Under pathological conditions, it appears that mitochondrial membrane storage sites are responsible solely for the regulation of intramitochondrial calcium homeostasis (*52*) since they can apparently maintain extramitochondrial $[Ca^{2+}]$ only at levels 5 to 10 times higher than normal cytosolic levels (*10*).

Although mitochondrial regulation of cytosolic $[Ca^{2+}]$ appears somewhat untenable, recent observations that ruthenium red prevents the oxidative stress and associated cell injury in the calcium-omission model (*53*) does suggest a role for mitochondrial $Ca^{2+}$ cycling in the generation of oxidative cell injury associated with calcium depletion. We have previously suggested that mitochondrial GSH is a compartmentalized pool, sequestered from cytosolic GSH, that is critical in protection against cytotoxicity (*54,55*). After the initial $Ca^{2+}$ redistribution within the calcium-depleted hepatocyte, mitochondrial GSH diminishes in parallel with cytosolic GSH (*19,35*). Alterations in the mitochondrial GSH redox system correlate with changes in mitochondrial $[Ca^{2+}]$ (*56*), probably as a result of secondary alterations of mitochondrial membrane sulfhydryl groups that are involved in $Ca^{2+}$ retention (*57*). Both $CCl_4$ and formaldehyde toxicities have been shown to be strongly related to mitochondrial functional changes secondary to changes in mitochondrial thiols and $[Ca^{2+}]$ (*58,59*). Additionally, exposure of renal mitochondria to oxygen-free radicals during disruption of calcium homeostasis reduces mitochondrial ATPase activity and other functions that are related to mitochondrial membrane damage (*60*). Together, these studies indicate a major, if not critical, role for the mitochondrion in oxidation-associated cell injury.

The alteration in calcium homeostasis inherent to the calcium-omission model can be further perturbed by the addition of the $Ca^{2+}$ ionophore A23187. The ionophore allows additional movement of $Ca^{2+}$ across the cell membranes. At low micromolar concentrations, A23187 affects only intracellular membrane $Ca^{2+}$ flux and induces greater toxicity in hepatocytes depleted of calcium. The finding that this toxicity is more closely correlated with the loss of mitochondrial GSH, rather than with cytosolic GSH, and is blocked by ruthenium red, which further supports the mitochondrion as the target organelle in oxidation-induced cytotoxicity resulting from extracellular calcium omission (*54*).

Thomas and Reed (*61,62*) have observed that ruthenium red and $La^{3+}$, which block the $Ca^{2+}$ translocation through the mitochondrial uniport, totally prevented malondialdehyde formation, GSH and protein thiol (ProSH) oxidation, and α-tocopherol loss induced by $Ca^{2+}$ omission. Accordingly, these agents also prevented leakage of intracellular $K^+$. Similar protective effects were provided by the $Ca^{2+}$ chelator EGTA. In these studies, the absence of extracellular $Ca^{2+}$ resulted in a marked decline of the mitochondrial transmembrane potential ($\Delta\varphi$), which could be prevented by ruthenium red, EGTA, vitamin E, and the iron chelator desferrioxamine. In contrast, oxidative stress induced by treatment with the redox active agent paraquat (PQ) and BCNU had little effect on $\Delta\varphi$; the malondialdehyde formation and $K^+$ leakage were not affected by ruthenium red or $La^{3+}$. Oxidative stress induced by $Ca^{2+}$ omission or PQ and BCNU also led to a marked loss of cellular adenosine triphosphate (ATP) (Fig. 1), which was prevented by ruthenium red only in the $Ca^{2+}$-omission model. These results indicate that the incubation of rat hepatocytes in the absence of extracellular $Ca^{2+}$ creates an unusual oxidative stress, which markedly affects mitochondrial function. The ability of vitamin E and desferrioxamine to inhibit the loss of $\Delta\varphi$ indicates

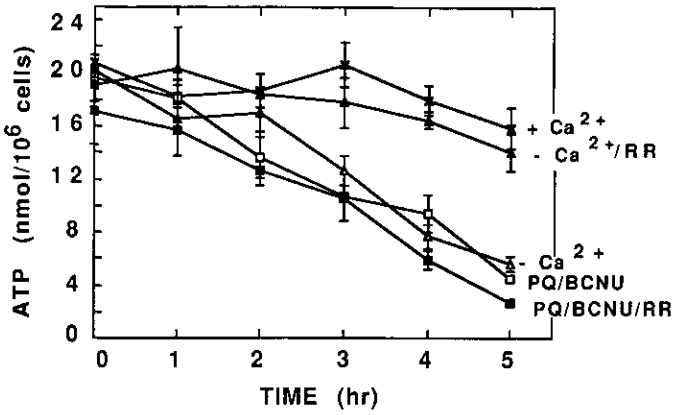


FIGURE 1. Effect of ruthenium red on hepatocyte ATP loss induced by paraquat/BCNU treatment or extracellular $Ca^{2+}$ omission.

that oxidative damage is involved in producing mitochondrial dysfunction. Furthermore, the potent inhibitory effects of ruthenium red and $La^{3+}$ suggest that the $Ca^{2+}$ movement through the uniport, perhaps indicative of mitochondrial $Ca^{2+}$ cycling, plays a major role in generating this oxidative stress and promoting cell injury (*61,62*). Also, because the studies on the oxidative stress induced by PQ have demonstrated its lack of effect on mitochondrial $\Delta\varphi$, the type of oxidative stress associated with calcium depletion is believed to be fundamentally distinct from that incurred with redox-active chemicals (*61,62*).

## Chemically Induced Cell Injury and Maintenance of Protein Thiols

The calcium-omission model places a stress on the protective systems of the hepatocytes by the experimental alteration of intracellular calcium homeostasis and enhancement of oxidative stress, and thence the alteration of the contents of cellular thiols and α-tocopherol, without the use of toxic chemicals. The design of this model is extremely useful for determining the relationships between these cellular systems in the presence of select inhibitors and enhancers of oxidative stress mechanisms. As described above, the stressed hepatocytes in this model become predisposed to the toxic effects of numerous chemicals, including those of such chemical diversity as $CCl_4$, bromobenzene, adriamycin (ADR), EMS, and the cationic ionophore A23187 (*18,20*). Despite the differences in mechanisms of initiation of toxicity, most of these compounds induce cell membrane damage to isolated hepatocytes (stimulation of lipid peroxidation and production of plasma membrane blebs). Accordingly, protection against the toxicity of EMS, A23187, and ADR/BCNU is proportional to the α-tocopherol contents of the cells (*20*).

In addition to α-tocopherol, a balanced intracellular thiol redox system is also important for cell viability. A component of this redox system is the status of sulfhydryl groups essential for the activities of many enzymes, including the membrane-bound $Ca^{2+}$ translocases (*63,64*). It has been proposed that GSH maintains cell viability via the maintenance of membrane ProSH groups, including those of the critical $Ca^{2+}$ translocases through thiol-disulfide exchange reactions (*41,43,44*). Thus, it was of interest to investigate the relationship between thiol status and α-tocopherol during protection against chemically enhanced oxidative stress. Cytotoxicity was induced in the calcium-omission model by two chemical schemes designed to deplete intracellular GSH by different mechanisms. The first involved the indirect depletion of GSH by adriamycin (ADR)-mediated generation of reactive oxygen species. ADR, an anthracycline quinone compound, is known to undergo redox cycling between the quinone and the semiquinone radical to produce $H_2O_2$ and $O_2^{\cdot}$ (*65,66*). $H_2O_2$ is reduced to $O_2$ and $H_2O$ by GSH peroxidase, with the oxidation of GSH cofactor to GSSG. Since ADR-mediated depletion of GSH is only observed during the inhibition of GSH regeneration, BCNU was added along with the quinone to inhibit GSH reductase (*32*). The second mechanism of GSH depletion used ethacrynic acid (EA) to directly conjugate intracellular GSH (*67*).

At 100 μM EA and 350 μM ADR/100 μM BCNU, the intracellular GSH was lowered to nondeductible levels at 1 hr and below 5% of initial levels of 3 hr, respectively (*68*). This was accompanied by decreased cellular α-tocopherol content and followed by losses in ProSH levels and cell viability. Although depletion of GSH was independent of cell $Ca^{2+}$ concentration, the losses in all three parameters were maximal in calcium-depleted cells. Supplementation with α-tocopherol succinate to both calcium-depleted and calcium-adequate cells elevated cellular α-tocopherol levels. In contrast to that observed in cells exposed to the simple $Ca^{2+}$ omission-induced stress described above, intracellular GSH concentrations were unaffected by α-tocopheryl succinate treatment and remained below detectable levels. This lack of anti-oxidant ability to maintain intracellular GSH during its chemical depletion and stimulation of lipid peroxidation was not unexpected and had been observed by others (*69*).

In spite of this severe depletion of GSH, ProSH levels continued to reflect the calcium-dependent α-tocopherol contents of the cells, such that at the highest α-tocopherol content (approximately 1.0 nmole/$10^6$ cells in calcium-depleted cells), ProSH remained above 75% of initial levels. At these levels of α-tocopherol and ProSH, the toxic effects of GSH-depleting agents were completely prevented (*68*). By loading both calcium-depleted and calcium-adequate cells with equal, intracellular concentrations of α-tocopherol (0.8–1.0 nmole/$10^6$ cells) during the 5-hr incubation period, both ProSH content and cell viability were maintained at initial levels, regardless of the depleted intracellular GSH stores or the status of cell calcium (*70*). This finding demonstrates that during the total depletion of intracellular GSH, the levels of ProSH in isolated hepatocytes, in parallel with cell

viability, are dependent on cellular α-tocopherol content. Although the depletion of intracellular GSH levels below 20% of physiological levels generally has been considered to be detrimental to the cell (*71–74*), these recent findings place further limits on the significance of the GSH threshold when the α-tocopherol level is elevated (*75*).

The most interesting outcome of the above experiments is the phenomenon of α-tocopherol-mediated maintenance of ProSH. The fact that cell viability directly correlated with ProSH levels strongly supports the hypothesis of Orrenius et al. that the loss of ProSH groups is one of the critical factors leading to cell death (*41*). However, in their experimental model, chemical modulation of ProSH alters the mechanisms involved in the maintenance of calcium homeostasis, thereby increasing cytosolic [$Ca^{2+}$]. This is believed by them to be the ultimate step leading to damage of the cytoskeletal structure and cell membranes (*47*). This compares to the present model whereby, regardless of altered calcium homeostasis, maintenance of ProSH groups prevents the loss of cell integrity. Interestingly, the loss of cellular ProSH can be prevented by a wide variety of anti-oxidants and metal in chelators—all of which control free radical-mediated oxidative events (Fig. 2). It is unclear whether α-tocopherol-mediated maintenance of ProSH prevents the release of compartmentalized $Ca^{2+}$ into the cell cytosol during lowered total-cell calcium concentrations and, thereby, prevents associated membrane degradative processes, or whether damage is prevented in spite of elevated cytosolic [$Ca^{2+}$].

The hypothesis that α-tocopherol prevents oxidative stress and associated injury by maintenance of ProSH has been only alluded to by other investigators using erythrocyte models of oxidative stress (*76–78*). The experiments described above with isolated hepatocytes provide the first substantial evidence for

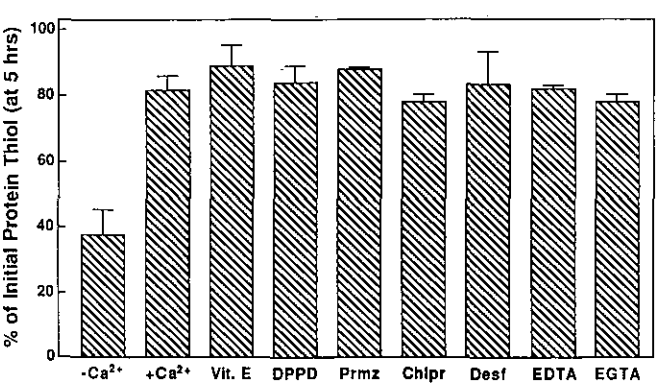


FIGURE 2. Effects of anti-oxidants and metal ion chelators on protein thiol status at 5 hr in isolated hepatocytes incubated in the absence of extracellular $Ca^{2+}$. The agents used were: vitamin E (Vit. E), *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), and promethazine (Prmz), all at 50 μM; chlorpromazine (Chlpr) at 100 μM; and desferrioxamine (Desf), EDTA, and EGTA all at 2.5 mM.

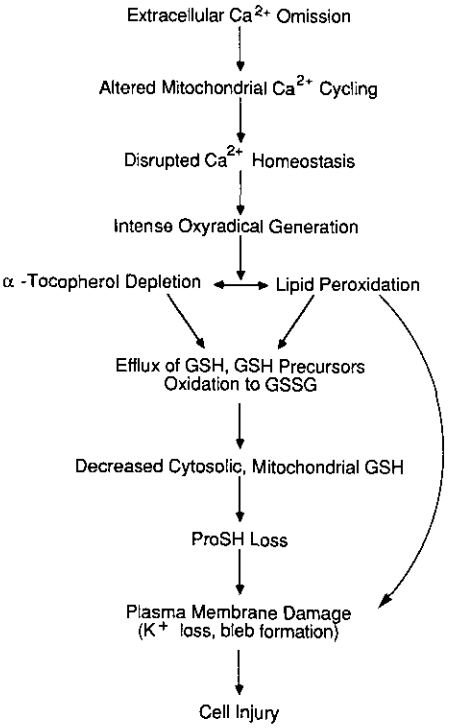


FIGURE 3. Calcium-omission model of oxidative stress to isolated hepatocytes: sequence of events leading to cell injury.

such a contention. The ability of α-tocopherol to prevent oxidative damage to an integral membrane protein has received very little attention, even though it has been noted that vitamin E deficiency alters the activities of numerous membrane-bound enzymes (*79*). For example, in rabbit skeletal muscle, α-tocopherol has been shown to stabilize $Ca^{2+}$-ATPase activity of sarcoplasmic reticulum and prevent the loss of transport of $Ca^{2+}$ ions during damage by thermally irradiated unsaturated fatty acids (*80*). The loss of activity of this enzyme by oxidizing agents has been advocated to result from the direct oxidation of SH groups, rather than being secondary to lipid peroxidation (*81*). It would appear then, that α-tocopherol maintains this $Ca^{2+}$ ATPase activity via direct maintenance of sulfhydryls and not secondary to its inhibition of lipid peroxidation. Whichever mechanism is operative, the close similarity between this enzyme and that of the endoplasmic reticulum suggests a commonality in α-tocopherol action.

The omission of extracellular $Ca^{2+}$ from incubations of isolated hepatocytes, as outlined above, may therefore be considered to induce a type of oxidative stress that is characterized by losses of α-tocopherol, GSH, and ProSH, thereby predisposing the cells to the damaging effects of toxic chemicals, as outlined schematically in Figure 3. In this scheme, the enhancement of chemically mediated toxicity to isolated hepatocytes when incubated without extracellular $Ca^{2+}$ is an important aspect of the calcium role in cytotoxicity.

Further research is needed to elucidate the mechanism of induced oxidative stress due to calcium omission. Lipid peroxidation may only be indicative of the intensity of the generation of oxyradicals as mitochondrial calcium homeostasis is lost.

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