AN ABSTRACT OF THE THESIS OF

Melani K. Savage for the degree of Doctor of Philosophy in Toxicology presented on January 28, 1994.

Title: Status of Mitochondrial Glutathione and Energy Levels During Cyclosporin A-Sensitive Permeability Transition Induced by Calcium and Inorganic Phosphate.

Redacted for Privacy

Abstract approved: _____

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The status of glutathione was studied in isolated rat liver mitochondria during permeability transition that was induced with Ca^{2+} and inorganic phosphate. The treatment of mitochondria with Ca^{2+} and inorganic phosphate resulted in large amplitude swelling and the rapid and nearly complete release of mitochondrial glutathione. The release of glutathione was prevented by cyclosporin A, a potent inhibitor of permeability transition. This suggests that glutathione release occurred via the putative Ca^{2+}-dependent inner membrane pore associated with permeability transition. Thus, glutathione efflux from mitochondria may occur under toxicological and pathological conditions in which mitochondria are exposed to elevated Ca^{2+} in the presence of near physiological concentrations of inorganic phosphate.

Incubation of mitochondria in the presence of Ca^{2+}, inorganic phosphate, and glutathione, followed by the addition of cyclosporin A, provided a mechanism to load mitochondria with exogenous glutathione at a greater rate of uptake than untreated mitochondria. This implies that opening and closing of the pore provided a mechanism for uptake of glutathione by mitochondria.

During Ca^{2+} and inorganic phosphate induced permeability transition, large amplitude swelling of the mitochondria occurs secondary to solute release, and is often used as an indicator of permeability transition. The addition of metabolic inhibitors of either electron transport or ATP synthesis prevented large amplitude swelling by an unknown mechanism, but did not prevent inner membrane permeability. These findings indicate that permeability transition can occur without large amplitude swelling. Monitoring the cyclosporin A-sensitive release of a concentrated matrix solute, such as glutathione, may be a useful indicator of permeability transition.

The status of pyridine and adenine nucleotides during Ca^{2+} and inorganic phosphate induced permeability transition was also examined. Under these conditions, ATP and ADP were rapidly depleted with concomitant formation of AMP. Pyridine nucleotides were rapidly oxidized intramitochondrially, followed by partial release from the mitochondrial matrix, presumably via the putative Ca^{2+}-dependent inner membrane pore. Conditions that allow maintenance of ADP may be important in preventing large amplitude swelling and may diminish solute release associated with the permeability transition.

Status of Mitochondrial Glutathione and Energy Levels
During Cyclosporin A-Sensitive Permeability Transition
Induced by Calcium and Inorganic Phosphate

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed January 28, 1994

Commencement June 1994

APPROVED:

Distinguished Professor of Biochemistry and Biophysics in charge of major

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Date thesis is presented January 28, 1994

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I. INTRODUCTION

The main theme of this thesis is understanding mitochondrial permeability transition and mitochondrial glutathione status in relationship to mitochondrial energy status.

Mitochondria contain a pool of glutathione which is discrete from the cytosolic compartment. Although little is understood about its regulation, changes in mitochondrial glutathione in-$vitro$ have been linked with a disruption in intracellular Ca^{2+} homeostasis. Under these conditions, loss of mitochondrial glutathione occurs by an unidentified mechanism, which correlates with loss of cell viability.

This thesis explores one possibility by which mitochondrial Ca^{2+} homeostasis may alter mitochondrial glutathione and energy status. The mechanism of such an alteration involves activation of a putative Ca^{2+}-dependent, cyclosporin A-sensitive mitochondrial inner membrane pore. Activation or opening of a putative pore is referred to as permeability transition. Many different agents, in the presence of Ca^{2+}, can "induce" permeability transition. Examples include inorganic phosphate, t-butyl hydroperoxide, and some heavy metals.

Chapter II provides background perspectives and current literature pertinent to the research presented in the following chapters. The chapter

begins with a discussion of mitochondrial function in aerobic metabolism and Ca^{2+} transport. The second part of the chapter introduces the concept of mitochondrial permeability transition, and ties that concept to the role of disrupted Ca^{2+} homeostasis and mitochondrial glutathione status in irreversible cell injury. Next is a discussion of the relationship of mitochondrial glutathione and endogenous oxidative stress. At the end is a brief section describing the purpose of this research. The main section of this chapter is a contribution to a book entitled "Proceedings of the Paul Hochstein Festschrift Conference" which is to be published shortly. The title of the manuscript submitted is "Oxidative Stress and Mitochondrial Permeability Transition".

A presentation of the research findings begin in Chapter III, which describes the rapid and nearly complete release of mitochondrial glutathione during Ca^{2+}- and inorganic phosphate-induced permeability transition. Also explored in Chapter III is the ability to load isolated mitochondria with exogenous glutathione by the controlled opening and closing of this putative Ca^{2+}-dependent pore. This chapter has been published (Arch. Biochem. Biophys. 290: 51-56, 1991.)

Chapter IV demonstrates that permeability transition can occur without large amplitude swelling. Large amplitude swelling has been a generally accepted characteristic of permeability transition. Following the addition of metabolic inhibitors, large amplitude swelling is alleviated in permeability transition induced mitochondria; however, mitochondrial glutathione is released to varying degrees, suggesting that mitochondrial glutathione release may be a sensitive indicator of permeability

transition. This chapter will be submitted to Archives of Biochemistry and Biophysics.

Chapter V describes a novel finding involving the effect of inorganic phosphate induced permeability transition on pyridine and adenine nucleotides. Pyridine nucleotide redox status was previously thought to be unaffected by inorganic phosphate during permeability transition, and is often used in comparison to agents, such as *t*-butylhydroperoxide, that alters this redox ratio; however, the findings presented in this chapter demonstrate that pyridine nucleotides undergo extensive oxidation in the presence of inorganic phosphate. Also demonstrated is the rapid depletion of ATP and ADP, with concomitant formation of AMP. This chapter will be submitted to Biochemical and Biophysical Research Communications.

Chapter VI explores whether pyridine nucleotides, which are normally impermeable to the inner mitochondrial membrane, efflux during permeability transition. Also examined in this chapter is the effect of metabolic inhibitors on pyridine and adenine nucleotide status during Ca^{2+} and Pi-induced permeability transition. This chapter will be submitted to Biochemistry Journal.

II. BACKGROUND PERSPECTIVES

Mitochondria

Background Information

The primary function of mitochondria is aerobic respiration and energy metabolism. Mitochondria are cytosolic organelles present in nearly all eukaryotic cells of higher animals and plants, and certain microorganisms including algae, protozoa, and fungi. An accepted theory on the evolutionary origin of mitochondria is the endosymbiotic theory which hypothesizes that invasion of bacteria into ancestral prokaryotic cells resulted in present day aerobic respiration (1). [A common misconception is that mitochondria are present in all eukaryotic cells. More than a thousand species of protozoa and a few fungi have no mitochondria (2). Red blood cells of mammals also lack mitochondria.]

The tissue most frequently used as a laboratory source of freshly isolated mitochondria is the rat liver; each parenchymal cell contains an average of 1300 mitochondria comprising 17% of the total cellular volume (3). As a tribute to their small size (approximately 4.4 μm X 0.45 μm), 7.2 X 10^9 mitochondria per milligram of protein are present in a typical suspension of isolated mitochondria (4).

Mitochondrial Structure

Mitochondria are highly compartmentalized organelles with two membranes (Figure II. 1). The outer membrane contains pores that allow equilibration of the inner membrane space with the

extramitochondrial environment, whereas the inner membrane is impermeable to most substances. Transport of most substances into and out of the matrix is mediated by numerous specific intermembrane protein carriers (Figure II. 2). Approximately 70% of the inner membrane is composed of proteins that are mainly transmembrane carriers or part of the respiratory chain (Figure II. 2, 3). The remaining 30% are phospholipids, primarily phosphatidylcholine and phosphatidylethanolamine, with a small proportion as cardiolipin (5).

Mitochondrial Calcium Transport

Mitochondria play an important role in the regulation of several ions, primarily H^+, Ca^{2+}, but also Na^+, K^+, Cl^-, and Mg^{2+}. The central role of mitochondria in regulating intracellular Ca^{2+} is interesting, since mitochondria have a high capacity to take up Ca^{2+}, as well as release Ca^{2+} (6-8). The finding that the endoplasmic reticulum has a higher affinity for Ca^{2+} (although lower capacity) has led some to propose that the endoplasmic reticulum is a more important participant in the dynamic regulation of intracellular Ca^{2+} (9). Proteins, such as calmodulin, are also capable of responding to changing Ca^{2+} levels and, therefore, may also contribute to the regulation of this ion.

Mitochondrial Ca^{2+} uptake and release occur by separate mechanisms (Figure II. 4) (10). Uptake is mediated by the electrophoretic uniporter, located on the inner membrane of mitochondria (11-12). It is driven by the membrane potential (~180 mV, negative inside) that is set up through the extrusion of H^+ from the matrix to the inner membrane space. The uptake of Ca^{2+} via the uniporter is noncompetitively

inhibited by the glycoprotein stain, ruthenium red (14). In addition, lanthanide ions are also inhibitors of the Ca^{2+} uniporter (15).

The Ca^{2+} efflux pathway primarily involves the Na^+-Ca^{2+} antiporter, which is driven by the exchange of Na^+ for H^+ (Figure II. 4) (10, 16). Functioning of this pathway depends upon the pH component of the proton motive force, and also appears to be regulated by the redox state of pyridine nucleotides, with oxidation favoring Ca^{2+} release (10, 17). A second Ca^{2+} efflux mechanism may also exist, involving direct Ca^{2+}-$2H^+$ exchange; but it is poorly understood at this time (Figure II. 4) (10, 13).

Due to separate processes of uptake and release of Ca^{2+}, mitochondria can presumably cycle Ca^{2+} by uptake, release and subsequent reuptake (10, 18). This process probably does not occur to any significant extent in normal mitochondria, as cycling of Ca^{2+} is an energy dissipating process that may cause a collapse of the membrane potential. However, under pathological conditions where intracellular Ca^{2+} homeostasis is disrupted, it is hypothesized that the mitochondria do cycle Ca^{2+}, thereby promoting irreversible cell injury (18-19).

A role for disrupted Ca^{2+} homeostasis in irreversible cell injury has been clearly shown under a variety of experimental conditions, ranging from chemically induced cytotoxicity to ischemia and reperfusion injury (20-25). The degree to which mitochondria and their ability to sequester Ca^{2+} contribute to cellular injury, especially *in vivo*, is less clear.

However, the ability of mitochondria to accumulate Ca^{2+} *in vitro* can result in pathological consequences to mitochondria under certain

experimental conditions. This is the major thrust of this thesis. One pathway appears to be the activation of a Ca^{2+}-dependent pore in the inner membrane of mitochondria. The opening of this pore results in disruption of the inner membrane permeability barrier required for normal functioning. Among other things, opening or activation of this pore allows the release of mitochondrial Ca^{2+}, as well as other matrix solutes. Although the physiological functions of this pore remains obscure, its pathological relevance, as discussed below, may be important.

Mitochondrial Permeability Transition

Background Information

Ca^{2+} damage to mitochondria was recognized shortly after work with isolated mitochondria began in the 1950's (26). Hunter and Haworth proposed that loading of Ca^{2+} into the mitochondria caused the opening of a hydrophilic channel in the inner membrane, allowing the passage of small molecules less than 1200 Da (27-29). It is now well accepted that mitochondria from a variety of tissues contain a Ca^{2+}-dependent inner membrane pore. The Ca^{2+}-dependent pore is approximately 20 Å in diameter when opened, resulting in the permeability of the inner membrane barrier (10, 30-33). This process is referred to as mitochondrial permeability transition.

Characteristics of Permeability Transition

Some of the well known biochemical characteristics of the Ca^{2+}-dependent permeability transition include: nonspecific inner membrane permeability to small solutes with molecular weight less than 1200 Da; large amplitude swelling; and the uncoupling of oxidative phosphorylation (10, 33). More recently, it has become apparent that proteins appear to traverse the inner membrane pore, although at a much slower rate than small matrix solutes (34).

In vitro activation or regulation of the pore occurs in the presence of Ca^{2+} and a second agent, termed an "inducing agent". Many inducing agents have been identified, and vary greatly in both structure and function (10, 35-37). Examples of inducing agents include near

physiological levels of inorganic phosphate, fatty acids, some heavy metals, organic sulfhydryl reagents, and oxidants, such as t-butyl hydroperoxide and hydrogen peroxide (10). The pore is thought to be activated under several different conditions involving (1) oxidative stress due to the oxidation of pyridine nucleotides by agents such as t-butyl hydroperoxide (38-41); (2) depletion of adenine nucleotides by pyrophosphate and phosphoenolpyruvate (42-44); (3) and by agents such as inorganic phosphate which are thought to have an intramitochondrial site of action (45-49). These different inducing agents, in the presence of Ca^{2+}, may act however through a common mechanism to induce inner membrane permeability by altering the conformation of the putative pore structure, as discussed below.

During permeability transition, Ca^{2+} as well as other solutes are rapidly released from the mitochondrial matrix, presumably via diffusion through pore opening (28-33). Following the release of matrix solutes, a colloidal osmotic pressure arises in the mitochondrial matrix, due to the high concentration of proteins that are slow to equilibrate (10, 34)). In order to correct the osmotic imbalance, entrance of H_2O results in massive swelling of the mitochondria (10).

Mitochondrial swelling under these conditions is termed large amplitude swelling. Although large amplitude swelling is a secondary event, it occurs within a short time (3-10 min) and is easily detected by monitoring the loss in absorbance of mitochondrial suspensions (50). Monitoring of mitochondrial swelling at 540 nm is a convenient assay, and it is often utilized as an indicator of permeability transition (51-52).

Inhibition of Permeability Transition by Cyclosporin A

A recent significant finding is that the immunosuppressive drug, cyclosporin A (CsA), is a potent inhibitor of the Ca^{2+}-induced permeability transition (53-56). CsA, a cyclic peptide, is a fungal metabolite which suppresses T-cell lymphocyte function by binding to the cellular receptor, cyclophilin or peptidyl-prolyl-*cis-trans*-isomerase (PPI) (57-58). CsA is currently in use as a potent clinical agent for preventing rejection of transplanted organs (59).

Studies by Fournier et al. (60) showed that CsA treatment promotes retention of accumulated Ca^{2+} within isolated rat liver mitochondria. Work by Crompton and coworkers (53) revealed that CsA is a potent inhibitor of the permeability transition induced by either inorganic phosphate or *t*-butyl hydroperoxide in the presence of Ca^{2+}. Broekemeier et al. (54) tested CsA as an inhibitor of the inner membrane pore in the presence of many different inducing agents and found that very low concentrations of CsA acted to protect against permeability transition. These studies all demonstrate the potency of CsA in preventing the permeability transition with concentrations as low as 100 pmoles CsA/mg mitochondrial protein.

Possible Mechanism of CsA Inhibition of Permeability Transition

The inhibition of pore opening is linear to the concentration of CsA, suggesting a 1:1 stoichiometry to some component of the pore which is present at approximately 100 pmoles/mg protein (61-63). A likely mitochondrial target for CsA is the matrix protein PPI, or cyclophilin. PPI has been identified in the mitochondrial matrix in concentrations of 100

pmoles/mg protein (61,63-64). As a peptide bond isomerase, PPI inverts the *cis-trans* configuration at proline residues and therefore is thought to play a role in protein folding (65-66).

The role of PPI in permeability transition is thought to involve the binding of PPI to the putative pore structure or a related component, thereby stimulating or catalyzing pore formation (61, 63). Halestrap et al. propose that PPI binds to a proline residue on the adenine nucleotide translocase dimer in the presence of Ca^{2+} and an inducing agent. This is thought to result in a conformational change of the carrier that promotes pore activity (61, 63).

Proposed Mechanisms of Inner Membrane Permeability

Several mechanisms for the inner membrane permeability defect have been proposed. Perhaps the most widely accepted view is that the inner membrane permeability is nonspecific in nature. Studies by Halestrap and co-workers (33, 61, 63-64) have focused on the connection between the adenine nucleotide translocase and pore formation. They have proposed the adenine nucleotide translocase dimer as the putative pore structure (61). They suggest that the binding of negative effectors such as Ca^{2+}, PPI, and inducing agents results in a conformational change of the adenine nucleotide translocase dimer from the "m" conformation (nonpermeable state) to the "c" conformation. It is proposed that the "c" conformation favors pore formation and agents, such as atractyloside, which are known to induce the "c" conformation of the adenine nucleotide translocase, promote inner membrane permeability (33, 61). This results in a conformational change of the adenine nucleotide

translocase which enables functioning as a nonspecific pore. Conversely, compounds which stabilize the "m" conformation, such as bongkrekic acid and ADP, prevent inner membrane permeability (33, 61).

Collapse of the membrane potential is a second mechanism thought to mediate the nonspecific permeability transition. Bernardi and colleagues (67-69) report that permeability transition can be controlled by collapse of the membrane potential. They suggest that collapse of the membrane potential is a cause, rather than a consequence of the permeability transition.

As the third possible mechanism, Richter et al. (70-73) have proposed that pore formation is not required for the inner membrane permeability. Their findings suggest two things; 1) that an inner membrane defect provides for a specific Ca^{2+} release pathway and 2) that mono ADP-ribosylation of critical inner membrane proteins is the responsible mechanism of permeability transition. They hypothesize that Ca^{2+} cycling, in the presence of oxidants such as t-butyl hydroperoxide, results in NAD(P)H oxidation followed by the hydrolysis of NAD+ to nicotinamide and ADP-ribose. Subsequent ADP-ribosylation of one or more critical proteins results in the specific release of Ca^{2+}. This work is also supported by Weis et al. who report that n-acetyl-p-benzoquinone imine induces mitochondrial Ca^{2+} release by the hydrolysis of oxidized pyridine nucleotides. (74)

Of all three mechanisms discussed, it appears that the current, and probably most accepted, understanding of permeability transition is that the pore structure is an allosteric inner membrane protein, perhaps the adenine nucleotide translocase dimer or some related component. In this

view, the putative pore structure has several different regulatory sites; this possibly explains why many different compounds, including Ca^{2+}, inorganic phosphate, oxidants, sulfhydryl reagents, heavy metals, ADP, ATP, atractyloside, and bongkrekic acid, either inhibit or promote the permeable state. Whether permeability transition occurs may depend upon which sites are modified.

Physiological Relevance of a Ca^{2+}-Dependent Pore

Whether the process of pore formation has a physiological function remains an unanswered and intriguing question. It is proposed that regulated opening and closing of an inner membrane pore could provide a way to load or release mitochondrial metabolites for which no other transport means is available (61, 63). Pathological functions of such a pore may also play a role in mitochondrial self-destruction during oxidative stress or Ca^{2+} overload to assure destruction of damaged tissue and may therefore be a component of programmed cell death or apoptosis (33). If the pore has a physiological function, it is likely that permeability transition, as observed experimentally, represents pathological consequences, perhaps associated with disrupted Ca^{2+} homeostasis. At this time, however, no evidence exist for the reversibility of permeability transition.

Cellular Injury, Calcium Homeostasis, and Mitochondrial Glutathione

Cellular Studies of Permeability Transition

Several studies have explored whether permeability transition occurs during irreversible cell injury caused by chemically induced cytotoxicity as well as ischemia and reperfusion injury. In these studies, the prevention of cell injury by CsA is causally linked to permeability transition occurring at the cellular level. Imberti et al. (76) reported that oxidant-induced hepatocyte injury is prevented by the combined actions of CsA and a phospholipase inhibitor (either trifluoperazine, mepacrine, or dibucaine), both known inhibitors of oxidant induced mitochondrial permeability transition (76). In another recent report by Kass and co-workers (19), CsA protected hepatocytes from pro-oxidant injury, possibly by preventing Ca^{2+} cycling and subsequent mitochondrial dysfunction. Pretreatment of hepatocytes with CsA followed by exposure to either t-butylhydroperoxide, cumene hydroperoxide, or 3,5-dimethyl-N-acetyl-p-benzoquinone imine prevented the loss of cell viability. Determination of intracellular Ca^{2+} pools suggested that mitochondrial Ca^{2+} depletion was an early event which was prevented by CsA (19).

Broekemeier et al. (78) demonstrated that CsA prevented hepatotoxicity of t-butyl hydroperoxide in the presence of high extracellular Ca^{2+} (10 mM Ca^{2+}) but not at physiological Ca^{2+} concentrations (2.5 mM). Recent studies by Snyder et al. (79) demonstrated with cultured hepatocytes that CsA protected against the toxic consequences of the mitochondrial poison, 1-methyl-4-phenylpyridinium. Their findings showed that atractyloside, in the

presence of CsA, eliminated the observed protective effects of CsA, leading to their conclusion that permeability transition is linked to the loss of cell viability in this model (79). It was also shown recently that low concentrations of CsA prevented irreversible damage to isolated myocytes induced by prolonged hypoxia and subsequent reoxygenation (80).

Disrupted Ca^{2+} Homeostasis and Mitochondrial Glutathione

Mechanisms of acute irreversible cell injury and death have long been a subject of interest. Pathologists have recognized for many years that cells of necrotic tissue accumulate Ca^{2+} (81). It was later proposed that a wide variety of toxic agents ultimately act by a final common pathway, characterized by an increased influx of Ca^{2+} across the plasma membrane, resulting in cellular pathogenesis and death (82-83).

As the role of Ca^{2+} in mediating cellular death was further investigated, studies suggested that the disruption of intracellular Ca^{2+} homeostasis, rather than the influx of Ca^{2+}, was directly linked to the expression of toxicity (84-85). Pro-oxidant compounds such as menadione, t-butyl hydroperoxide, and acetaminophen were shown to directly affect intracellular Ca^{2+} homeostasis by inhibiting the Ca^{2+}-ATPase of either the plasma membrane (menadione and acetaminophen) or the endoplasmic reticulum (t-butyl hydroperoxide) (84-85). Whether oxidative stress results in the loss of Ca^{2+} homeostasis or whether disrupted Ca^{2+} homeostasis causes oxidative stress, remains to be fully understood. However, the two processes appear linked in some way.

Several studies have suggested that mitochondrial glutathione plays a critical role in cell viability during conditions involving disrupted

Ca^{2+} homeostasis (86-87, 92-95). Meredith and Reed first proposed a role for mitochondrial glutathione in cytotoxicity after observing that depletion of cytosolic glutathione did not affect cell viability; whereas, depletion of mitochondrial and cytosolic glutathione correlated with the onset of cell injury (86). Leakage of the cytosolic enzyme lactate dehydrogenase, a marker of irreversible cell injury, increased markedly when mitochondrial glutathione was depleted to less than 10% of the initial value (87). Other reports have shown that cytotoxicity, as measured by lipid peroxidation, liver necrosis, and loss of cellular enzymes *in vivo* and *in vitro* occurred only if the cellular glutathione levels fell below 10-15% of the initial value (88-91). This value corresponds to the percentage of total glutathione associated with the mitochondria.

Studies have shown that isolated hepatocytes incubated in the absence of extracellular Ca^{2+}, under an atmosphere of 95% O_2/5% Co_2, undergo an acute oxidative stress (92-95). This was indicated by an increase in lipid peroxidation, a decrease in both cytosolic and mitochondrial glutathione, and a loss of vitamin E as well as an increase in both K^+ and lactate dehydrogenase leakage. Cells were protected from injury when incubated in the presence of chelators of either Ca^{2+} or Fe^{3+}, antioxidants, ruthenium red, and lanthanides (92-95). The effect of the latter two compounds implies that the electrogenic influx of Ca^{2+} into the mitochondria is involved. Thus the absence of extracellular Ca^{2+} induces a potent and acute oxidative stress, possibly by the disruption of mitochondrial Ca^{2+} handling.

The results from these studies (92-95) suggest that mitochondria are target organelles for the expressed toxicity as suggested by the near complete loss of mitochondrial glutathione by an unknown mechanism, which correlated with the onset of irreversible cell injury. A second indication that mitochondria are likely targets for the injury associated with the Ca^{2+} omission model of oxidative stress was the loss of the mitochondrial membrane potential (95). Most of the agents that prevented loss of mitochondrial glutathione and irreversible cell injury also prevented the collapse of the membrane potential, suggesting a role for mitochondrial Ca^{2+} homeostasis.

Glutathione

Discovery and Functions

Glutathione was discovered a little more than a century ago. In 1888, deRey-Pailhade (96-97) reported that yeast cells contain a substance responsible for the reduction of sulfur to hydrogen sulfide. He named this substance "philothion" from the Greek words for "love" and "sulfur." He suggested this substance must have an important biological role due to its ubiquity in the diverse biological samples he examined.

It was soon proposed by Heffter (98) and Arnold (99) that the compound responsible for the reducing properties of the yeast cell was cysteine. Further work revealed that the substance was water soluble and possibly a dipeptide containing glutamate and cysteine. Hopkins renamed this compound glutathione and later found that glycine was also present (100-101). The structure of glutathione (Figure II. 5) was deduced from chemical studies and confirmed by synthesis (102-103). Reference to glutathione or GSH indicates the reduced form of glutathione throughout this manuscript.

Today, we know that glutathione, a nonprotein thiol, is present in millimolar concentration within cells of animals, plants, and microorganisms. Glutathione often functions as a first line of cellular defense against reactive oxygen species, radiation- induced free radicals, and reactive xenobiotic metabolites (104-105). Although glutathione is most often considered in light of cellular protection and detoxification, it participates in the formation of toxic cellular metabolites, particularly with several halogenated compounds (106-107). The focus of this

remaining discussion will be regarding biosynthesis of glutathione, cellular protection of glutathione against reactive oxygen species, and its particular role in mitochondria.

Biosynthesis of Glutathione

Enzymatic biosynthesis of glutathione is energy dependent and occurs from its constitutive amino acids in the cytosolic compartment of perhaps all mammalian tissues (111). It is thought that glutathione synthesis in the liver is limited by the availability of cysteine (0.2-0.5 mM) (108). γ-Glutamyl-cysteine synthetase, in the presence of ATP, catalyzes the rate-limiting step in which glutamic acid and cysteine are covalently linked through the atypical γ-carboxyl-derived peptide bond (109) (Equation II. 1A). This unusual linkage makes glutathione resistant to hydrolytic cleavage of proteases and aminopeptidases and protects the thiol on cysteine from rapid oxidation.

The glycine residue, thought to protect glutathione from the actions of γ-glutamyl-cyclotransferase, is added enzymatically, forming the tripeptide, in the presence of glutathione synthetase and ATP (109) (Equation II. 1B). Breakdown of glutathione occurs extracellularly by the enzymatic action of γ-glutamyl transpeptidase and dipeptidases that occur mainly at the luminal surface of the brush border membrane in the kidney (109).

γ-glutamylcysteine synthetase

A. L-glutamate + L-cysteine + ATP ----> L-γ-glutamyl-L-cysteine + ADP + Pi

glutathione synthetase

B. L-γ-glutamyl-L-cysteine + glycine + ATP ----> glutathione + ADP + Pi
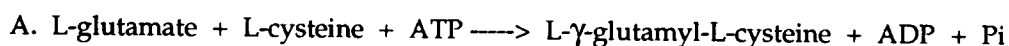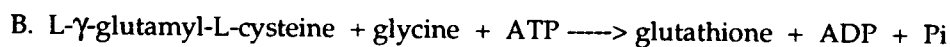
Equation II. 1. Biosynthesis of Glutathione

Glutathione Compartmentation and Function in Mitochondria

In hepatocytes, glutathione is found in the cytosol and mitochondria as separate, nonequilibrated pools (86, 104), whereas nuclear glutathione is in equilibrium with the cytosol (110). Glutathione is present in millimolar concentrations as reduced glutathione (GSH) with minor fractions present as either glutathione disulfide (GSSG), mixed disulfides of glutathione and protein thiols, or thioethers (111).

It is thought that glutathione synthesis takes place in the cytosol because activity of γ-glutamylcysteine synthetase and glutathione synthetase have not been detected in mitochondrial fractions. It therefore appears that cytosolic glutathione is transported into the mitochondria. Studies of hepatic mitochondrial glutathione transport by Kurosawa et al. and Martensson and Meister have previously demonstrated that glutathione uptake is energy-dependent, requiring both the mitochondrial membrane potential and the presence of the pH gradient (112-113). Mitochondrial glutathione transport in extrahepatic tissues

may be different, as suggested by McKernan et al. (114). They reported that uptake of glutathione by renal cortical mitochondria is electroneutral, involving counter ion transport with dicarboxylic acids and independent of the membrane potential (114).

Several studies have shown that mitochondrial glutathione functions as a discrete pool from cytosolic glutathione as first suggested by Vignais and Vignais (115). A report by Jocelyn (116) demonstrated that mitochondrial glutathione is impermeable to the inner membrane following isolation of mitochondria, suggesting that mitochondrial glutathione status is highly regulated and possibly energy-dependent. Studies by Meredith and Reed (86) demonstrated different rates of hepatic glutathione turnover between the cytosol and mitochondria, supporting the earlier contention of separate intracellular glutathione pools. Their findings indicated that the half-life for mitochondrial glutathione is 30 hours vs. 2 hours for cytosolic glutathione. Although mitochondria contain approximately 10-15% of the total cellular glutathione pool, the concentration of mitochondrial glutathione (10 mM) is higher than that observed in the cytosol (7 mM) (117).

The observation that mitochondrial glutathione is more concentrated than cytosolic glutathione may suggest that its biological role in mitochondria is of significance to cellular homeostasis. Perhaps the most significant function of mitochondrial glutathione is the detoxification of endogenously formed hydrogen peroxide (118-120) to water via the glutathione redox cycle (Figure II. 6). Although catalase activity was recently demonstrated in heart mitochondria, (122), liver mitochondria do not contain catalase (121), and therefore we may assume

that a primary function of mitochondrial glutathione is for the detoxification of endogenously produced H_2O_2 (discussed below). The glutathione redox cycle requires the enzymes GSH peroxidase (selenium containing) and GSSG reductase along with the cofactors GSH and NADPH (Figure II. 6) (123-124).

Another important function of mitochondrial glutathione is maintenance of protein thiols (104, 111). Mitochondria contain about 100 nmol of total thiol per mg of protein with nearly 95% being associated with protein and the remaining 5% as nonprotein thiol, mostly glutathione (125-126). Sulfhydryl groups are essential for the activity of nearly all metabolic carriers associated with the inner membrane of mitochondria. Oxidation of these essential sulfhydryls generally leads to inhibition of transporting capacity that can result in mitochondrial dysfunction (126).

The ratio of GSH:GSSG in mitochondria is approximately 18:1 under normal (untreated) conditions. Unlike cytosolic GSSG, mitochondrial GSSG is not effluxed from the matrix compartment (127). Olafsdottir and Reed (127) demonstrated that during oxidative stress induced with *t*-butyl hydroperoxide, GSSG is accumulated in the mitochondrial matrix and is eventually reduced back to GSH. However, as the redox state of the mitochondria increased, an increase in protein mixed disulfides was also observed. It was concluded that mitochondria are more sensitive to redox changes in GSH:GSSG than the cytosol and therefore mitochondria may be more susceptible to the damaging effects of oxidative stress. These findings lend credence to the suggestions that, under certain experimental conditions, irreversible cell injury due to

oxidative challenge may result from irreversible mitochondrial dysfunction.

Endogenous Oxidative Stress of Mitochondria

Nearly 90% of the total O_2 consumed by mammalian species is delivered to mitochondria where a 4-electron reduction to H_2O by the electron transport chain is coupled to ATP synthesis (Figure II. 3) (118-120). Nearly 4% of the mitochondrial O_2 is incompletely reduced, due to the leakage of electrons along the respiratory chain. Incomplete reduction forms toxic reactive oxygen species such as the superoxide radical ($O_2\cdot$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical ($HO\cdot$) (118-120). Without mitochondrial antioxidant defense systems such as the glutathione redox cycle (Figure II. 6), aerobic metabolism, an essential process for life in many species, would likely be impossible due to reactive oxygen species.

The superoxide radical undergoes disproportionation to H_2O_2 and O_2 via the Mn-containing superoxide dismutase. This reaction can also take place nonenzymatically, but at a rate approximately four orders of magnitude less at pH 7.4 (128). Richter (129) calculated that during normal metabolism, one rat liver mitochondrion produces 3×10^7 superoxide radicals per day. It is estimated that superoxide and hydrogen peroxide steady state concentrations are in the picomolar and nanomolar range, respectively (130). Jones and co-workers (131) have estimated the hepatocyte steady state H_2O_2 concentration to be as high as 25 μM. If H_2O_2 is not detoxified to H_2O, formation of $HO\cdot$ can occur by metal (iron) catalyzed Haber Weiss or Fenton type reactions (Equation II. 2) (132). The

HO· species is one of the most reactive and short lived biological radicals and has the potential to initiate lipid peroxidation of biological membranes (132-133), although not as effectively as other radicals, e.g., the ROO· radical (134). Unless termination reactions occur, the process of lipid peroxidation will propagate, resulting in potentially high levels of oxidative stress. Therefore, detoxification of endogenously produced H_2O_2 is critical for redox maintenance of mitochondrial as well as cellular homeostasis.

A. Haber Weiss Reaction $O_2^· + H_2O_2 + Fe^{3+} ----> O_2 + OH^- + HO· + Fe^{2+}$

B. Fenton Reaction $H_2O_2 + Fe^{2+} ----> HO· + Fe^{3+}$
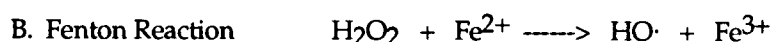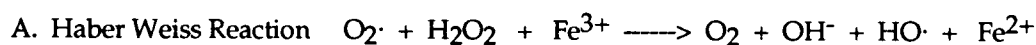
Equation II. 2.

Statement of Purpose

Previous studies have suggested that disrupted Ca^{2+} homeostasis due to the absence of extracellular Ca^{2+} results in the loss of mitochondrial glutathione through an unknown mechanism. The goal of the present work is to further investigate the role of Ca^{2+} in regulating glutathione levels in isolated rat liver mitochondria. In particular, this work has explored the role of mitochondrial permeability transition in this process.

We selected inorganic phosphate as the inducer of permeability transition. In the Ca^{2+} omission model of cellular oxidative stress, no exogenous treatment is needed, other than the removal of physiological

Ca^{2+}. The use of inorganic phosphate therefore seemed to possibly provide a mimic of the cellular model which was our initial goal.

In summary, with this research we hoped to further characterize the role of mitochondrial glutathione and mitochondrial Ca^{2+} homeostasis and explore the mechanism(s) of how disrupted Ca^{2+} homeostasis influences electron transport processes, cellular energy production, and transport of GSH. The major questions addressed here were (1) Does Ca^{2+}- and inorganic phosphate-induced permeability transition affect mitochondrial glutathione status? (2) Does opening of the Ca^{2+}-dependent pore provide a mechanism to load mitochondria with glutathione? (3) What effect do respiratory inhibitors have on permeability transition and glutathione status? (4) What are the effects of Ca^{2+}- and inorganic phosphate-induced permeability transition on mitochondrial pyridine nucleotides and adenine nucleotides?

INNER
MEMBRANE

OUTER MEMBRANE

CRISTA

MEMBRANE
CONTACT
POINT

MITOCHONDRIAL
DNA

RIBOSOMES

MATRIX

OUTER MEMBRANE
PORE

INNER MEMBRANE
SPACE
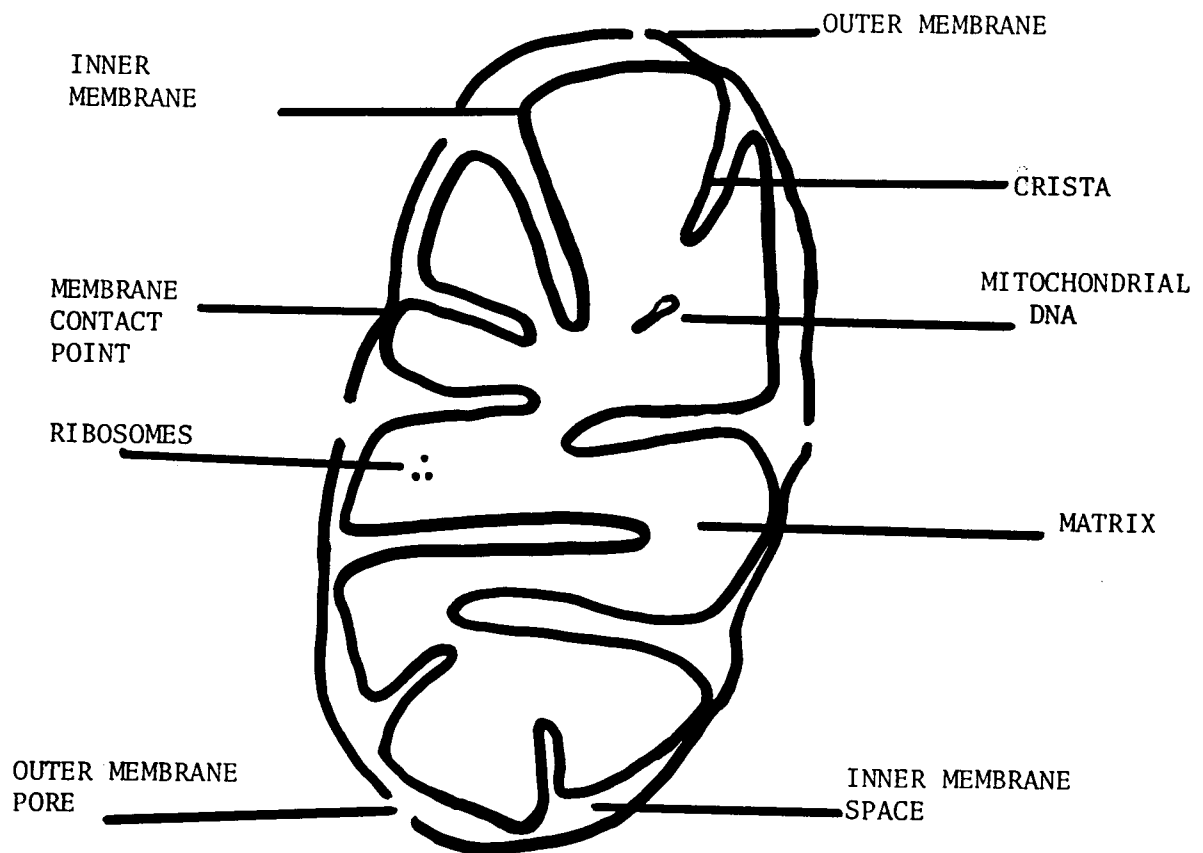
Figure II. 1. Main structural features of the mitochondrion.

Inner
Membrane

Extramitochondrial
Environment

Matrix

ADP^{-3}

ATP^{-4}

Pi$^-$

OH$^-$

dicarboxylate^{-2}

Pi^{-2}

tricarboxylate^{-2}

malate^{-2}

pyruvate$^-$

OH$^-$

glutamate

OH$^-$

glutamate

aspartate$^-$

L-ornithine$^+$

H$^+$

acylcarnitine

L-carnitine
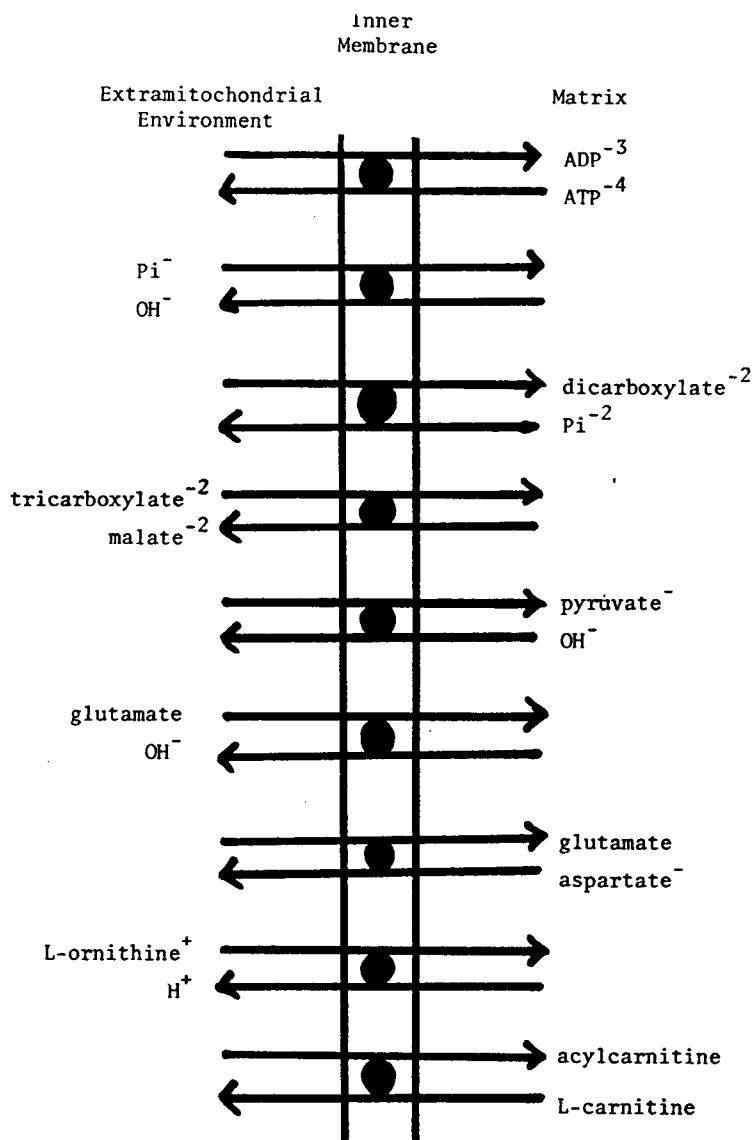
Figure II. 2. Intermembrane carriers of mitochondria.
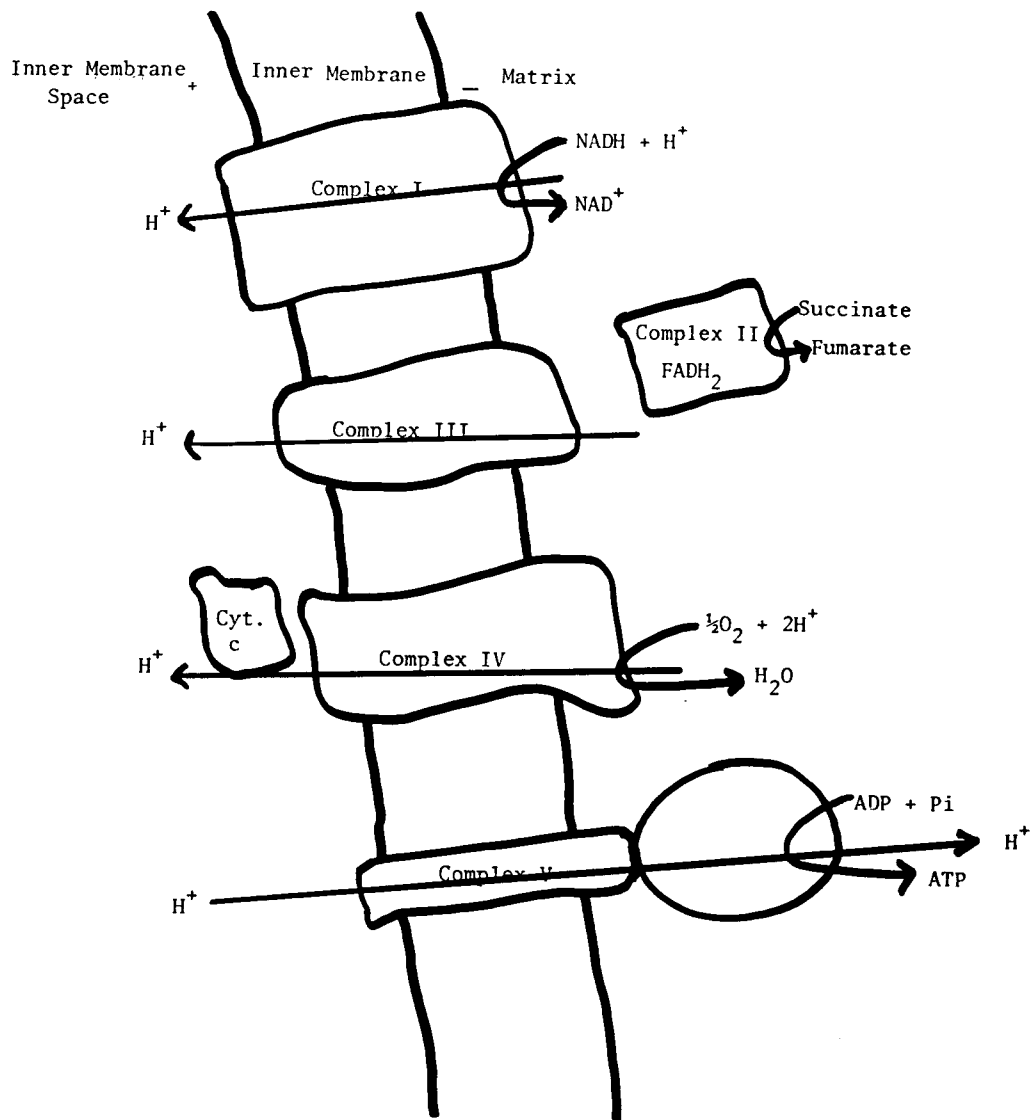
Figure II. 3. The respiratory chain of mitochondria.
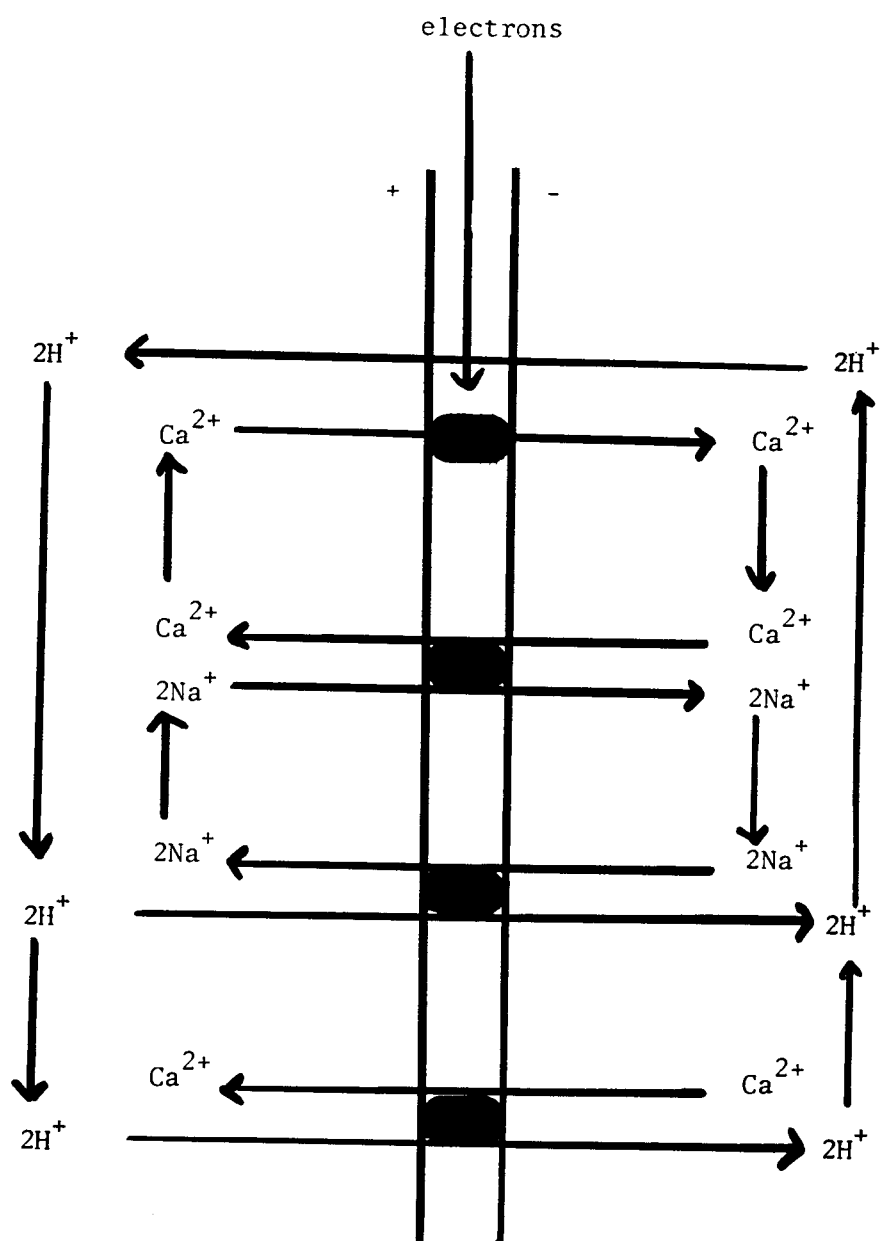
Figure II. 4. Mitochondrial Ca^{2+} transport.
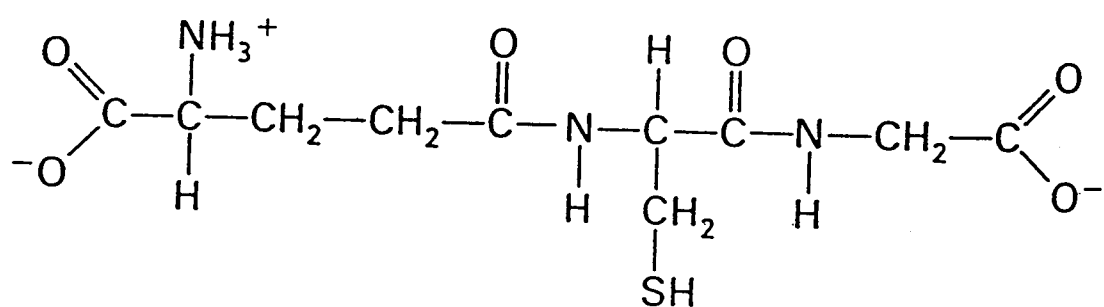
Figure II. 5. Structure of glutathione.

ROH
H$_2$O

GSH

NADPH

NADH

Substrate

ATP

GSH
Peroxidase

GSSG
Reductase

Energy Linked
Transhydrogenase

Respiratory
Chain
Complexes

H$_2$O$_2$
ROOH

GSSG

NADP$^+$

NAD$^+$

Oxidized
Substrate

ADP + Pi

H$^+$
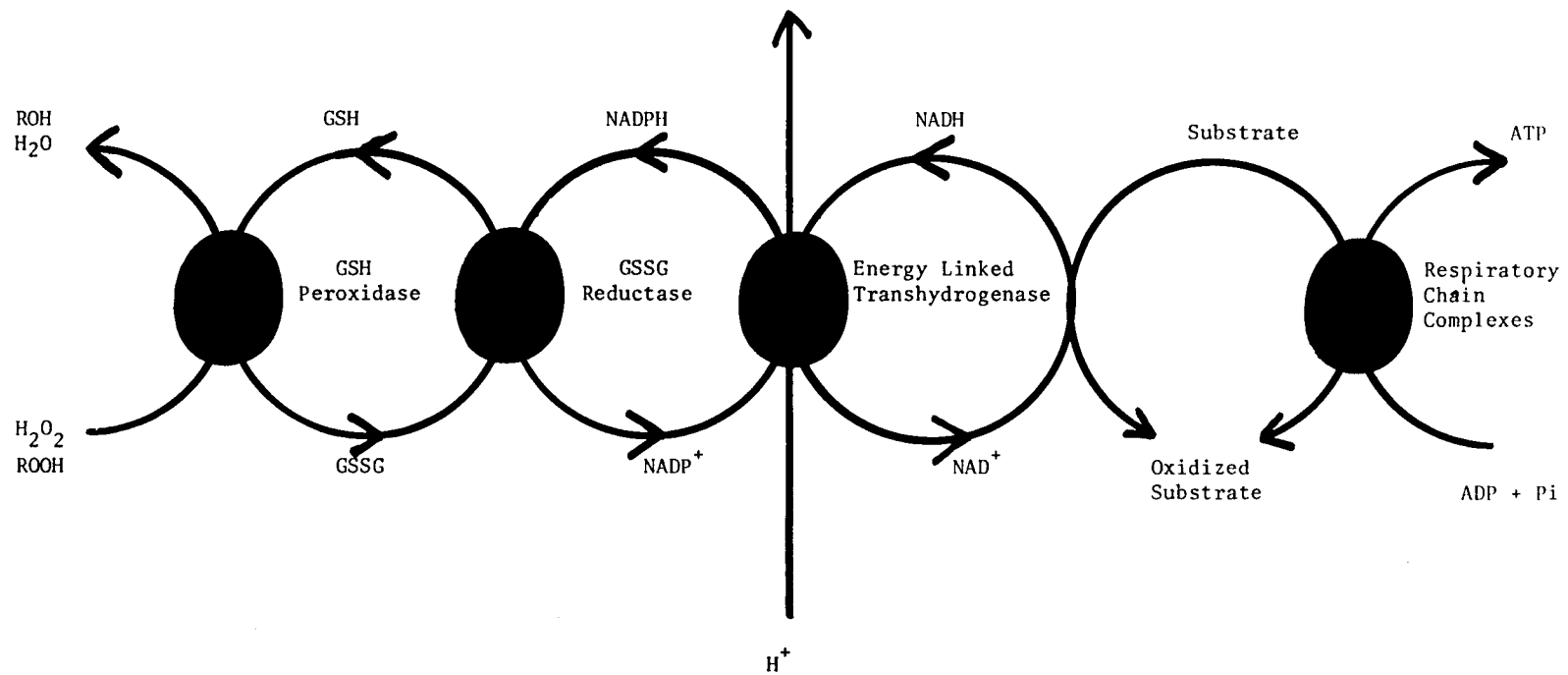
<u>Figure II. 6.</u> Mitochondrial glutathione redox cycle.

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III. CALCIUM- AND PHOSPHATE-DEPENDENT RELEASE AND LOADING OF GLUTATHIONE BY LIVER MITOCHONDRIA

Abstract

The status of glutathione was studied in isolated rat liver mitochondria under conditions which induce a permeability transition. The transition is characterized by an increased inner membrane permeability to some low molecular weight solutes and by large amplitude swelling. Addition of 70 μM Ca^{2+} and 3 mM Pi to mitochondria resulted in mitochondrial swelling and extensive release of glutathione that was recovered in the extramitochondrial medium as glutathione. Both swelling and the efflux of mitochondrial glutathione were prevented by CsA. Incubation of mitochondria in the presence of Ca^{2+}, Pi, and glutathione followed by the addition of CsA provided a mechanism to load mitochondria with exogenous glutathione that was greater than the rate of uptake by untreated mitochondria. Thus, glutathione efflux from mitochondria may occur under toxicological and pathological conditions in which mitochondria are exposed to elevated Ca^{2+} in the presence of near physiological concentrations of Pi through a nonspecific pore. Opening and closing of the pore could also provide a mechanism for the uptake of glutathione by mitochondria.

Introduction

Isolated mitochondria from a variety of tissues can undergo a Ca^{2+}-dependent permeability transition (1-9). Previously identified characteristics of the transition include permeability of the inner mitochondrial membrane to several low molecular weight (< 1200 Da) solutes, loss of coupled functions, and large amplitude swelling (1-4). The transition occurs when mitochondria are treated with Ca^{2+} in the presence of an inducing agent. Several structurally and functionally different inducing agents have been identified. Examples of inducing agents include near physiological concentrations (10) of inorganic phosphate (Pi) (4), hydroperoxides (2), some heavy metals (11), and sulfhydryl reagents (5).

The increased permeability is thought to occur through a Ca^{2+}-dependent nonspecific inner membrane pore through which ions and small molecules can diffuse very quickly from the mitochondrial matrix (1-3). Cyclosporin A (CsA), an immunosuppressive cyclic peptide, is a potent inhibitor of the permeability transition and solute movement via this nonspecific pore (4, 12-13). In a recent report, Szabo´ and Zoratti (14) suggested that the Ca^{2+}-dependent pore of the permeability transition and the giant channel of the inner mitochondrial membrane as observed with patch clamp techniques are the same species. This suggestion is based on the common feature of inhibition by CsA.

Rat liver mitochondria contain approximately 10 mM glutathione (307 Da) verses 7 mM glutathione in the cytosol (15-16). The cytosolic glutathione pool may supply the mitochondrial glutathione pool via a

specific transport system since glutathione synthesis has not been observed with mitochondria. Early studies by Jocelyn et al. (17) indicated that the retention of mitochondrial glutathione is a highly regulated process. This observation was based on the finding that isolated mitochondria retained their glutathione, indicating that under normal isolation conditions, the inner mitochondrial membrane is impermeable to glutathione (17).

At present, very little is understood about the mechanism(s) of glutathione movement in isolated mitochondria or in mitochondria contained in cells. We therefore investigated the effect of the permeability transition on glutathione status in isolated rat liver mitochondria to determine if opening the pore provides a mechanism for glutathione release and uptake.

Experimental Procedures

Materials

All chemicals were purchased from commercial sources and were of the highest quality available. CsA was a generous gift from Dr. Sue Kohlhepp (Providence Hospital, Portland, OR). CsA was dissolved in 100% ethanol (0.5 mM stock) and stored at 4°C. $[^3H]H_2O$ (2 mCi/ml), $[^{14}C]$sucrose (0.5 mCi/ml), $[^{35}S]GSH$ (55.2 Ci/mol), and scintillation cocktail (formula 983) were purchased from New England Nuclear.

Mitochondrial Preparation

Liver mitochondria were isolated from 325-375 g male Sprague-Dawley rats by the method of Schnaitman and Greenwalt (18) and washed twice in an isolation medium containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, and 0.5 mg/ml bovine serum albumin (defatted), pH 7.0. Mitochondrial protein was measured by the method of Bradford (19) or Peterson (20) with bovine serum albumin as protein standard. Cytosolic contamination in the final mitochondrial suspension was negligible as determined by measuring the activity of the cytosolic enzyme, lactate dehydrogenase (21). The mitochondrial matrix enzyme, citrate synthase was determined to assess mitochondrial leakage (22).

Mitochondrial Incubations

Washed mitochondrial pellets were suspended in 5-7 ml of incubation medium containing 213 mM mannitol, 71 mM sucrose, 3 mM HEPES (Na^+), 10 mM succinate (Na^+), and 1µM rotenone (0.5 mM stock

dissolved in 100% ethanol). Mitochondria were suspended in 25 ml Erlenmeyer flasks at protein concentrations of 1 mg/ml. After making various additions to the buffer, initial time points were taken immediately following the addition of mitochondria. The ensuing mitochondrial suspensions were rotated (100 rpm) in a Lab-Line Orbit Environ-Shaker at 25°C and exposed to atmospheric air for the incubation period.

Samples (1 ml), taken at the times indicated in the figure legends, were transferred to 1.5 ml microcentrifuge tubes and centrifuged for 30 s at 13,000 g. The supernatants were removed for further biochemical analysis. The pellets were washed once in incubation buffer containing the treatment compounds. The washed mitochondria were suspended (and pelleted) in 0.2 ml 10% (w/v) perchloric acid (containing the internal standard, gamma-glutamyl-glutamate) for analysis of mitochondrial glutathione.

Biochemical Analyses

Both intra- and extramitochondrial glutathione and glutamate levels were determined by the HPLC method of Reed et al. (23). Briefly, supernatant and perchloric acid samples were derivatized for HPLC analysis by the addition of iodoacetic acid (20 mg/ml H_2O containing cresol purple as a pH indicator) followed by 2,4-fluorodinitrobenzene (1% v/v in 100% ethanol). The derivatized samples were stored in the dark for 24 hr at 4°C prior to HPLC analysis as previously described (23).

Total intramitochondrial Ca^{2+} content was determined in the 10% perchloric acid mitochondrial extracts. Intramitochondrial samples were taken from the perchloric acid layer and mixed with an equal volume of

0.1% $LaCl_3$ to inhibit interference by other ions and then analyzed for total

intramitochondrial Ca^{2+} with a Perkin-Elmer model 403 atomic

absorption spectrophotometer (acetylene and air). Inorganic phosphate

was determined by the method of Fiske and Subbarow (24). Samples (1.0

ml) were centrifuged at 13,000 g for 30 s and the pellets resuspended in 0.12

ml 5% trichloroacetic acid. Samples were kept on ice prior to being

assayed. 0.1 ml was transferred to a glass tube containing 0.7 ml 5%

trichloroacetic acid. 0.2 ml acid molybdate (1.25 g ammonium molybdate

in 100 ml of 2.5 N sulfuric acid) was added, followed by the addition of 0.05

ml Fiske Subbarow reducer (Sigma). The solution was vortexed and the

absorbance at 660 nm was determined with an Aminco DW 2000

spectrophotometer following a 10 min incubation.

Mitochondrial swelling was determined spectrophotometrically by

monitoring the decrease in absorbance at 540 nm with an Aminco DW

2000 spectrophotometer operated in split beam mode (25). Mitochondrial

volumes were determined using $[^3H]H_2O$ and $[^{14}C]$sucrose as described in

earlier methods (26-27). These measurements are based on the

assumptions that H_2O is permeable to all mitochondrial compartments

and sucrose is permeable to only the outer membrane in normal

mitochondria. The glutathione permeable space was determined with

$[^{35}S]$GSH. Briefly, mitochondria (1 mg protein/ml) were incubated in 25

ml Erlenmeyer flask containing either $[^3H]H_2O$, $[^{14}C]$sucrose, or $[^{35}S]$GSH.

For incubations with $[^{35}S]$GSH, 1 mM cold glutathione was added to the

incubation buffer. Aliquots (1 ml) were taken at the time points indicated

in the figure legends and centrifuged immediately. Supernatant samples

(0.05 ml) were taken for scintillation counting on a Packard Liquid

Scintillation Spectrometer (Model 2450). The pellet was washed immediately in appropriate buffer and then resuspended in 0.2 ml 10% perchloric acid. Following centrifugation, 0.1 ml samples were taken for scintillation counting.

Results

The permeability transition in isolated mitochondria is a well

characterized Ca^{2+}-dependent phenomenon that includes such changes as

large amplitude swelling and movement of solutes from the

mitochondrial matrix compartment, presumably due to permeability

changes of the inner mitochondrial membrane. Figure III. 1 shows that

mitochondria (1 mg/ml) treated with 70 μM Ca^{2+} and 3 mM Pi (CaPi)

undergo a rapid loss in absorbance at 540 nm, consistent with

mitochondrial swelling (25). Maximal swelling occurred within 4 min

and was inhibited by treatment with 0.5 μM CsA, a potent inhibitor of the

Ca^{2+}-dependent nonspecific pore (4, 12-13). CsA added between 0-4 min

prevented further swelling from occurring, although mitochondrial

absorbance did not recover to control values (data not shown).

Swelling also occurred in mitochondria treated with 3 mM Pi alone

(Figure III. 1), probably due to the presence of endogenous Ca^{2+} (data

shown below) that is retained or acquired during the isolation procedure.

Addition of 1 mM EGTA, a Ca^{2+} chelator, in the presence of 3 mM Pi

prevented mitochondrial swelling (data not shown), verifying the

dependence of pore formation on the presence of Ca^{2+}.

During the permeability transition, some low molecular weight ions

and molecules (<1200 Da) are released from the mitochondrial matrix (3).

We therefore investigated the effect of the permeability transition on

mitochondrial glutathione (307 Da). Figure III. 2 shows that treatment

with CaPi resulted in extensive release of mitochondrial glutathione

within 5 min that was recovered in the extramitochondrial buffer as

glutathione (Figure III. 2B). Measurement of citrate synthase activity during the 30-min incubation showed that this matrix enzyme is retained, indicating that mitochondrial lysis could not account for the glutathione released. The addition of 0.5 μM CsA (at time zero) in the presence of CaPi treated mitochondria completely inhibited the release of glutathione (Figure III. 2A, B) suggesting that glutathione release occurred through the Ca^{2+}-dependent pore.

Treatment with 3 mM Pi alone also induced the permeability transition and resulted in extensive glutathione release (Figure III. 2A,B), with nearly complete loss from mitochondria occurring in 20 min. EGTA (1 mM) was added to determine if the presence of endogenous Ca^{2+} contributed to the release of glutathione under these conditions. Our findings showed that removal of Ca^{2+} by chelation prevented glutathione release (data not shown) as it also protected against mitochondrial swelling.

We also examined the effects of 70 μM Ca^{2+} alone on mitochondrial glutathione release (data not shown). During the 30 min incubation period, Ca^{2+}- treated mitochondria underwent only slight swelling (less than 10% increase in volume) and released approximately 23% of their glutathione that was recovered in the incubation medium. This partial release of mitochondrial glutathione may be explained by the presence of endogenous Pi.

Measurement of intramitochondrial Pi (Figure III. 3) showed that untreated (control) mitochondria contained about 5 nmoles Pi/mg protein. With the addition of 70 μM Ca^{2+}, the mitochondrial Pi levels increased slightly over control values. With the addition of 3 mM Pi or

CaPi, mitochondria accumulated nearly 6 and 8 times, respectively, the level of Pi retained by control mitochondria. For mitochondria treated with CaPi plus CsA, the Pi accumulation was nearly 15 times greater than control values and nearly twice the Pi concentration for CaPi treated mitochondria (Figure III. 3). The accumulation of Pi was a somewhat unexpected result, particularly for the CaPi-treated mitochondria.

Mitochondria undergoing the permeability transition have been shown to lose matrix components such as Ca^{2+}, Mg^{2+}, and other solutes (1-3). We measured intramitochondrial Ca^{2+} to confirm these results (Figure III. 4) and our findings showed that Ca^{2+} is released rapidly by mitochondria undergoing the permeability transition. The addition of CsA under these conditions prevented the loss of Ca^{2+}. Measurement of mitochondrial glutamate by HPLC showed qualitatively the same pattern of results as measurement of Ca^{2+} and glutathione, with complete loss of mitochondrial glutamate only occurring in mitochondria treated with CaPi (data not shown).

Comparison of the extent of glutathione loss with the decrease in absorbance at 540 nm (swelling) showed that glutathione loss correlated with swelling for Ca^{2+} alone, Pi alone, and most of the data for CaPi (Figure III. 5). With CaPi, the swelling occurred so rapidly that the last 20-30% of glutathione release occurred only as swelling approached 100%. Thus, the results indicate that rapid and complete loss of mitochondrial glutathione occurred under conditions where CaPi induced a permeability transition.

Induction of the permeability transition has been shown to be associated with an increase in the sucrose permeable space. This is

determined by incubating mitochondria with radiolabeled sucrose and comparing the amount of sucrose associated with the mitochondrial pellet following centrifugation (9, 28-29). We performed similar experiments to compare the volume of sucrose-permeable space to the water-permeable space. Mitochondria were incubated with either $[^3H]H_2O$ or $[^{14}C]$sucrose and centrifuged through a layer of dibutylpthalate to separate intra- and extramitochondrial components. Our findings indicated that CaPi increased the sucrose-permeable space [from 2.49 µl/mg (± 0.391) to 4.62 µl/mg (± 0.241)]. Addition of $[^{35}S]$GSH instead of $[^{14}C]$sucrose indicated that the glutathione permeable space is similarly increased [2.52 µl/mg (± 0.138) to 5.35 µl/mg (± 0.241)].

Addition of CsA to mitochondria that had undergone the permeability transition was found to result in blocking the further movement of sucrose (31). Thus, $[^{14}C]$sucrose could be trapped in mitochondria by initially incubating with $[^{14}C]$sucrose under conditions which caused the transition followed by addition of CsA to prevent further movement across the membrane. We performed similar experiments with $[^{35}S]$GSH to determine whether glutathione could be similarly loaded or trapped into mitochondria. Addition of CsA to mitochondria following a brief incubation with CaPi and 1 mM exogenous glutathione provided a method to load exogenous glutathione that was not removed upon washing into the mitochondrial matrix (data not shown).

Experiments to determine whether that increase in matrix glutathione was due to transport or to trapping by closing the pore were performed. The permeability transition was induced by incubation with

CaPi in the presence of 1 mM glutathione and 0.3 μCi/μmol [^{35}S]GSH. CsA was added at either time zero or at the time the sample was taken for analysis (Figure III. 6). After centrifugation to remove the mitochondria from the incubation media, mitochondria were resuspended in the appropriate media without [^{35}S]GSH and washed by recentrifugation. Using this protocol, only about 5% of the scintillation counts associated with the pellet could be attributed to counts in the extramitochondrial water as ascertained by measuring the amount of [^{3}H]H$_2$O remaining associated with the pellet following the same procedure. The amount of glutathione that was taken up by control mitochondria was about 0.5 nmol glutathione/min/mg protein (Figure III. 6). In the presence of CaPi, no time-dependent increase in glutathione was observed. However, addition of CsA to mitochondria undergoing the permeability transition resulted in a time-dependent increase in the amount of glutathione associated with the mitochondria. This amount was nearly twofold greater than the amount of glutathione transported by control mitochondria (Figure III. 6). Experiments in which CsA was added along with CaPi at zero time (permeability transition inhibited) showed somewhat less glutathione uptake than did mitochondria (control) without CaPi. Thus, the uptake of glutathione by transport systems under conditions which do not activate the pore or in which the pore is blocked was less than the uptake of glutathione by the controlled opening and closing of the pore.

Discussion

The present study provides evidence for a mechanism of both release and loading of mitochondrial glutathione. By inducing the permeability transition with CaPi mitochondria released nearly 100% of their glutathione within a 5 min incubation period. Addition of 3 mM Pi alone also induced the permeability transition, as indicated by mitochondrial swelling and nearly complete release of mitochondrial glutathione within 20 min. We hypothesize that release of matrix glutathione occurs through a Ca^{2+}-dependent nonspecific pore which is opened during the transition, allowing movement of some low molecular weight molecules. CsA, an inhibitor of the pore, prevented loss of glutathione in the presence of either Pi alone or CaPi. Treatment with CsA provided a method to load mitochondria with exogenous glutathione by first inducing the transition, and then adding CsA to close or inhibit the pore.

Since the function of the CaPi-dependent pore is not known, it is not clear whether activation of the pore is necessarily physiological or deleterious. In a recent report, Halestrap and Davidson (30) suggested that this pore is the adenine nucleotide transporter that has been converted to a different functional state by Ca^{2+} and Pi. They reported that CsA probably binds to the mitochondrial matrix enzyme, peptidyl-prolyl cis-trans isomerase (PPI) and prevents the enzyme from interacting with the adenine nucleotide translocase. Their study suggested that the interaction of PPI with the adenine nucleotide translocase in the presence of Ca^{2+} and an inducing agent results in nonspecific pore opening. They further

suggested that an occasional opening and closing of the pore could provide a mechanism for the mitochondria to eliminate waste products for which no specific transport system exists. Such an occasional opening and closing of the pore also could provide a means to load mitochondria with cytoplasmic components, including glutathione. We directly compared the amount of glutathione that was loaded with the pore closed verses the pore opened and found that the rate of loading was greater through the pore. However, it must be emphasized that sustained opening of the pore, such as would occur with a sustained elevation of Ca^{2+}, is likely to cause extensive loss of mitochondrial components and result in irreversible damage.

Our data indicated that the presence of a near physiological concentration of Pi (3 mM) is sufficient to induce the permeability transition. This is presumably due to the presence of 6-10 nmol Ca^{2+} per milligram of protein retained by the mitochondria during the isolation procedure. Whether this Ca^{2+} is free or bound remains to be determined. However, by chelating the Ca^{2+} with the addition of 1 mM EGTA, mitochondrial swelling and glutathione release were both prevented, indicating the dependence of pore formation on the presence of available Ca^{2+}. The presence of 70 µM Ca^{2+} (alone) did not result in any appreciable swelling; however, these mitochondria released approximately 23% of their glutathione.

Control mitochondria contained about 5 nmol Pi/mg protein while Ca^{2+} treated mitochondria nearly doubled their Pi concentration. The increased levels of Pi may reflect ATP hydrolysis since the uptake of Ca^{2+}

via the uniport is an energy dependent process that can lead to dissipation of the mitochondrial membrane potential.

Ca^{2+} and other matrix components measured (glutathione and glutamate) were released when mitochondria were treated with CaPi. However, Pi was unexpectedly retained within the matrix under conditions which induced the permeability transition. Possible explanations for Pi retention include formation of a phosphate precipitate or interaction of Pi with molecules that are retained within the matrix under these conditions. Such interactions may include ionic or covalent association of Pi with molecules such as protein(s) retained within the matrix compartment.

Pi retention raises the question of whether the Ca^{2+}-dependent pore has some selectivity. To our knowledge, this is the first evidence that raises the question of selectivity of the Ca^{2+}-dependent pore. Previous findings suggests that the pore is nonspecific due to the variety of molecules and ions which are permeable.

In summary, the present study demonstrates a Ca^{2+}-dependent mechanism for the rapid and nearly complete release of mitochondrial glutathione. Evidence presented suggests that the release of matrix glutathione occurs through a Ca^{2+}-dependent pore of which CsA is a potent inhibitor. The addition of CsA prevented glutathione release as well as mitochondrial swelling. Understanding how CsA prevents permeability changes and consequently release of matrix solutes may provide mechanistic insight as to how this type of mitochondrial response occurs. In addition we have shown that glutathione can also be trapped or

loaded in mitochondria by opening and closing of the pore with CaPi and CsA respectively.
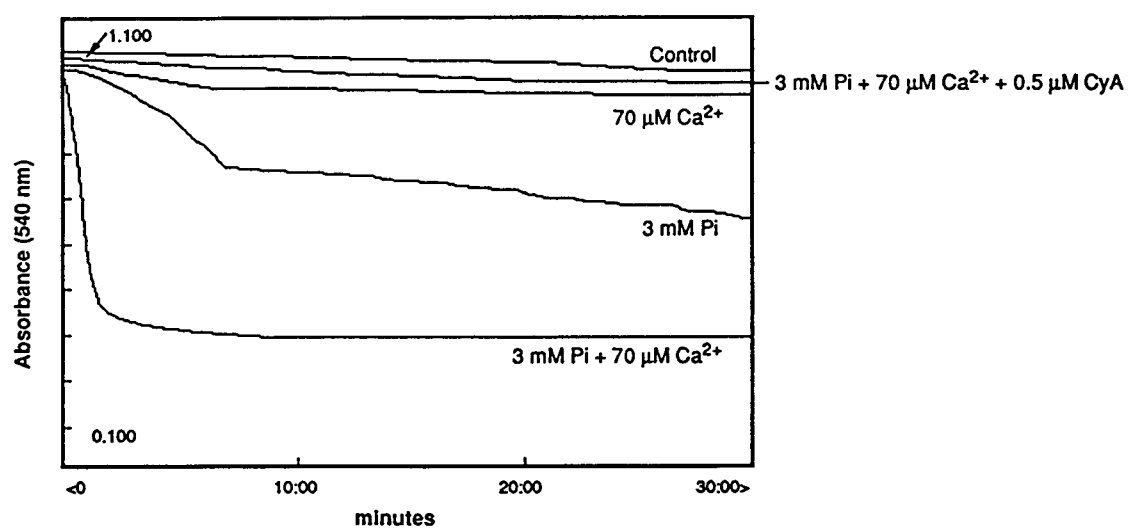
Figure III. 1. Mitochondrial swelling. Mitochondrial suspensions (1 mg/ml) were monitored continuously for 30 min at absorbance 540 nm using a Aminco DW 2000 spectrophotometer in split beam mode in the presence of treatments listed. Values are from a single representative experiment.

Figure III. 2. The effect of permeability transition on mitochondrial glutathione. Mitochondria (1 mg/ml) were incubated in the absence (control) or presence of 70 µM Ca^{2+} + 3mM Pi (CaPi); 3 mM Pi (Pi); or 70 µM Ca^{2+} + 3 mM Pi + 0.5 µM CsA (CaPiCsA) for 30 min. Samples (1 ml) were taken at the various times indicated and analyzed for (A) intra- and (B) extramitochondrial glutathione as described in Experimental Procedures. Values are expressed as means of nmol glutathione per mg mitochondrial protein ± S.E.M. n = 4.

A
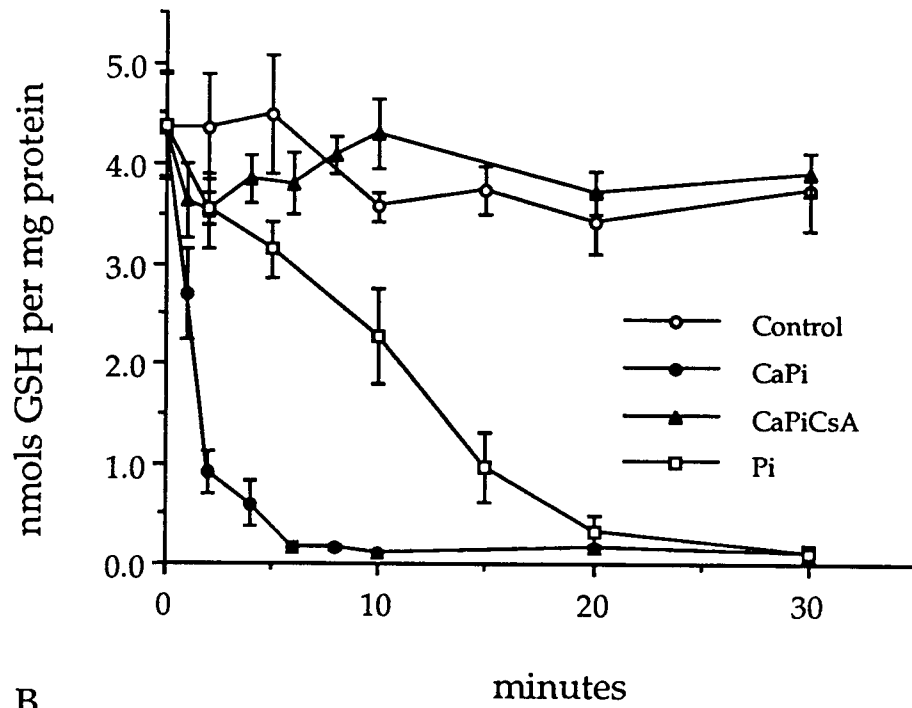
B
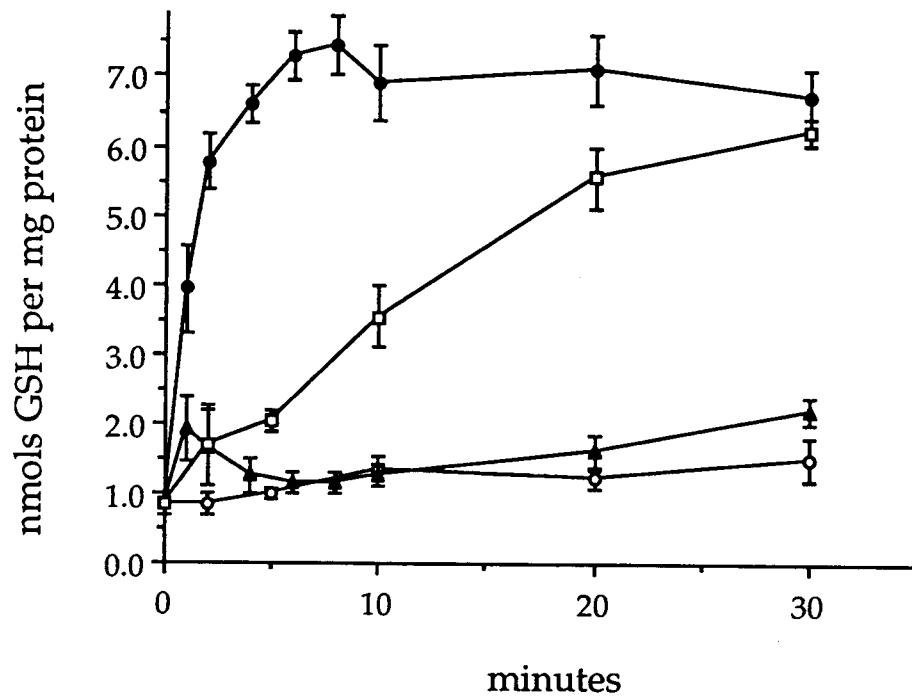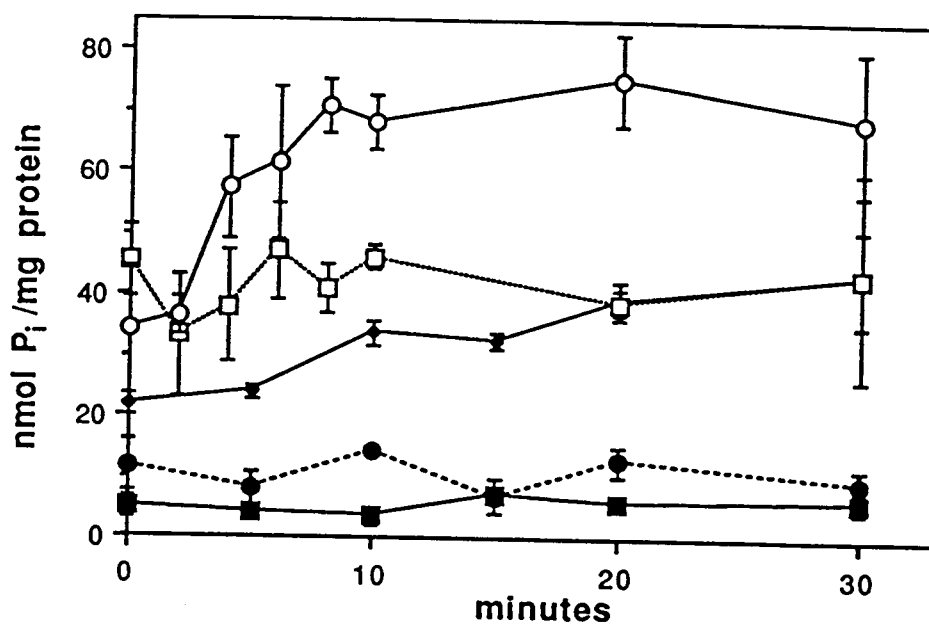
Figure III. 3. Mitochondrial inorganic phosphate concentrations. Pi levels were determined in mitochondria incubated in the absence (control, closed square) or presence of 70 μM Ca2+ (closed circle); 3 mM Pi (diamond); 70 μM Ca2+ + 3 mM Pi (open square); or 70 μM Ca2+ + 3 mM Pi + 0.5 μM CsA (open circle). Samples (1 ml) were assayed for Pi at the various times indicated as described in Experimental Procedures. Values are expressed as means ± S.E.M. n = 4.
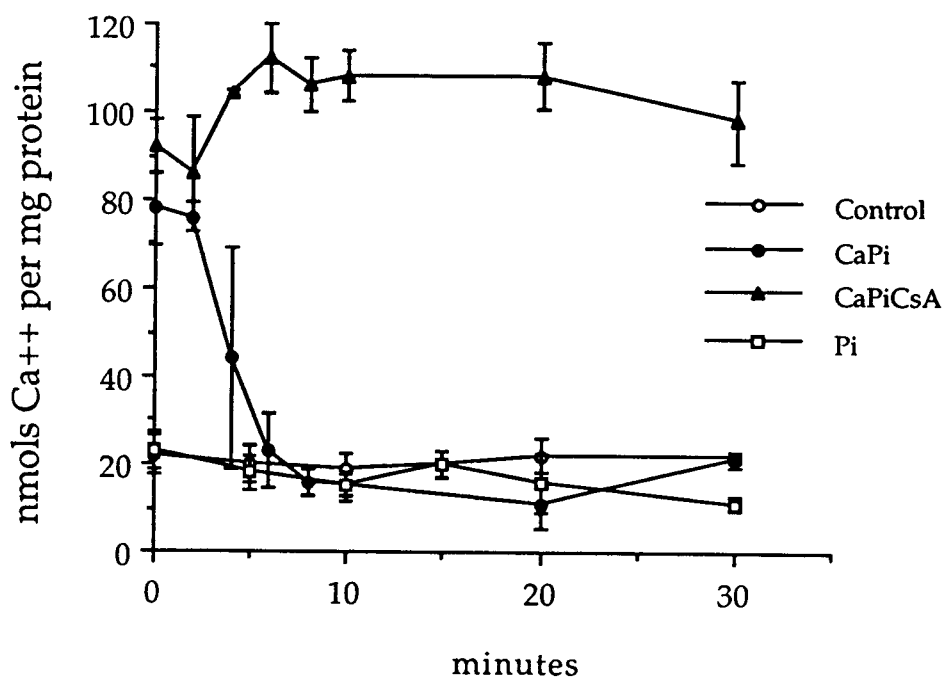
Figure III. 4. Total intramitochondrial Ca^{2+} concentrations. Ca^{2+} levels were determined in the absence (control) or presence of 70 μM Ca^{2+} + 3 mM Pi (CaPi); 3 mM Pi (Pi); or 70 μM Ca^{2+} + 3 mM Pi + 0.5 μM CsA (CaPiCsA). Samples (1 ml) were taken at the various times indicated and analyzed for total intramitochondrial Ca^{2+} as described in Experimental Procedures. Values are expressed as means ± S.E.M. n = 4.
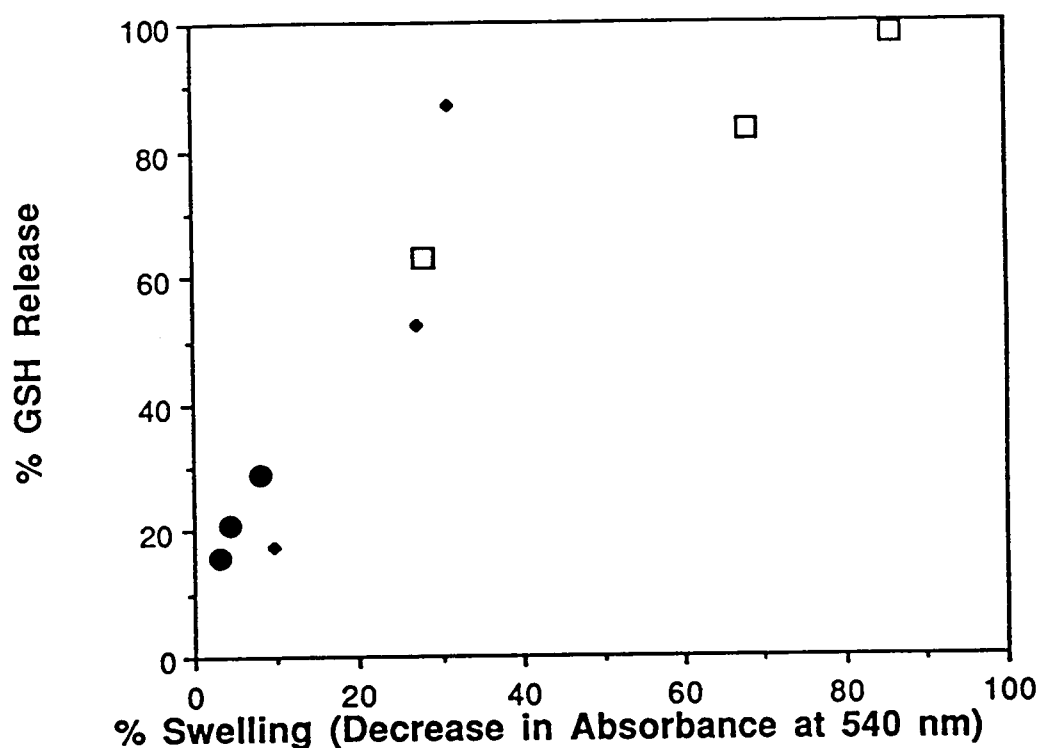
Figure III. 5. Correlation of mitochondrial swelling and glutathione loss. Data are expressed as percent mitochondrial glutathione loss vs percent mitochondrial swelling in the presence of either 70μM Ca^{2+}(circle); 3 mM Pi (diamond); or 70 μM Ca^{2+} + 3 mM Pi (square). Mitochondrial samples were analyzed at 5, 10, and 15 min for both swelling and GSH loss. For each treatment, the earliest time point correlates with the least amount of swelling and GSH loss whereas the latest time point correlates with the greatest amount of swelling and GSH loss. Data are derived from the average of 2 representative experiments.
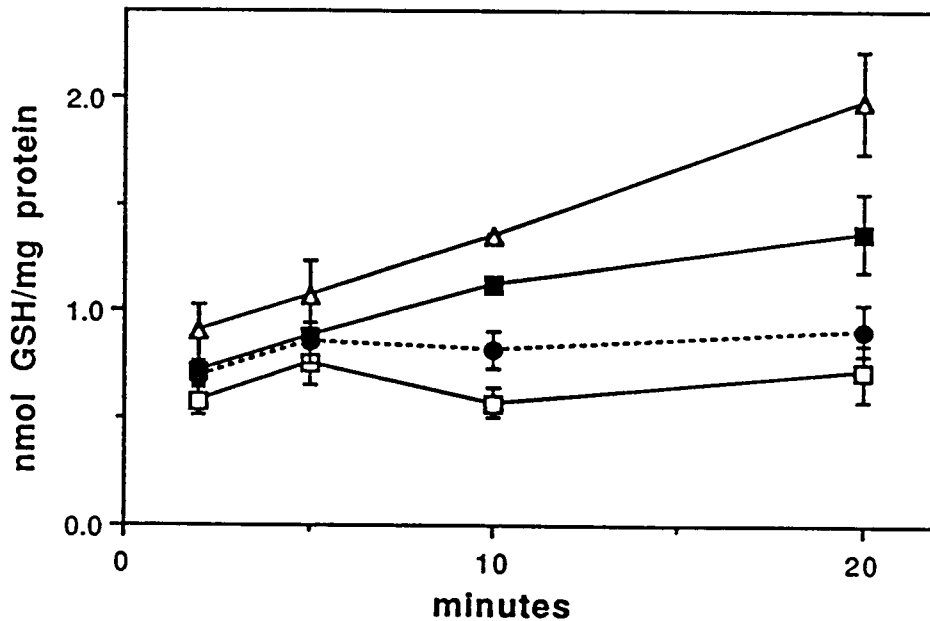
Figure III. 6. Effect of the permeability transition on mitochondrial glutathione uptake. Mitochondria (1 mg/ml) were incubated with 1 mM glutathione + 0.3 μCi/μmol ^{35}S-glutathione (control, closed square) in the presence of 70 μM Ca^{2+} + 3 mM Pi (permeability transition induced, open square); or 70 μM Ca^{2+} + 3 mM Pi + 0.5 μM CsA (permeability transition inhibited, closed circle). To trap glutathione in mitochondria undergoing the permeability transition, 0.5 μM CsA (open triangle) was added when the sample was taken for analysis as described in Experimental Procedures. Values are expressed as means $\pm$ S.E.M. n = 3.

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IV. MITOCHONDRIAL GLUTATHIONE AND CACLIUM RELEASE BY A CYCLOSPORIN A-SENSITIVE MECHANISM OCCURS WITHOUT LARGE AMPLITUDE SWELLING

Abstract

Treatment of isolated mitochondria with calcium and inorganic phosphate induces inner membrane permeability that is thought to be mediated through a nonselective, calcium-dependent pore. The inner membrane permeability results in the rapid escape of small matrix solutes such as glutathione and calcium, loss of coupled functions, and large amplitude swelling. In the current study, we have identified conditions of permeability transition without large amplitude swelling, a parameter often used to assess inner membrane permeability. The addition of oligomycin, antimycin, or sulfide to incubation buffer containing calcium and inorganic phosphate abolished large amplitude swelling, but did not prevent inner membrane permeability as demonstrated by the release of glutathione and calcium. Release of both glutathione and calcium were inhibited by the addition of cyclosporin A, a potent inhibitor of permeability transition. Transmission electron microscopy (TEM) analysis, combined with biochemical data, indicate that permeability transition can be observed in the absence of large amplitude swelling. Collapse of the membrane potential was observed both with and without large amplitude swelling. We conclude that CsA-sensitive permeability transition can occur without obvious morphological changes and, therefore, that

monitoring of the CsA-sensitive release of endogenous matrix solutes, such as GSH, may be a sensitive and useful indicator of permeability transition.

Introduction

Mitochondrial glutathione (GSH) is critical to cell viability, especially under experimental conditions involving a disruption of cellular Ca^{2+} homeostasis (1-5). These studies demonstrate a direct relationship between the loss in mitochondrial GSH and a decrease in cell viability; however, the mechanism by which GSH depletion occurs remains uncertain.

Only 10-15% of the cellular GSH resides in liver mitochondria, where it is concentrated (10 mM vs. 7 mM in the cytosol) (13) and functions in cellular defense against reactive oxygen species, particularly H_2O_2 (14-15). Endogenous production of reactive oxygen species normally occurs during aerobic respiration, when approximately 4% of the O_2 consumption results in the formation of H_2O_2 (14-15). Since liver mitochondria do not contain catalase (16), detoxification of H_2O_2 occurs through the GSH peroxidase/reductase redox cycle (17-18). Loss of mitochondrial GSH could therefore be detrimental to cell viability, particularly if challenged oxidatively.

We have previously demonstrated a Ca^{2+} dependent mechanism for the rapid and nearly complete depletion of mitochondrial GSH (6). Treatment of isolated mitochondria with Ca^{2+} and Pi permeabilizes the inner membrane of mitochondria, releasing small matrix solutes such as GSH and Ca^{2+} (6-10). Permeability transition is thought to occur due to activation, or perhaps deregulation, an inner membrane protein that functions as a putative pore. Following permeabilization, mitochondria undergo large

amplitude swelling. Both inner membrane permeability and swelling are prevented by nanomolar concentrations of the immunosuppressive cyclic peptide, cyclosporin A (CsA) (6-7, 11-12).

The transport of Ca^{2+} by mitochondria is driven by the transmembrane potential (19-21). The inward movement of Ca^{2+} decreases the transmembrane potential which is restored either by increasing respiratory chain activity or ATP hydrolysis. Since mitochondrial Ca^{2+} uptake is closely linked with permeability transition, we have decided to examine the effects of treatment with the metabolic inhibitors, antimycin, oligomycin, and sulfide, on inner membrane permeability, large amplitude swelling, and the transmembrane potential. Antimycin A and sulfide are electron transport inhibitors at complex III and cytochrome oxidase, respectively. Oligomycin inhibits ATP synthase and eventually stops all electron transport by coupled mitochondria. (22)

As discussed below, our findings indicate that Ca^{2+}- and Pi-dependent permeability transition can occur without large amplitude swelling in the presence of antimycin, sulfide, or oligomycin. These findings suggest that under certain experimental conditions assessment of mitochondrial integrity via the light scatter assay or TEM analysis may not accurately reflect the biochemical alterations due to changes in mitochondrial inner membrane permeability. This information may be relevant to cellular or isolated mitochondrial studies relying on TEM for assessment of mitochondrial integrity, or studies of isolated mitochondria relying on the light scatter assay as a gauge of inner membrane permeability.

Experimental Procedures

Materials

All chemicals were purchased from commercial sources and were of the highest quality available. CsA was a generous gift from Dr. Sue Kohlhepp (Providence Hospital, Portland, OR). CsA was dissolved in 100% ethanol (0.5 mM stock) and stored at 4°C. Oligomycin (A,B,C), antimycin A, and sodium sulfide were purchased from Sigma Chemical Company (St. Louis, MO). Aqueous stock solutions of sodium sulfide (0.5 M) were prepared the day of each experiment. Stock solutions of oligomycin (3 mM) and antimycin (1 mM) were dissolved in ethanol. [^{3}H]TPMP (triphenylmethylphosphonium) (1mCi/ml) and scintillation cocktail (Formula 983) were purchased from New England Nuclear.

Preparation of Mitochondria

Isolated mitochondria were prepared by differential centrifugation according to the method of Schnaitman and Greenwalt (23). Briefly, livers from male Sprague-Dawley rats (325-375 g) (Simonsen Labs, Gilroy, CA) were excised and homogenized in isolation buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.5 mM EGTA, and 0.5 mg/ml bovine serum albumin, pH 7.4. Mitochondria were washed twice in isolation buffer devoid of EGTA, pH 7.0. After the final wash, the mitochondrial pellets were suspended (25-30 mg/ml) in incubation buffer containing 213 mM mannitol, 71 mM sucrose, 10 mM succinate, and 3 mM HEPES, pH 7.0.

Mitochondrial protein was determined spectrophotometrically by the method of Bradford (24) with bovine serum albumin as the standard.

Mitochondrial Incubations

Aliquots of suspended mitochondria were added to 10-ml Erlenmeyer flasks (1 mg protein/ml) containing incubation buffer plus the various treatments described in the figure legends. After briefly swirling the flask, 1.0 ml samples were taken immediately following the addition of mitochondria. These samples constitute the initial time points. Mitochondrial suspensions were exposed to atmospheric air throughout the remainder of the incubation period. Aliquants (1ml) of mitochondrial suspensions, taken at the times indicated in the figure legends, were transferred to 1.5-ml microcentrifuge tubes and immediately centrifuged for 30 s at 13,000 g. The supernatants were removed for further biochemical analysis where indicated. The remaining mitochondrial pellet was resuspended in 10% perchloric acid (PCA) and analyzed for GSH, Ca^{2+}, or [^3H]TPMP as described below.

Biochemical Analysis

GSH was determined by HPLC analysis according to the method of Reed et al. (26) with modifications as described previously (6). Ca^{2+} levels were quantitated by atomic absorption spectrophotometry as previously described (6). Assessment of the mitochondrial membrane potential was determined by uptake of [^3H]TPMP (27). Briefly, [^3H]TPMP was added to incubation flasks to produce a final specific

activity of 9 μC/umol. Aliquots (1 ml) of the suspended mitochondria were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 13,000 g for 30 s. The supernatant was discarded and the pellet carefully resuspended in incubation buffer devoid of [^{3}H]TPMP and centrifuged to remove any remaining extramitochondrial [^{3}H]TPMP. The final pellet was resuspended in 200 μl of 10% PCA, centrifuged, and prepared for scintillation counting on a Packard liquid scintillation spectrophotometer (Model 2450).

Large amplitude swelling was assessed spectrophotmetrically by monitoring the decrease in absorbance at 540 nm with an Aminco DW 2000 spectrophotometer operated in split beam mode (25). Concentration response to each metabolic inhibitor was established on the basis of the effect on swelling.

TEM Analysis

Samples (1 mg) for TEM were fixed in 3.0% glutaraldehyde, pH 7.2, for approximately 4 hrs. Mitochondria were allowed to settle out by gravity and rinsed twice with 0.2M sodium cacodylate buffer. The rinsed samples were centrifuged at 12,500 g for 10 min. Following centrifugation, the pellet was gently removed and minced into pieces, 1 mm^3. Samples were postfixed in 1.0% OsO$_4$ for 1 hr, rinsed through a graded acetone series, infiltrated with, and embedded in, epoxy resin. Ultrathin sections (60-80 nm) were cut and stained with uranyl acetate and bismuth subnitrate. Specimens were examined with a Ziess EM 10/A transmission electron microscope at an accelerating voltage of 60 kV.

Results

Effect of Metabolic Inhibitors on Mitochondrial Large Amplitude Swelling, GSH, and Ca^{2+}

Incubation of mitochondria with 70 µM Ca^{2+} and 3 mM Pi (CaPi) resulted in the rapid decrease in absorbance at 540 nm due to large amplitude swelling (Figure IV. 1E). Addition of 0.5 µM CsA, a potent inhibitor of permeability transition, prevented large amplitude swelling induced by CaPi, appearing similar to untreated control samples (Figure IV. 1A) (6-7). Similarly, addition of either 0.5 mM sulfide, 3 µM oligomycin, or 1 µM antimycin also inhibited CaPi induced large amplitude swelling that is generally associated with permeability transition (Figure IV. 1B,C,D).

Mitochondria treated with CaPi undergo a rapid and nearly complete release of GSH within 5-min as previously demonstrated (6), and as shown in Figure IV. 2. The addition of either 0.5 mM sulfide, 3 µM oligomycin, or 1 µM antimycin ameliorates CaPi induced loss of matrix glutathione to varying degrees (Figure IV. 2). Sulfide and oligomycin are the most potent inhibitors of GSH loss within the first 10-min of incubation, with antimycin being the weakest.

The GSH was recovered in the extramitochondrial buffer as GSH (data not shown) which was prevented following the addition of 0.5 µM CsA (Figure IV. 3). Detection of oxidized GSH (GSSG) did not occur above untreated control values (<0.5 nmol GSH equivalent/mg) (Data not shown). The degree to which the compounds inhibit CaPi-induced

GSH loss through a concentration dependent manner was not examined.

Treatment of mitochondria with the electron transport inhibitors antimycin or sulfide decreases the membrane potential which drives Ca^{2+} ions into the mitochondria via the Ca^{2+} uniport. Diminished Ca^{2+} uptake corresponds to treatment with electron transport inhibitors. This is shown in Figure IV. 4, where in the presence of CaPi plus 0.5 mM sulfide or 1 μM antimycin, uptake of Ca^{2+} is reduced approximately 65% as compared to mitochondria treated with CaPi alone. Mitochondria treated with 3 μM oligomycin (Figure IV. 4), an inhibitor of ATP synthesis, show an initial uptake of Ca^{2+} greater than CaPi alone. The increased uptake of Ca^{2+} is presumably due to the initial overshoot of the membrane potential because of inhibition of proton translocation at the ATP-synthase.

After initially taking up Ca^{2+}, mitochondria treated with CaPi alone rapidly released Ca^{2+} through a CsA-sensitive mechanism as previously reported and demonstrated in Figure IV. 4 (6). Regardless of the initial Ca^{2+} uptake in the presence of the metabolic inhibitors, Ca^{2+} are gradually released to levels similar to that observed with CaPi treatment alone (Figure IV. 4). Release of Ca^{2+} is prevented following the addition of 0.5 μM CsA (data not shown).

TEM Analysis

Since large amplitude swelling was not observed in the presence of metabolic inhibitors (Figure IV. 1), mitochondria were prepared and analyzed by TEM to determine if ultrastructural changes could be

observed under these conditions. Figure IV. 5A shows untreated mitochondria following a 10-min incubation. They have an electron-dense and well defined ultrastructure, indicating they are functionally and structurally intact and highly energized (28).

Figure IV. 5B shows mitochondria undergoing permeability transition following a 5-min incubation in the presence of CaPi. A major proportion of the population has undergone large amplitude swelling as evidenced by the loss in electron density and an increase in size. By 10-min (Figure IV. 5C), nearly the entire population of mitochondria has undergone large amplitude swelling. Addition of 0.5 µM CsA prevented the CaPi induced response as shown in Figure IV. 5D following a 20-min incubation.

Mitochondrial samples treated for 10-min with CaPi plus either 0.5 mM sulfide, 3 µM oligomycin, or 1 µM antimycin, (Figure IV. 5E,F,G, respectively,) do not exhibit morphological changes to the degree observed with CaPi treatment alone following a 5- or 10-min incubation (Figure IV. 5B,C). However, some mitochondria in these populations appear somewhat less electron dense than untreated mitochondria (Figure IV. 5A), and are perhaps in the orthodox conformation as opposed to the condensed conformation. These two conformations have been studied by Hackenbrock (28) who has proposed that they are related to either the metabolic state of the mitochondria, or the energization of the inner mitochondrial membrane.

To determine if a correlation exists between GSH release and the morphological alterations observed with TEM analysis, these

parameters were compared with the various treatment regimens. The degree of morphological alteration was assessed by counting, in random areas (2" X 2") of the electron micrographs, the number of morphologically altered mitochondria, and dividing this number by the total mitochondria within the area. Criteria used to identify altered mitochondria were loss of matrix density, enlargement (swelling), and similarity to mitochondria treated with CaPi (Figure IV. 5B,C). As shown in Table 1, the degree of morphological alteration does not correlate well with the amount of GSH released (correlation coefficient = 0.70).

Finally, to assess mitochondrial energy status, the membrane potential was assayed by the uptake and retention of the radiolabeled lipophilic cation, [^{3}H]TPMP. As shown in Figure IV. 6, the membrane potential was depleted almost immediately following CaPi treatment alone, or CaPi plus either 0.5 mM sulfide, 1μM antimycin, or 3 μM oligomycin. A stable membrane potential, similar to that observed with untreated samples, was observed following the addition of 0.5 μM CsA to CaPi-treated mitochondria in spite of high matrix Ca^{2+} levels (70 nmols Ca^{2+}/mg protein) (6).

<u>Discussion</u>

The major findings of this report is the dissociation of inner membrane permeability from large amplitude swelling. As large amplitude swelling results from the permeabilization of the inner membrane, it is often used to assess whether permeability transition is occurring in mitochondrial samples. As a secondary event to permeability transition, large amplitude swelling occurs because matrix proteins are slow to equilibrate through the permeability defect as compared with smaller solutes. This is thought to create a colloid-osmotic pressure imbalance which drives the entrance of water, resulting in dilution of the matrix and massive swelling (9).

Several previous reports have suggested that both antimycin and oligomycin inhibit permeability transition (29-32). These conclusions are based upon the ability of these compounds to prevent large amplitude swelling. In this report, we have demonstrated the release of both matrix GSH and Ca^{2+} without large amplitude swelling. This occurs when either antimycin, oligomycin, or sulfide are coincubated in buffer containing CaPi (Figure IV. 1,2,4). Under these conditions, the addition of CsA completely prevented GSH release (Figure IV. 3), as well as Ca^{2+} release (data not shown). Protection by CsA, a potent inhibitor of the permeability transition, suggests that inner membrane permeability occurs in the absence of large amplitude swelling.

Analysis of mitochondrial samples by TEM also indicated that the morphological alterations observed with CaPi treatment are

prevented when metabolic inhibitors are present, yet biochemically, these systems are similar as reflected by the loss of matrix solutes. These findings suggest that severe mitochondrial injury, such as the loss of matrix GSH, can occur without obvious morphological changes. Therefore, it may be necessary to monitor solute release rather than relying solely on ultrastructural analysis such as TEM or the decrease in light scatter at 540 nm to assess inner membrane permeability.

Whether inner membrane permeability occurs only in a susceptible subpopulation or gradually throughout the entire population is not clear. If a susceptible subpopulation exists, then the release of matrix solutes (GSH and Ca^{2+}) presumably represents release of GSH and Ca^{2+} from the susceptible population and would most likely be accompanied by large amplitude swelling. However, a comparison of the percent of GSH released with the percent of mitochondria exhibiting morphological alterations does not seem to strongly support this argument. Following a 10-min incubation, mitochondria coincubated with CaPi plus either antimycin, oligomycin, or sulfide, released 73%, 65%, and 27%, respectively, of their GSH. Seemingly, if this were an all-or-none response, then the percent of GSH released would represent the total release of GSH from 73%, 65%, and 27% of the mitochondrial population, and some comparable degree of large amplitude swelling would be observed.

Since the degree of morphological alterations does not correlate strongly with the degree of GSH released (Table 1), low inner membrane permeability may be occurring throughout the population. Although mitochondria do not appear altered morphologically, they

may be experiencing some degree of permeability barrier disruption. Release of Ca^{2+} appears to occur from the entire population, as opposed to a subpopulation, as near maximal Ca^{2+} release occurred following treatment with the metabolic inhibitors (Figure IV. 4).

Mitochondria treated with oligomycin, antimycin, or sulfide in buffer containing CaPi appear to be in the orthodox conformation as opposed to the condensed (Figure IV. 5E,F,G) (28). Changes in morphology from the condensed to the orthodox conformation are generally associated with a decrease in mitochondrial energy status (28). Mitochondrial energy status is regulated by several different parameters including membrane potential, adenine nucleotides, pyridine nucleotides, the ratio of GSH:GSSG as well as the ratio of total thiols:total disulfides. Recent publications (33-34) suggest that the collapse of the membrane potential is the likely cause of permeability transition. Other studies have also suggested that membrane potential is important in regulating inner membrane permeability (35-37).

We assessed the membrane potential under the various conditions studied by measuring the uptake and retention of the radiolabeled lipophilic cation, [^{3}H]TPMP, which is directly related to the membrane potential (27). As shown in Figure IV. 6, exposure of mitochondria to CaPi resulted in an immediate 80% decrease in TPMP uptake that was prevented by CsA. Addition of CsA preserved the membrane potential similarly to untreated samples throughout a 20-min incubation, although these samples contain a high level of matrix Ca^{2+} (6).

Although the addition of the metabolic inhibitors to CaPi treated mitochondria prevented mitochondrial large amplitude swelling, and inhibited solute release to varying degrees, they did not prevent the collapse of the membrane potential. By the initial time point, the membrane potential had collapsed and remained so throughout the 20-min incubation.

The addition of antimycin or sulfide alone (without CaPi) to mitochondrial suspensions also resulted in the immediate collapse of the membrane potential (data not shown). However, treatment with sulfide or antimycin alone did not result in the release of GSH, nor were there any other indications of permeability transition. Addition of oligomycin alone did not collapse the membrane potential during the 20-min incubation (data not shown).

One possible interpretation of these results is that the collapse of the membrane potential alone may not result in permeability transition but may require other biochemical factor(s) or conditions as yet unidentified. Loss of the membrane potential appears to accompany the permeable state, but does not correlate with whether or not large amplitude swelling occurs. These findings suggest that collapse of the membrane potential accompanies and/or contributes to the inner membrane permeability but may not provide the sole trigger for the transition to the permeable state. The permeability transition may be regulated by several different events, and depending upon which have occurred, these may determine the severity of inner membrane permeability.

Schlogel et al. (38) recently reported that t-butylhydroperoxide induced Ca^{2+} release in mitochondria was due to the hydrolysis of NAD^+ and subsequent mono-ADP ribosylation of a critical protein that allows the specific release of Ca^{2+}; however, this release was not accompanied by mitochondrial swelling. It is tempting to speculate that the release of mitochondrial Ca^{2+} (38) may have occurred via a mechanism similar to the one we report here. The status of matrix components, such as GSH, were not reported in their study (38).

In summary, our major findings suggest that the absence of mitochondrial large amplitude swelling does not indicate an absence of an inner membrane permeability. We find that CsA-sensitive permeability transition can occur without large amplitude swelling, and that under these conditions, the loss of solute regulation of GSH and Ca^{2+} is indicated by the release of these molecules. Ultrastructural analysis of mitochondria by TEM, or monitoring of mitochondrial swelling spectrophotometrically at 540 nm, did not indicate the significant biochemical alterations occurring under the conditions studied. This observation maybe important because mitochondrial integrity is often assessed by TEM and the light scatter assay. We conclude that CsA-sensitive permeability transition can occur without obvious morphological changes. Therefore, monitoring the CsA-sensitive release of endogenous matrix solutes, such as GSH, may be a sensitive and useful indicator of permeability transition.

Table IV. 1. Comparison of Morphological Alterations and GSH Release *

	% Morphological Alterations**	%GSH Release	r=0.700
Control	6.75	0	
CaPi	100	95	
+CsA	0	0	
+Sulfide	18.5	27	
+Oligomycin	10.5	65	
+Antimycin	14.5	73	

*Data analyzed following a 10-min incubation under the various incubation conditions as previously described. Control = untreated mitochondrial samples; CaPi = 70 μM Ca2+ and 3 mM Pi;
+CsA = 0.5 μM CsA plus CaPi; +sulfide = 0.5 mM sulfide plus CaPi; +oligomycin = 3 μM oligomycin plus CaPi; and +antimycin = 1μM antimycin plus CaPi.
**The percent of mitochondria with morphological alterations was calculated by counting, in random areas (2" X 2") on the electron micrographs, the number of morphologically altered mitochondria, and dividing this number by the total mitochondria within the area. Criteria used to identify altered mitochondria were a loss of matrix density, enlargement (swelling), and similarity to mitochondria treated with CaPi.
***r=correlation coefficient calculated by linear regression analysis.
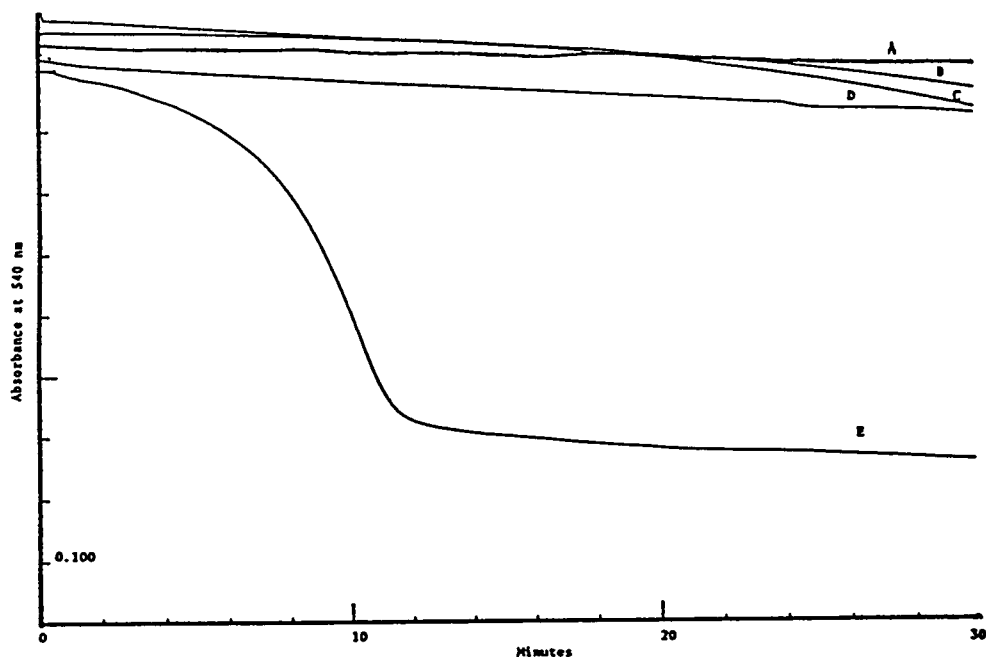
Figure IV. 1. Mitochondrial Swelling. Mitochondrial suspensions (1mg/ml) were monitored continuously for 20 min using an Aminco DW 2000 spectrophotometer. Traces A-E are representative of the following treatments; A. control or 70 μM Ca and 3 mM Pi (CaPi) plus 0.5 μM CsA; B. 0.5 mM sulfide CaPi; C. 1 μM antimycin plus CaPi; D. 3 μM oligomycin plus CaPi; E. CaPi.
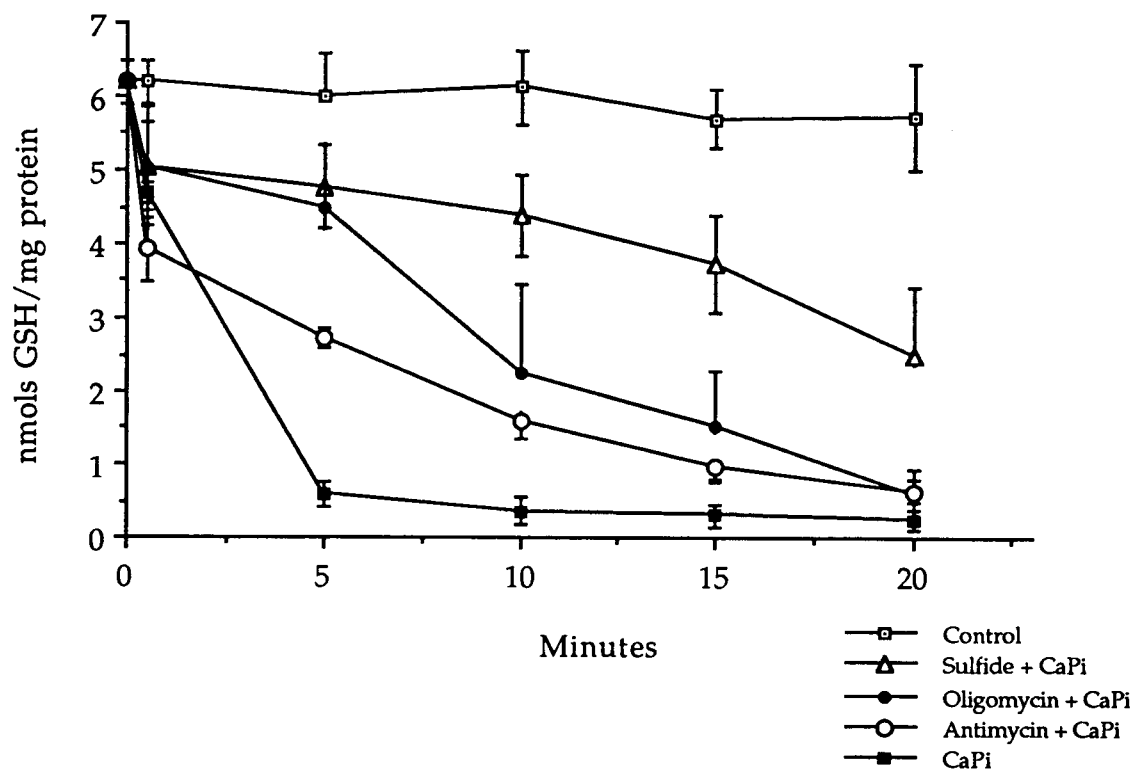
Figure IV. 2. Mitochondrial glutathione concentrations. Mitochondria (1mg/ml) were incubated in the absence (control) or presence of either 70 μM Ca^{2+} and 3 mM Pi (CaPi); 0.5 mM sulfide plus CaPi; 3 μM oligomycin plus CaPi; 1 μM antimycin plus CaPi. Samples (1 ml) were taken at the various times indicated and analyzed for GSH as described in Experimental Procedures. n=3-4 ± S.E.M.

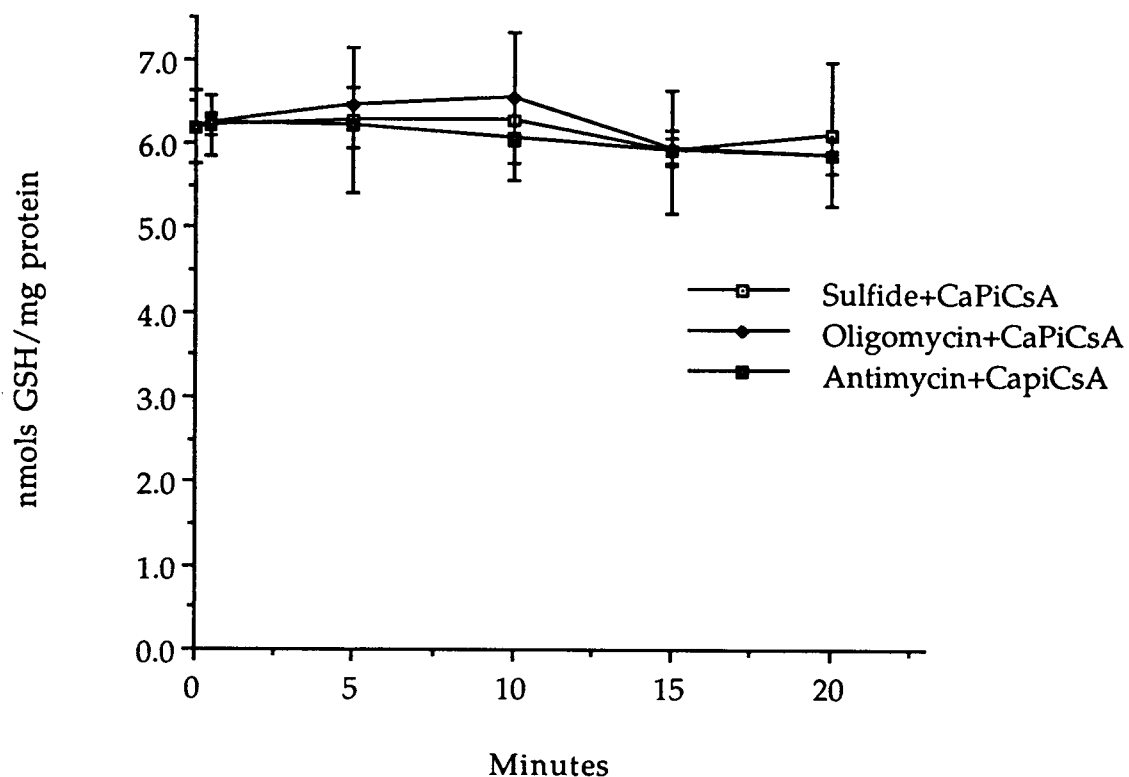Figure IV. 3. Protection with cyclosporin A against glutathione release. Mitochondria (1mg/ml) were incubated in the presence of either 0.5 mM sulfide plus 70 μM Ca^{2+} and 3 mM Pi plus 0.5 μ CsA (CaPiCsA), 3 μM oligomycin plus CaPiCsA or 1 μM antimycin plus CaPiCsA. Samples (1 ml) were taken at the various times indicated and analyzed for GSH as described in Experimental Procedures. n = 3-4 ± S.E.M.

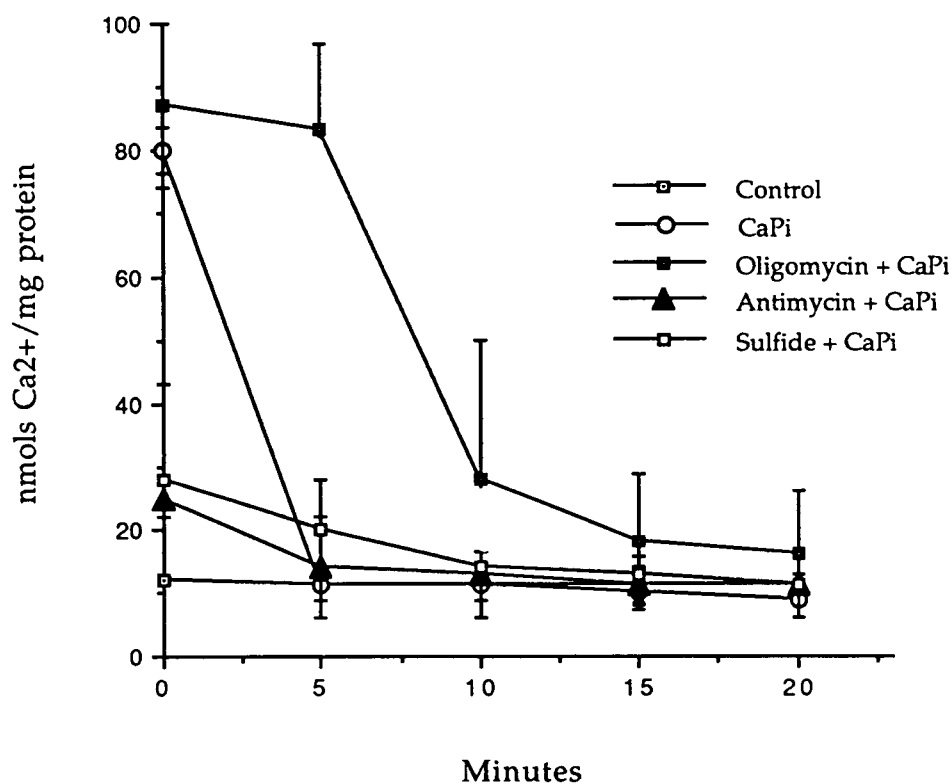**Figure IV. 4.** Calcium status following treatment with metabolic inhibitors. Mitochondria (1mg/ml) were incubated in the absence (controls) or presence of either 70 µM Ca^{2+} and 3 mM Pi (CaPi); 0.5 mM sulfide plus CaPi; 3 µM oligomycin plus CaPi; 1 µM antimycin plus CaPi. Samples (1 ml) were taken at the various times indicated and analyzed for Ca^{2+} as described in Experimental Procedures. n = 3-4 ± S.E.M.

Figure IV. 5. Ultrastructure of mitochondria in the absence and presence of metabolic inhibitors during permeability transition. Mitochondria (1mg/ml) were prepared for TEM as described in Experimental Procedures. (A) Control (untreated) mitochondria following a 10-min incubation; (B) Mitochondria incubated with 70 μM Ca^{2+} and 3 mM Pi (CaPi) for 5 min; (C) Mitochondria incubated with CaPi for 10 min; (D) Mitochondria incubated with CaPi plus 0.5 μM CsA for 20 min; (E) Mitochondria incubated with CaPi plus 0.5 mM sulfide for 10 min; (F) Mitochondria incubated with CaPi plus 3 μM oligomycin for 10 min; (G) Mitochondria incubated with CaPi plus 1μM antimycin for 10 min.
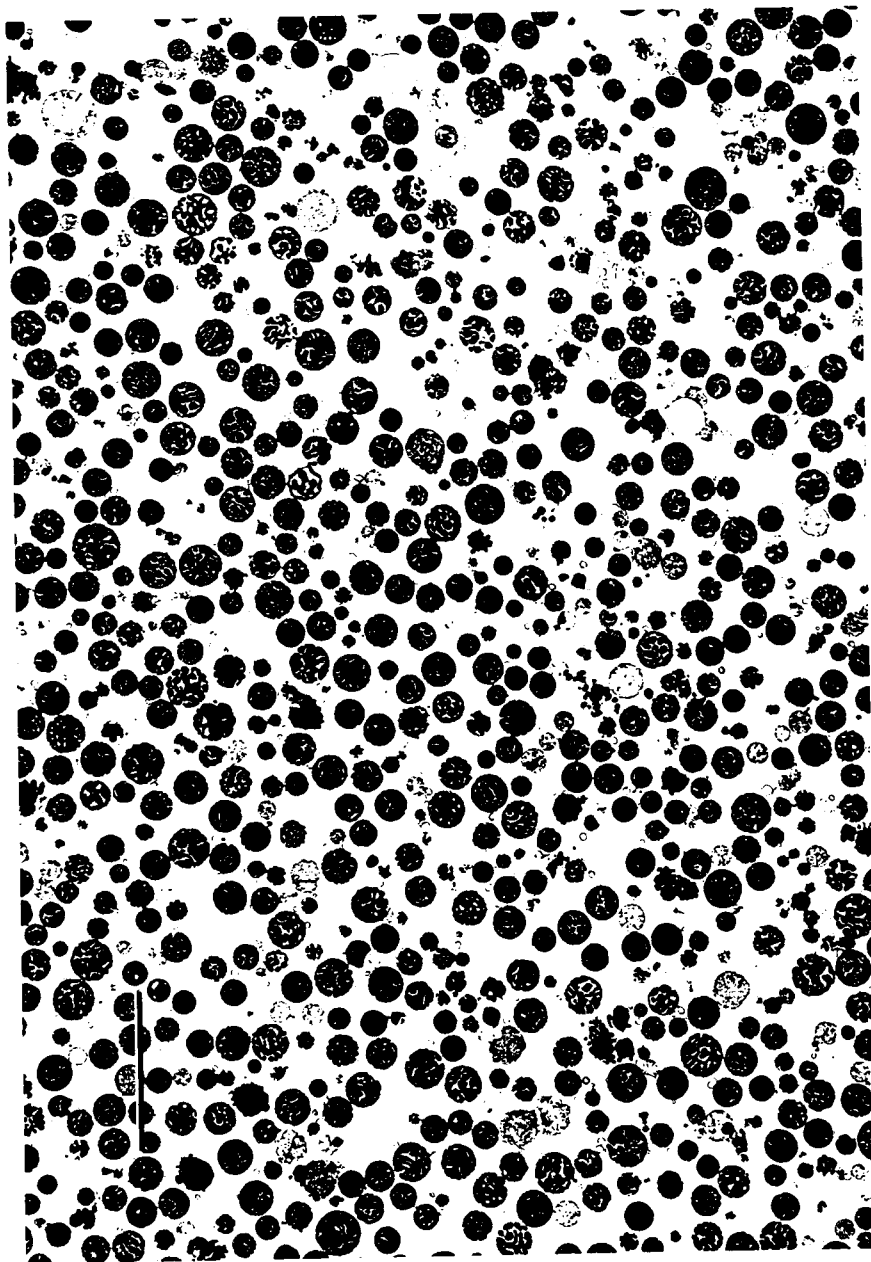
Figure IV. 5A.
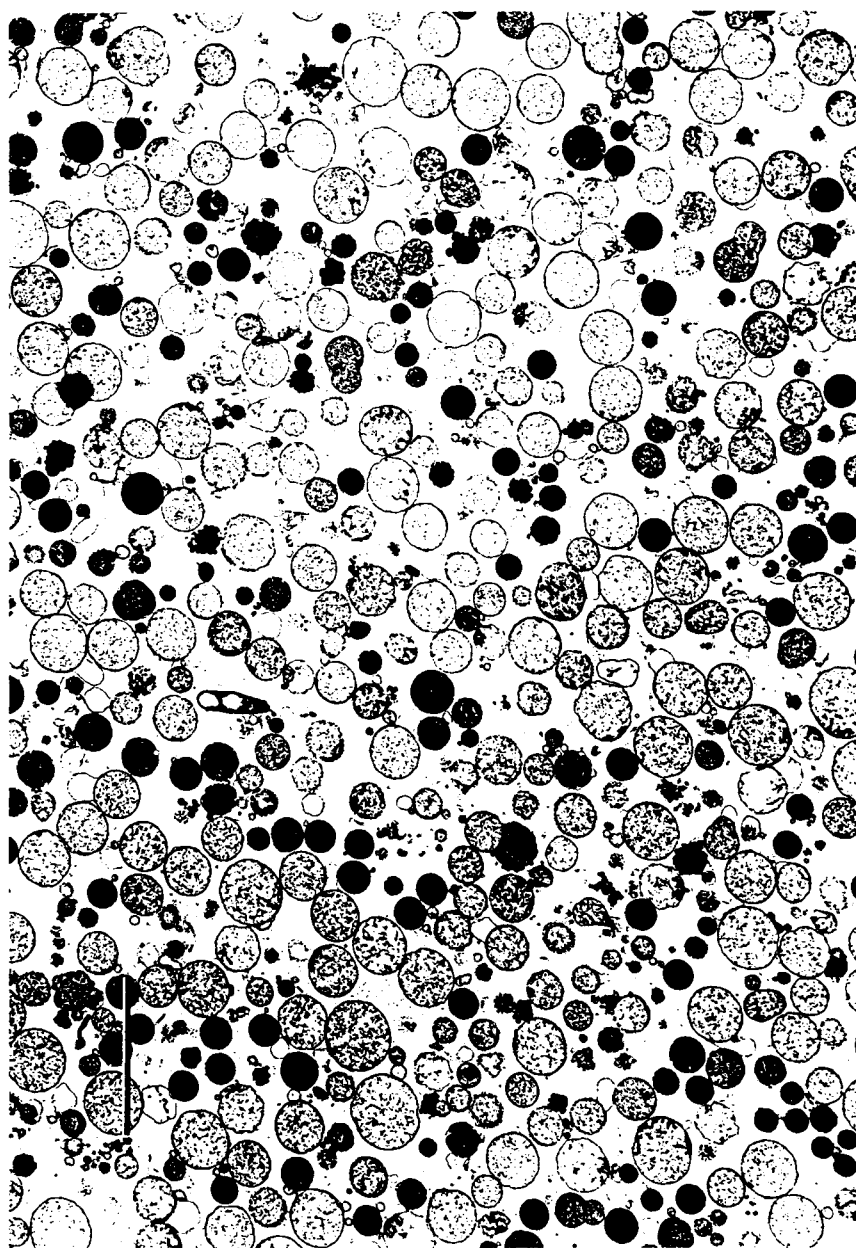
Figure IV. 5B.
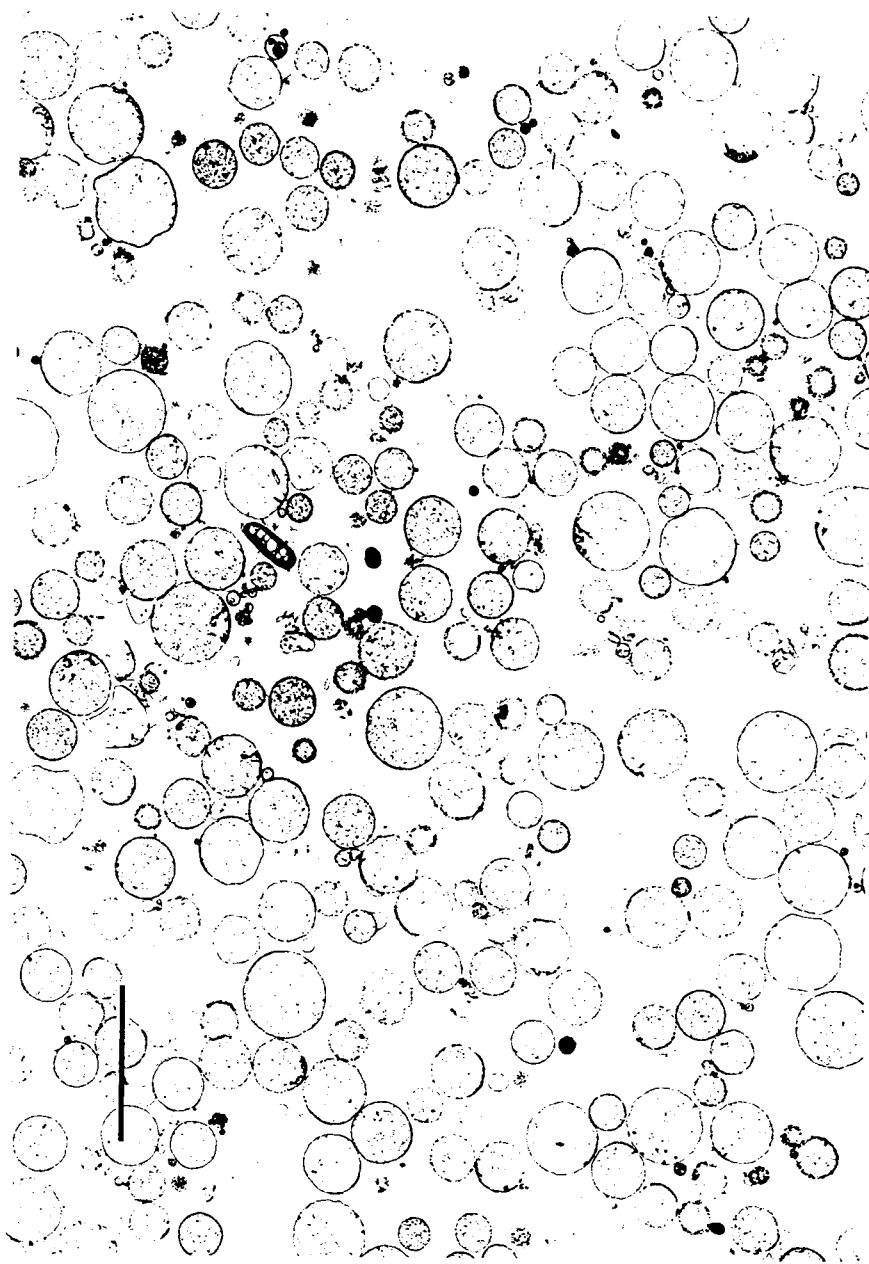
Figure IV. 5C.
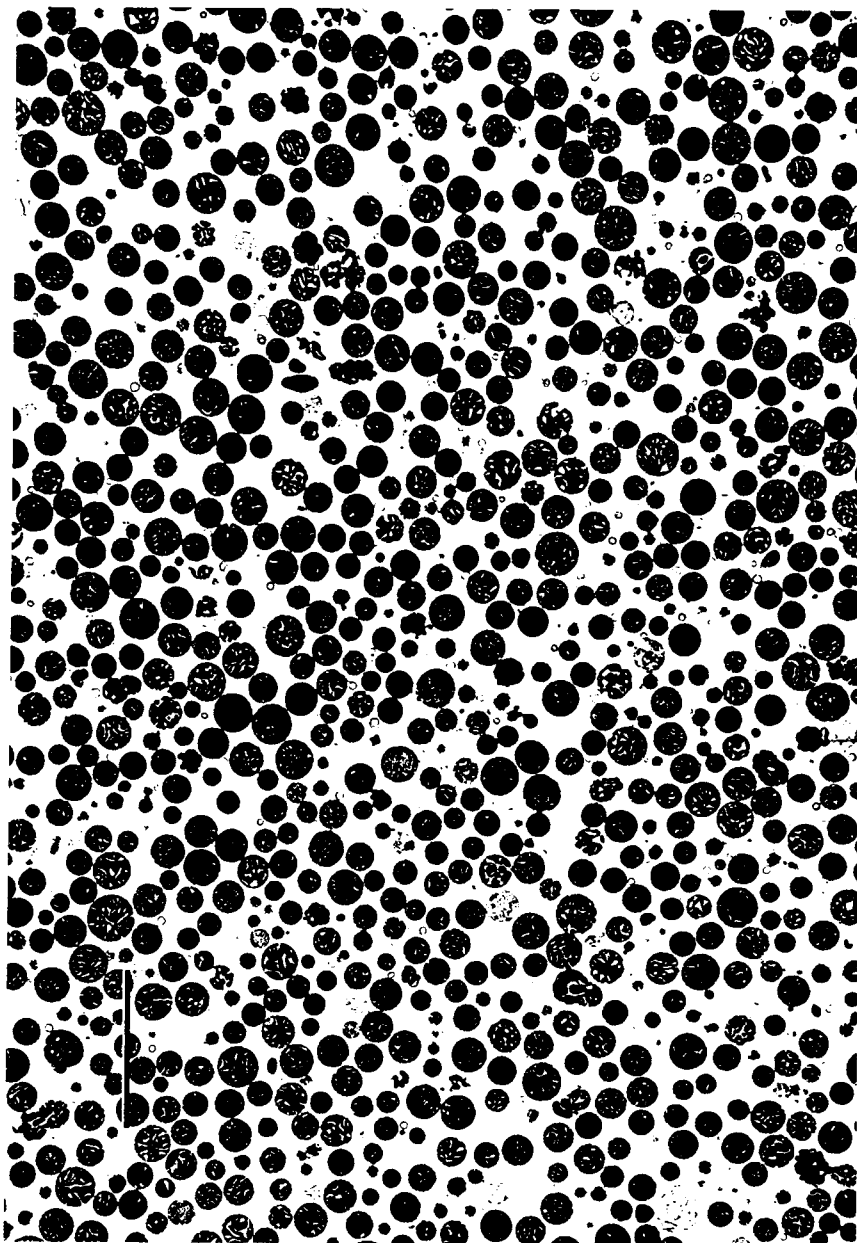
Figure IV. 5D.
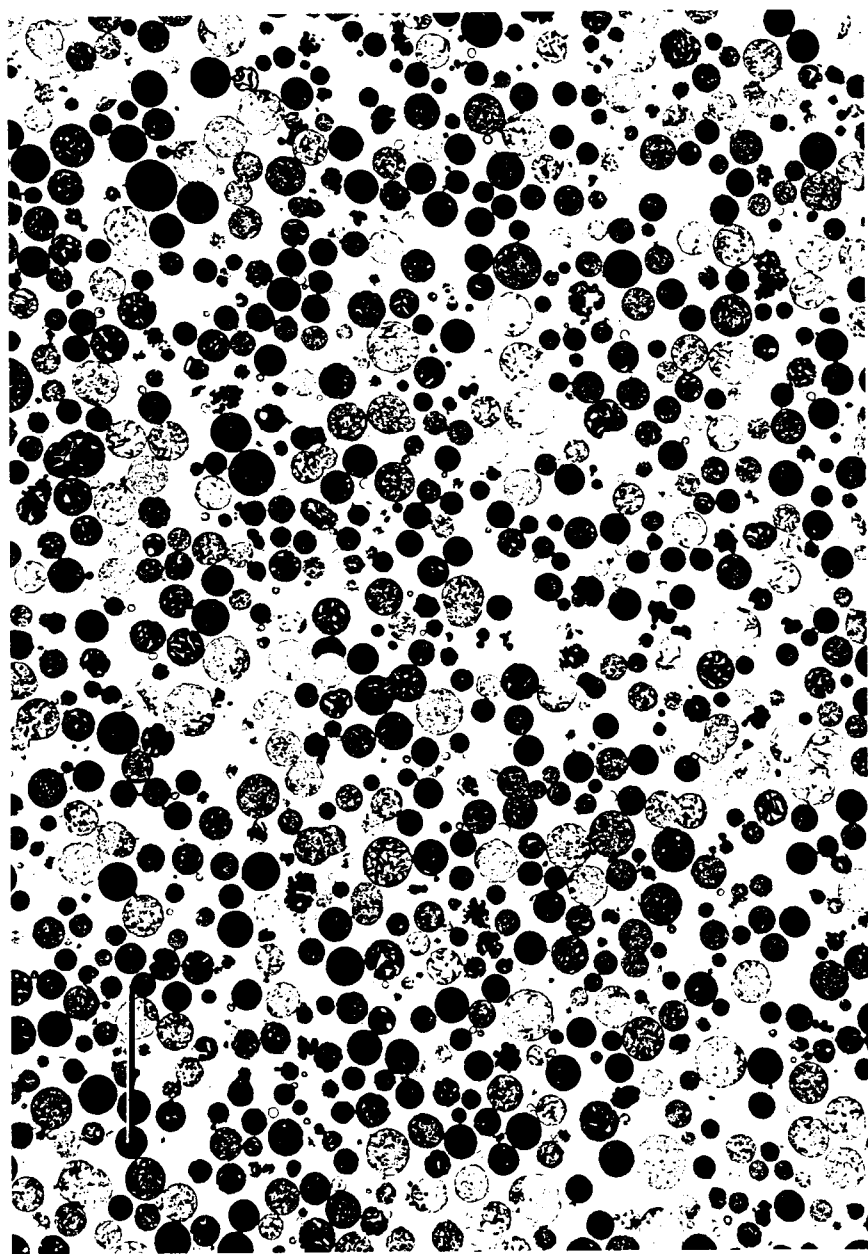
Figure IV. 5E.
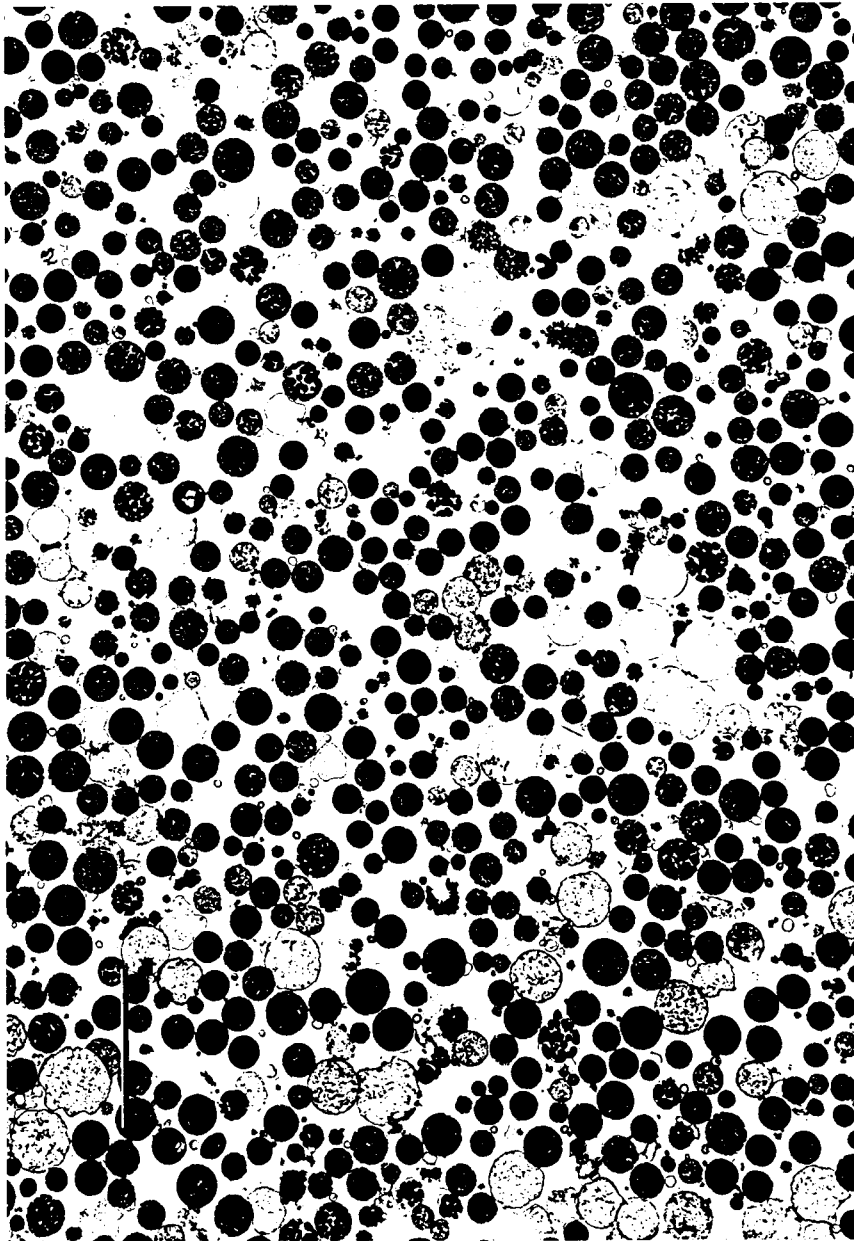
Figure IV. 5F.
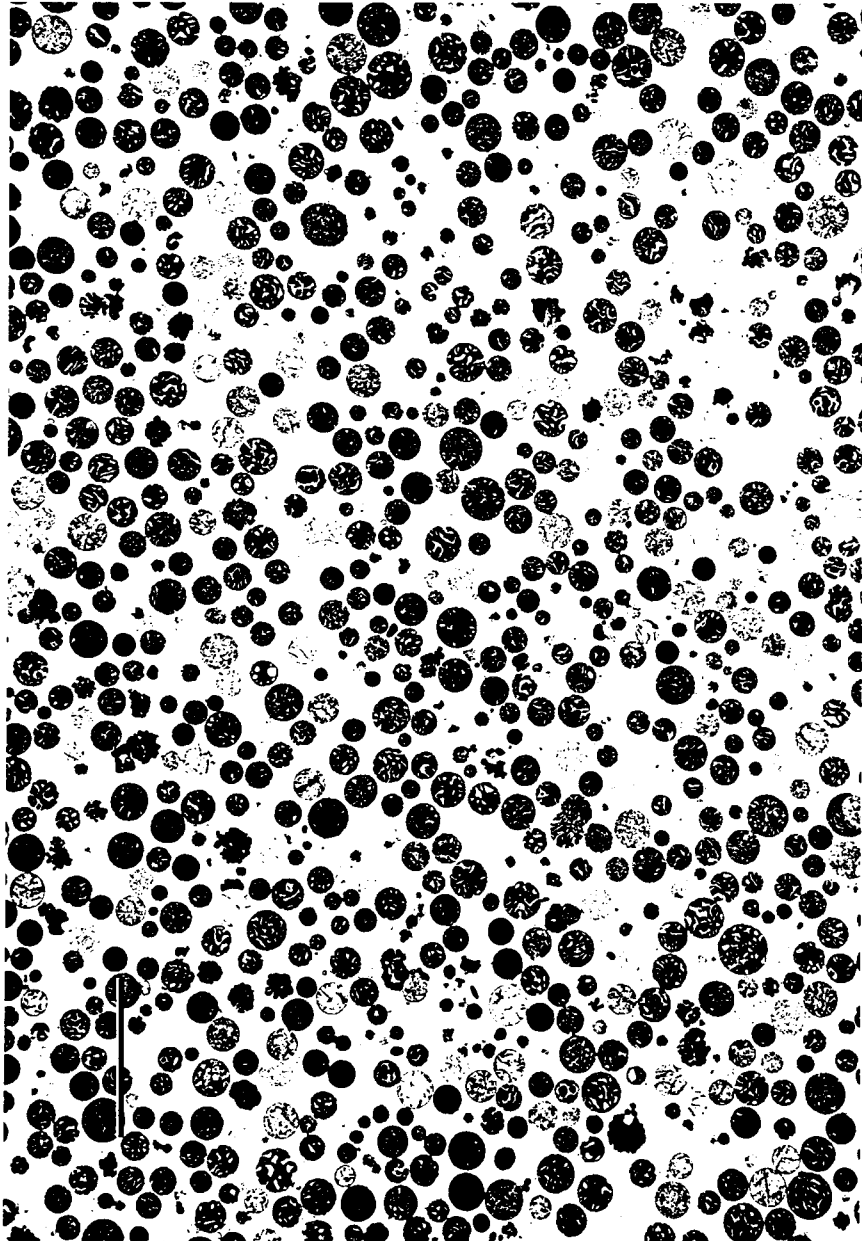
Figure IV. 5G.
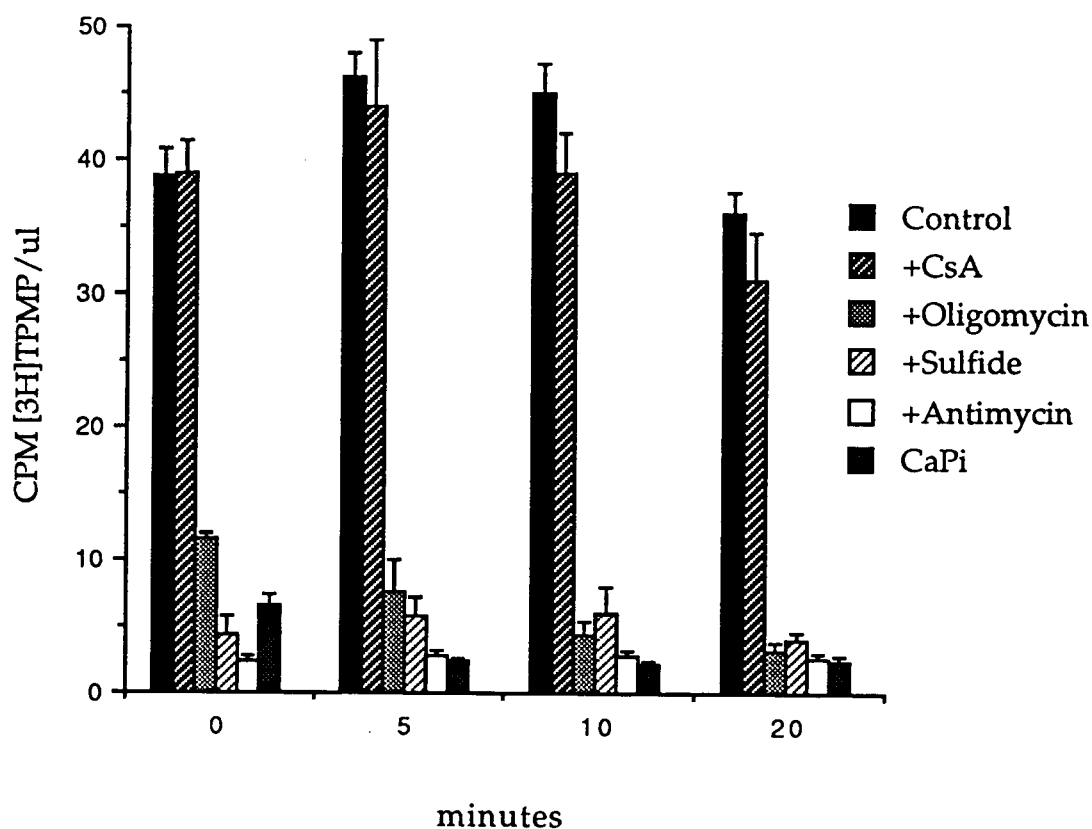
Figure IV. 6. Uptake of the cationic compound [^{3}H]TPMP (triphenylmethylphosphonium): assessment of the mitochondrial membrane potential. Mitochondria were incubated in the presence or absence of the various treatments with the addition of [^{3}H]TPMP. Samples were taken at the times indicated and prepared for analysis by liquid scintillation counting as described in the Experimental Procedures. n = 3 $\pm$ S.E.M.

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V. OXIDATION OF PYRIDINE NUCLEOTIDES AND DEPLETION OF ATP AND ADP DURING CALCIUM- AND INORGANIC PHOSPHATE-INDUCED MITOCHONDRIAL PERMEABILITY TRANSITION

Abstract

We have examined the pyridine and adenine nucleotide status during calcium- and inorganic phosphate-induced permeability transition. Calcium- and inorganic phosphate-induced permeability transition is accompanied by the rapid oxidation of pyridine nucleotides and depletion of ATP and ADP with conversion to AMP. The addition of cyclosporin A, a potent inhibitor of the permeability transition, prevented the oxidation of pyridine nucleotides and depletion of ATP and ADP.

Introduction

The inner membrane of isolated mitochondria becomes permeable when treated with Ca^{2+} and inorganic phosphate (Pi) due to the activation of a Ca^{2+}-dependent inner membrane pore. Activation or opening of this pore, referred to as permeability transition, results in the nonselective release of small matrix solutes, such as GSH and Ca^{2+}, loss of coupled functions, and large amplitude swelling (1-6). The immunosuppressive cyclic peptide, cyclosporin A (CsA) is a potent inhibitor of permeability transition and its consequences (2-4).

Many different compounds in the presence of Ca^{2+} induce permeability transition in isolated mitochondria by mechanisms which are thought to be numerous (7). Several studies have suggested that inducing agents such as t-butylhydroperoxide, hydrogen peroxide, acetoacetate, or oxalacetate induces permeability transition by the oxidation of pyridine nucleotides (7-10). Other agents, such as pyrophosphate and phosphoenol pyruvate, deplete adenine nucleotides and induce permeability transition (7). Pi is an example of an inducing agent that previously was thought not to change the redox status of the pyridine nucleotides or deplete ATP or ADP pools (10-14).

In this chapter, we have examined the status of NADH, NADPH, ATP, ADP, and AMP during Ca^{2+}- and Pi-induced permeability transition. Our findings indicate that Ca^{2+}- and Pi-induced permeability transition results in the rapid oxidation of NADH and NADPH and the depletion of ATP. Treatment with CsA prevented these changes in mitochondrial energy status.

Experimental Procedures

Materials

All chemicals were purchased from commercial sources and were of the highest quality available. CsA was a generous gift from Dr. Sue Kohlhepp (Providence Hospital, Portland, OR). CsA was dissolved in 100% ethanol (0.5 mM stock) and stored at 4°C.

Mitochondrial Preparation

Mitochondria were isolated from the livers of male Sprague-Dawley rats (325-375 g) according to the method of Schnaitman and Greenwalt (15). Differential centrifugation was used with isolation buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.5 mM EGTA, and 0.5 mg/ml bovine serum albumin, pH 7.4. Mitochondria were washed twice in isolation buffer devoid of EGTA, pH 7.0. After the final wash, mitochondria were suspended in 5-7 ml of incubation buffer containing 213 mM mannitol, 71 mM sucrose, 10 mM succinate and 3 mM HEPES, pH 7.0. Protein concentration was determined by the method of Bradford (15) with bovine serum albumin as the standard.

Mitochondrial Incubations

Mitochondria (1 mg protein/ml) were suspended in 10 ml Erlenmeyer flasks containing incubation buffer alone (untreated control) or 70 µM Ca^{2+} and 3 mM Pi or 70 µM Ca^{2+} and 3 mM Pi plus 0.5 µM CsA. Aliquants for initial time points were taken immediately following the

addition of mitochondria and prepared for analysis of pyridine and adenine nucleotides.

Biochemical Analysis

Pyridine and adenine nucleotides were measured by high-performance liquid chromatography (HPLC) according to the method of Jones (17). Briefly, 1 ml samples were transferred to microcentrifuge tubes containing either 0.5 ml 10% perchloric acid (PCA) for the extraction of NAD^+, $NADP^+$, ATP, ADP, and AMP or 0.1 ml 0.5 M KOH in 50% ethanol and 0.35% cesium chloride for the extraction of NADH and NADPH. Following extraction for 15 min on ice, samples were immediately frozen at -80°C. until analysis, which was conducted within 24 hrs of sample preparation. Just prior to analysis, samples were thawed and acid extracts neutralized with an equal volume of both 10 M KOH and 1 M K_2PO_4; samples were then centrifuged at 13,000 g for 30 s to remove debris and assayed for either reduced pyridine nucleotide (base extraction) or oxidized pyridine nucleotides and adenine nucleotides (acid extraction).

Results

Treatment of mitochondria with 70 µM Ca^{2+} and 3 mM Pi (CaPi) resulted in the rapid and extensive oxidation of NADH as shown in Figure V. 1. NADH decreased 61% and 89% within a 5- and 10- min incubation period, respectively (Figure V. 1A). The loss of NADH can be accounted for in the recovery of NAD^{+} (Figure V. 1B). Similarly, NADPH underwent oxidation to NADP^{+} (Figure V. 2A,B), although there was a slight delay in oxidation as compared to NADH (Figure V. 1A,B). Treatment with 0.5 µM CsA prevented oxidation of both NADH and NADPH as induced by CaPi treatment (Figure V.s 1 & 2).

The impact of CaPi on the adenine nucleotide status was also determined under these conditions as shown in Figure V. 3. By 5 min, ATP was depleted by nearly 70% and was accounted for by a transient rise in ADP (Figure V. 3B), and in the eventual formation of AMP (Figure V. 3C). Addition of 0.5 µM CsA to incubation buffer containing CaPi prevented ATP depletion. Addition of CsA resulted in a slight increase in the ATP concentration (Figure V. 3A) while ADP and AMP levels were somewhat lower than untreated control samples (Figure V. 3B,C).

Discussion

This report demonstrates that CaPi-induced permeability transition involves the oxidation of NADH and NADPH and the depletion of ATP and ADP. The addition of CsA, a potent inhibitor of permeability transition, prevented these changes in mitochondrial energy status. The effect of CaPi-induced inner membrane permeability has not previously been reported to involve redox changes in pyridine nucleotides or deplete ATP to our knowledge.

We have previously reported that mitochondrial GSH is rapidly released through the inner membrane pore during CaPi-induced permeability transition (3). The release of GSH occurs without formation of GSSG. It is possible that the GSH redox cycle is so efficient that detection of GSSG is not possible under these conditions, possibly explaining the oxidation of pyridine nucleotides. Under these conditions, there was also no detection of lipid peroxidation as determined by the thiobarbituric acid (TBA) assay (data not shown).

Carbonera et al. (14) reported that the addition of the antioxidant, *t*-butylhydroxytoluene (BHT) to Ca^{2+}- and Pi-treated mitochondria prevented permeability transition. They proposed that Pi may contribute to inner membrane permeability by increasing the formation of oxygen radicals due to enhancement of the electron leak. They reported no change in the mitochondrial GSH redox status. Novgorodov et al. (18) reported that BHT, in the presence of Ca^{2+} and Pi, prevented the collapse of the membrane potential and Ca^{2+} release. Although evidence of lipid peroxidation as measured by the TBA assay was not observed, they

proposed that initial steps in lipid peroxidation control Ca^{2+}- and Pi-induced permeability transition.

There is growing interest on the similarities between ischemic reperfusion injury, chemical toxicity, and mitochondrial permeability transition; a central component being the oxidation of pyridine nucleotides. Early studies by Lehninger (19) showed a strong relationship between the ability of mitochondria to retain Ca^{2+} in the presence of a high ratio of reduced:oxidized pyridine nucleotides. A recent study by Takeyama et al. (20) showed that O_2 metabolites derived from the xanthine-xanthine oxidase system triggered a CsA-sensitive and Ca^{2+}-dependent membrane transition that depleted pyridine nucleotides. Permeability transition induced with oxidants that are substrates for glutathione peroxidase, including t-butylhydroperoxide or hydrogen peroxide, is thought to be mediated by oxidation of pyridine nucleotides that are formed during the reduction of GSSG (21).

We observed that CaPi-induced permeability transition rapidly depleted ATP. Within 5 min, the ATP concentration was depleted nearly 70%, while ADP and AMP levels increase nearly 100%. The increase in ADP was transient, and after 5 min, the level decreases to below untreated control values while AMP levels increased, accounting for the loss of ATP and ADP.

Several studies indicate the importance of metabolic energy, particularly ATP and ADP, in maintaining cell viability under various conditions that are also prevented by CsA (4, 22, 23). Halestrap and Davidson (23) proposed that the permeability transition pore is the adenine nucleotide translocase that has changed conformation. Both ATP

and ADP are positive effectors against pore formation by stabilizing the nonpermeable state or "m" conformation. Agents inducing the "c" conformation of the carrier promotes inner membrane permeability. Lê-Qûoc and Lê-Qûoc (24) report that oxidation of pyridine nucleotides is correlated with the stabilization of the "c" conformation of the adenine nucleotide carrier and therefore promote inner membrane permeability.

In summary, we report that CaPi induced permeability transition was accompanied by the rapid oxidation of NADH and NADPH and the depletion of ATP. The oxidation of pyridine nucleotides may be related to early stages of inner membrane alterations associated with CaPi-induced permeability transition. The ability of CsA to maintain energy status during permeability transition may be important in understanding the mechanism(s) by which it prevents this mitochondrial injury as induced by inorganic phosphate.

Figure V. 1. Total mitochondrial NADH and NAD+. Mitochondria
(1 mg/ml) were incubated in the absence (control) or presence of either 70
μM Ca^{2+} and 3 mM Pi (CaPi) or CaPi plus 0.5 μM CsA (CaPiCsA).
Samples (1 ml) were taken at the times indicated and prepared for the
analysis of (A) NADH and (B) NAD+ as described in Experimental
Procedures. n = 3-4 ± SEM.

A

B
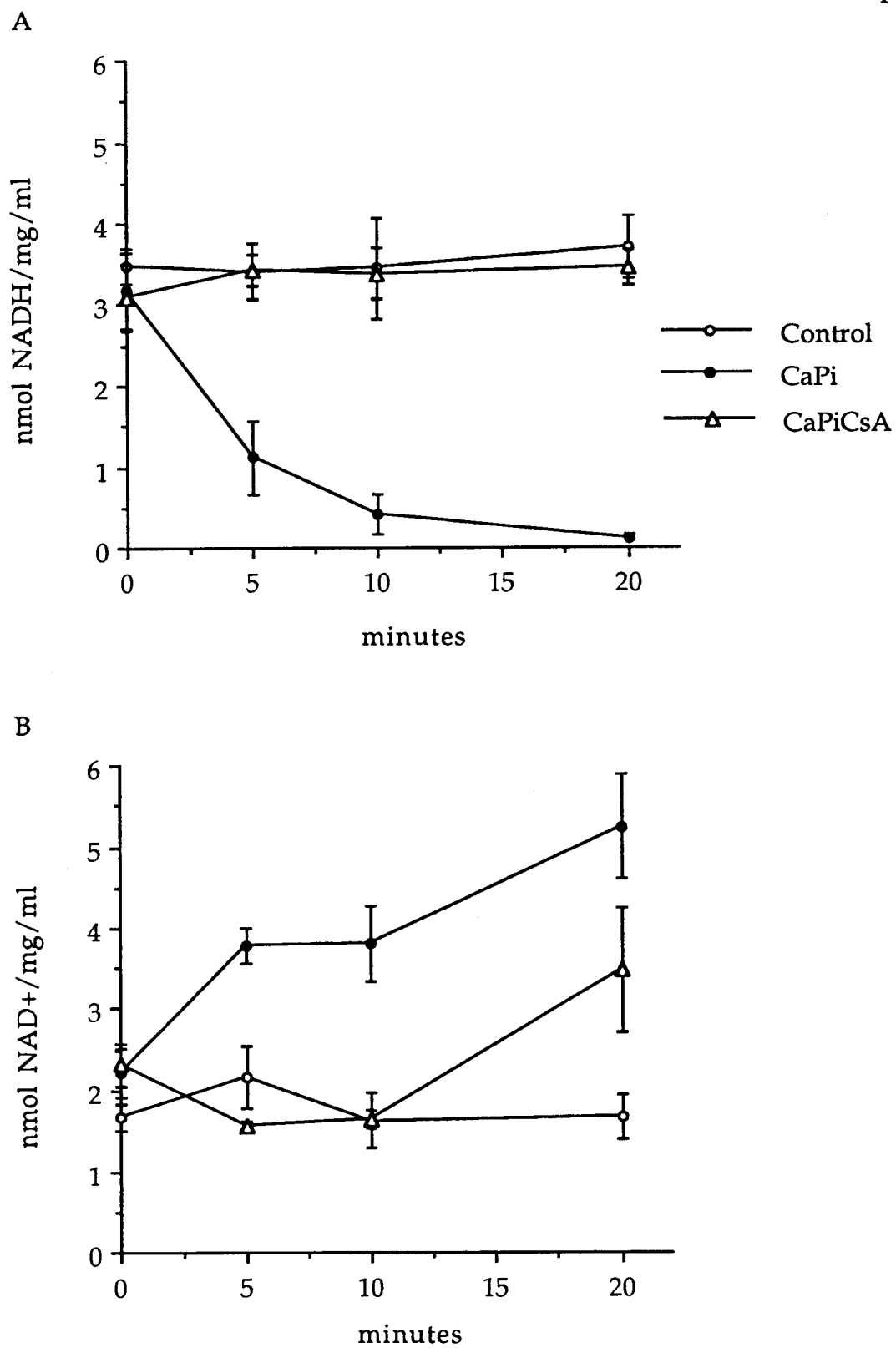
Figure V. 1.

Figure V. 2. Total mitochondrial NADPH and NADP$^+$. Mitochondria (1 mg/ml) were incubated in the absence (control) or presence of either 70 μM Ca^{2+} and 3 mM Pi (CaPi) or CaPi plus 0.5 μM CsA (CaPiCsA). Samples (1 ml) were taken at the times indicated and prepared for the analysis of (A) NADPH and (B) NADP$^+$ as described in Experimental Procedures. n = 3-4 $\pm$ SEM.

A

B

Figure V. 2.

Figure V. 3. Total mitochondrial ATP, ADP, and AMP. Mitochondria (1 mg/ml) were incubated in the absence (control) or presence of either 70 μM Ca^{2+} and 3 mM Pi (CaPi) or CaPi plus 0.5 μM CsA (CaPiCsA). Samples (1 ml) were taken at the times indicated and prepared for the analysis of (A) ATP, (B) ADP, and (C) AMP as described in Experimental Procedures. n = 3-4 $\pm$ SEM.
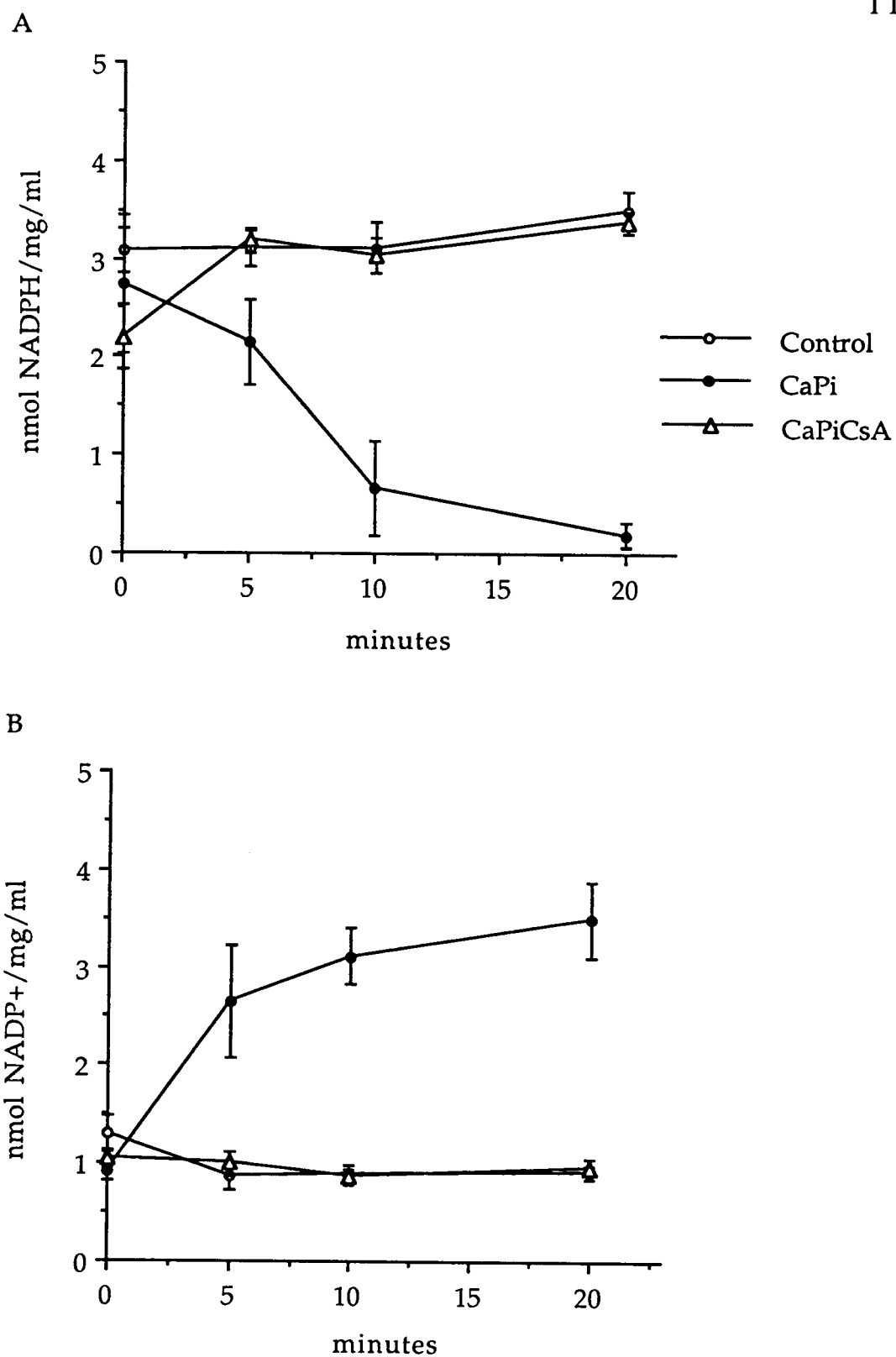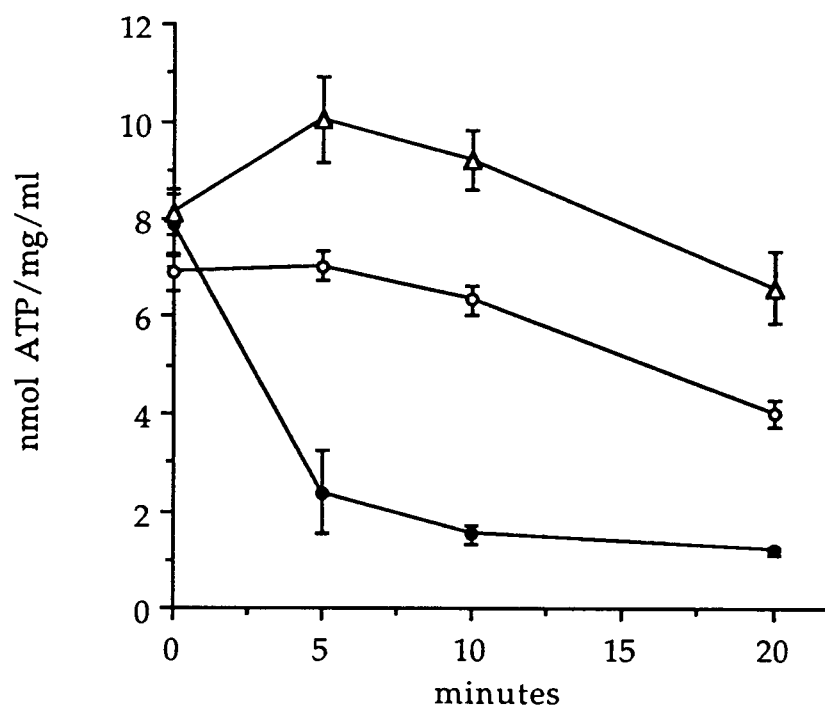
A

B
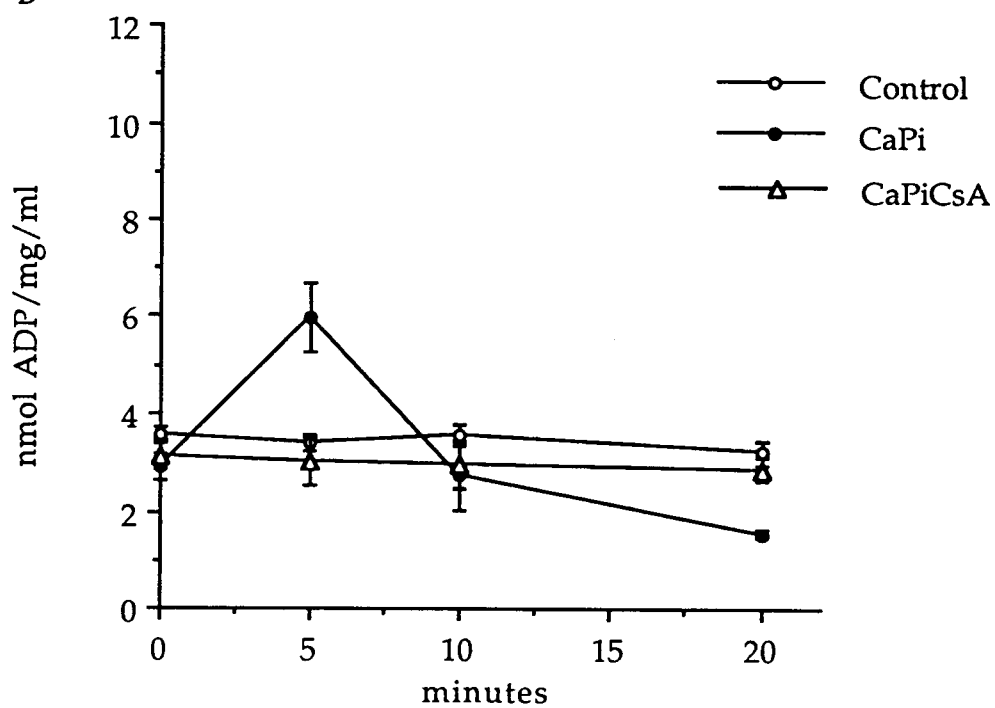
Figure V. 3.
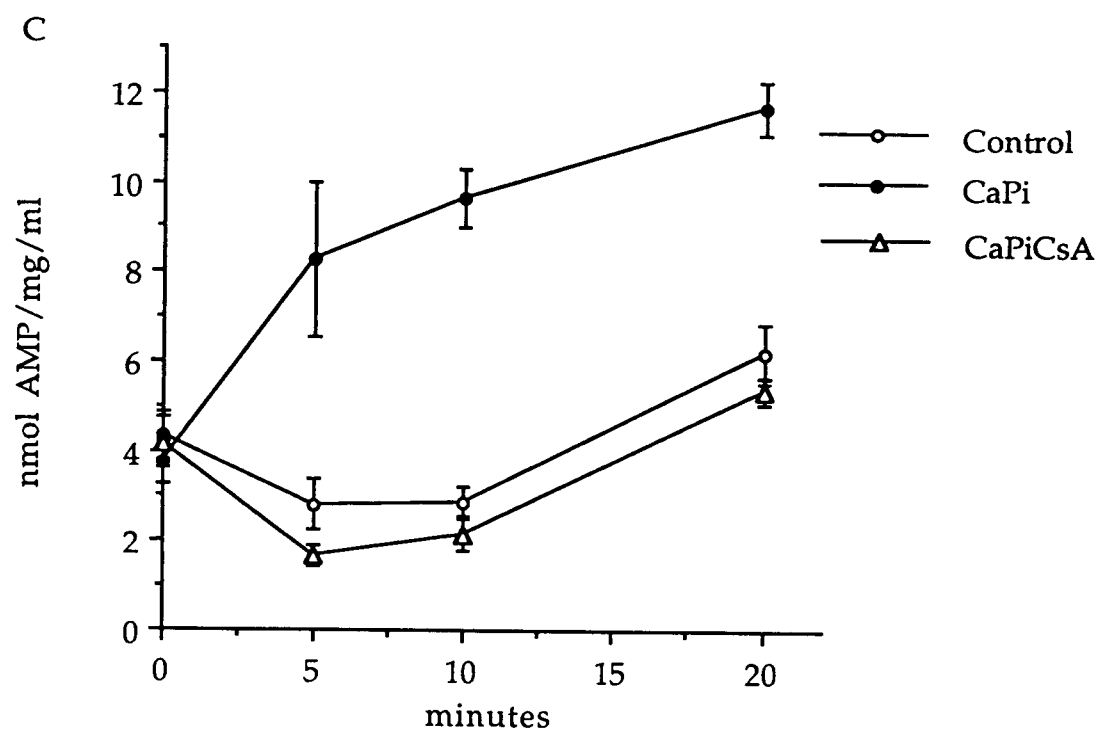
Figure V. 3.

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VI. RELEASE OF NAD$^{\pm}$ and NADP$^{\pm}$ DURING CALCIUM- AND PHOSPHATE-INDUCED PERMEABILITY TRANSITION OCCURS BOTH WITH AND WITHOUT LARGE AMPLITUDE SWELLING

Abstract

The mitochondrial inner membrane is normally impermeable to most substances, with the entrance and exit of molecules being a highly regulated process. However, the inner membrane of isolated mitochondria undergo acute (<5 min) alterations in the presence of Ca^{2+} and near physiological levels of Pi. The Ca^{2+}-dependent inner membrane alterations, referred to as permeability transition, are inhibited by cyclosporin A and characterized by inner membrane permeability to small (<1200 Da) solutes, uncoupling of the mitochondria, and large amplitude swelling. We recently reported that during permeability transition induced by calcium and Pi, NADH and NADPH are rapidly oxidized and that ATP, and ADP are rapidly depleted. Recently, we observed that the addition of either antimycin, oligomycin, or sulfide to Ca^{2+} and Pi treated mitochondria prevented large amplitude swelling of the mitochondria, but did not prevent cyclosporin A-sensitive inner membrane permeability. The purpose of our current study is to 1) determine if NAD(H) or NADP(H) (700-835 Da) are released during Ca^{2+}- and Pi-induced permeability transition since these molecules are relatively small and 2) determine the effects of antimycin, oligomycin, and sulfide on pyridine, as well as adenine nucleotide status in mitochondria treated with Ca^{2+} and Pi. Our findings indicate that during

Ca^{2+}- and Pi- induced permeability transition, NADH and NADPH oxidation occurs intramitochondrially, followed by the release of NAD^+ and $NADP^+$, presumably via the inner membrane pore. We also report that the addition of antimycin, oligomycin, or sulfide to Ca^{2+} and Pi treated mitochondria results in varying degrees of NADH and NADPH oxidation and release of NAD^+ and $NADP^+$ in the absence of large amplitude swelling. Our findings suggest that maintenance of endogenous adenine nucleotides, especially ADP, may be important in modulating permeability transition.

Introduction

The inner membrane of mitochondria isolated from a variety of tissues become permeable following activation or deregulation of a Ca^{2+}-dependent inner membrane pore (1-4). This process, referred to as permeability transition requires the presence of a second agent, termed an inducing agent (1,4). Many different inducing agents have been identified; examples include near physiological levels of inorganic phosphate (Pi), *t*-butylhydroperoxide, and sulfhydryl reagents (4). The mechanism(s) by which the various agents induce permeability transition is not completely understood; however, it is postulated that they may all act on a similar protein structure (1,4).

Opening or activation of this inner membrane pore with Ca^{2+} and Pi results in the release of small (<1200 Da) matrix solutes such as GSH (307 Da) and Ca^{2+}, large amplitude swelling, and a loss of coupled functions. (4, 9-11). Cyclosporin A (CsA), an immunosuppressive cyclic peptide, is a potent inhibitor of the permeability transition (5-6). The mechanism of CsA's inhibition is thought to involve the binding of CsA to the matrix protein, cyclophillin (7-8, 12).

Although the physiological relevance of such a pore remains uncertain, there is increasing evidence suggesting that the opening and closing of this inner membrane pore is a highly regulated process with several different control sites. This may explain why many structurally and functionally different inducing agents, including Pi, sulfhydryl reagents, oxidants, and heavy metals, induce the Ca^{2+}-dependent permeability transition (4).

Inducing agents such as *t*-butyl hydroperoxide and hydrogen peroxide are thought to mediate permeability transition due to the oxidation of NADH and NADPH (4, 13-14). The inducing agent Pi was not previously thought to alter pyridine nucleotide ratios and is therefore often studied in comparison to agents, such as *t*-butylhydroperoxide which induce changes in pyridine nucleotide ratios (15-17).

We have recently reported that during Ca^{2+} and Pi induced permeability transition both NADH and NADPH are extensively oxidized and ATP and ADP are depleted within several minutes (22). This is a novel finding since Pi induced permeability transition was not previously thought to alter pyridine nucleotide ratios or adenine nucleotide status. We have also observed conditions in which inner membrane permeability occurs without large amplitude swelling (27). These conditions were observed when mitochondria were treated with either antimycin, oligomycin, or sulfide in combination with Ca^{2+} and Pi (27).

The purpose of the current study is to 1) determine if NAD(H) or NADP(H) (700-835 Da) release occurs during Ca^{2+}- and Pi-induced permeability transition since these molecules are normally impermeable to the inner membrane and relatively small (<1200 Da) and 2) determine the status of both pyridine, and adenine nucleotides following treatment with either antimycin, oligomycin, or sulfide during Ca^{2+}- and Pi-induced permeability transition.

Experimental Procedures

Mitochondrial Preparation

Isolated mitochondria were prepared by differential centrifugation according to the method of Schnaitman and Greenwalt (18). Briefly, livers of male Sprague-Dawley rats (325-375 g) (Simonsen Labs, Gilroy, CA) were excised and homogenized in isolation buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.5 mM EGTA, and 0.5 mg/ml bovine serum albumin, pH 7.4. Mitochondria were washed twice in isolation buffer devoid of EGTA, pH 7.0.

After the final wash, the mitochondrial pellets were suspended (25-30 mg/ml) in incubation buffer containing 213 mM mannitol, 71 mM sucrose, 10 mM succinate, and 3 mM HEPES, pH 7.0. Mitochondrial protein was determined spectrophotometrically by the method of Bradford (19) with bovine serum albumin as the standard.

Mitochondrial Incubations

Mitochondria were added to 10-ml Erlenmeyer flasks (1 mg protein/ml) containing incubation buffer plus the various treatments described in the figure legends. After briefly swirling the flask, 1.0 ml samples were taken immediately following the addition of mitochondria. These samples constitute the initial time points. Mitochondrial suspensions were exposed to atmospheric air throughout the remainder of the incubation period. Aliquants (1ml) of mitochondrial suspensions, taken at the times indicated in the figure legends and prepared for analysis of pyridine and adenine nucleotides as described below.

Biochemical Analysis

Intra- and extra-mitochondrial pyridine and adenine nucleotides were measured according to the HPLC method of Jones (20) with the following modifications to accommodate mitochondrial samples. Briefly, 1 ml samples containing 1 mg protein were removed from incubation flask at the appropriate time point, transferred to microcentrifuge tubes and centrifuged at 13,000 g for 30 s to separate the suspended mitochondria from the incubation buffer. A sample (0.500 ml) of the supernatant (extra-mitochondrial) was transferred to microcentrifuge tubes containing either 0.100 ml 10% perchloric acid (PCA) for the analysis of ATP, ADP, AMP, $NADP^+$, and NAD^+ or 0.100 ml 0.5 M KOH in 50% ethanol and 0.35% cesium chloride for the analysis of NADH and NADPH, and then frozen immediately at -80° C. The remaining supernatant was carefully discarded by aspiration. The mitochondrial pellets were suspended in either 0.500 ml 10% PCA for extraction of intramitochondrial ATP, ADP, AMP, NADP+, and NAD+ or 0.100 ml 0.5 M KOH in 50% ethanol and 0.35% cesium chloride for extraction of NADPH and NADH. The samples were immediately iced for 15 min, sonicated for 5 s, and then frozen at -80° C. Prior to analysis, samples were thawed, the acid extracted samples neutralized with 10 mM KOH and 1 M KH_2PO_4, centrifuged at 13,000 g for 30 s to remove insoluble debris, and assayed for either NADH and NADPH (base extraction) or NAD^+, $NADP^+$, ATP, ADP, and AMP (acid extraction). All samples were assayed within 24 hr of collection.

The spectrophotometric determinations of pyridine nucleotides were monitored at 340 nm and 370 nm in an Aminco DW2000 spectrophotometer operated, in dual beam mode.

Results

Ca^{2+} and Pi-induced permeability transition is accompanied by the rapid and extensive oxidation of NADH and NADPH (22). Since pyridine nucleotides are relatively small molecules (<850 Da) and normally impermeable to the inner membrane, we hypothesize that release of pyridine nucleotides could occur through the putative inner membrane pore. To test this hypothesis, both intra- and extra-mitochondrial NAD(H) and NADP(H) levels were measured during permeability transition induced with $70\mu M$ Ca^{2+} and 3 mM Pi (CaPi).

As shown in Figures VI. 1A and 2A, mitochondrial NADH and NADPH pools are decreased roughly 50% within 5-min following treatment with CaPi. This decrease is not accounted for by an increase in extramitochondrial NADH and NADPH (Figures VI. 1A and 2A). The depletion of NADH and NADPH are accounted for as NAD^{+} and $NADP^{+}$ which are found both intra- and extra-mitochondrially (Figures VI. 1BC and 2BC). The addition of 0.5 μM CsA, a potent inhibitor of permeability transition, prevented the oxidation and release of NAD^{+} and $NADP^{+}$ (Figures VI. 1ABC and 2ABC).

To discount the possibility that NADH and NADPH are released from the matrix, and then subsequently oxidized extramitochondrially, we monitored spectrophotometrically the redox state of exogenous NADH and NADPH during CaPi induced permeability transition. The addition of CaPi to mitochondrial suspensions resulted in the oxidation of endogenous pyridine nucleotides as demonstrated in Figure VI. 3 by the decrease in absorbance and 340 and 370nm. Following oxidation of

endogenous NADH and NADPH, the subsequent addition of exogenous NADH and NADPH increased the absorbance, which was maintained throughout the incubation period, indicating that no further oxidation was occurring.

We have previously observed that the addition of either oligomycin (3 μM), sulfide (0.5 mM) or antimycin (1μM) to incubation buffer containing CaPi prevented mitochondrial large amplitude swelling but did not prevent inner membrane permeability (27). Inner membrane permeability was indicated by the CsA-sensitive release of matrix GSH and Ca^{2+}. This suggested that activation of the putative Ca^{2+}-dependent pore could occur without large amplitude swelling. As part of the present study, we therefore determined if mitochondrial energy status in the form of NADH, NADPH, ATP and/or ADP was preserved in the presence of the metabolic inhibitors. As shown in Figures VI. 4A and 5A, the addition of oligomycin (3 μM), sulfide (0.5 mM) or antimycin (1μM) to incubation buffer containing CaPi decreased NADH and NADPH to varying degrees.

Treatment with oligomycin and sulfide maintained NADH levels 40% and 20%, respectively, greater than CaPi treatment alone, whereas the addition of antimycin potentiated the loss of NADH by 20% (Figure VI. 4A). The observed loss of NADH and NADPH was not accounted for by the recovery of NADH and NADPH extramitochondrially (Figures VI. 4A and 5A). With all treatments, the NADH lost was recovered as NAD^+ and detected both intra- and extra-mitochondrially (Figures VI. 4ABC). Interestingly, the amount detected extramitochondrially does not correspond to the amount of NADH lost. For example, treatment with

antimycin resulted in increased formation of NAD^+ compared to CaPi treatment alone (Figures VI. 4 BC), although antimycin treated mitochondria released less NAD^+ (Figure VI. 4C).

The mitochondrial NADPH pool followed the same pattern of oxidation and release as observed with NADH following treatment with the metabolic inhibitors. The loss of NADPH was accounted for as $NADP^+$, which was recovered both intra- and extra-mitochondrially (Figures VI. 5A,B,C). The release of $NADP^+$ from the matrix followed the same pattern as that observed with NAD^+ following a 5-min incubation (Figure VI. 4C). The observed pattern is that treatment with CaPi resulted in the greatest release of NAD^+ and $NADP^+$, followed by treatment with antimycin + CaPi, oligomycin + CaPi, and then sulfide + CaPi.

Table 1 lists the pyridine nucleotide redox couples of NADH:NAD^+ and NADPH:$NADP^+$ following a 5-min incubation. Treatment with CaPi results in greatly diminished ratios of both NADH:NAD^+ and NADPH:$NADP^+$, as compared to untreated controls and CsA treated mitochondrial samples. However, the addition of antimycin to CaPi treated mitochondria resulted in the redox ratios of both NADH:NAD^+ and NADPH:$NADP^+$ less than that observed with CaPi treatment alone. The addition of oligomycin or sulfide to CaPi treated mitochondria preserved the NADH:NAD^+ and NADPH:$NADP^+$ redox couples compared to CaPi treatment alone.

Measurements of ATP, ADP, and AMP with the various treatments are reported in Table 2. Treatment with CaPi resulted in the depletion of ATP and ADP by 5 min, with 75% of the adenine pool recovered as AMP

(Table 2). Addition of 0.5 μM CsA eliminated this effect, resulting in increased levels of ATP, slightly increased levels of ADP, and decreased AMP levels compared to untreated control samples (Table 2).

The presence of the metabolic inhibitors in addition to CaPi preserved ATP and/or ADP levels compared to CaPi treatment alone (Table 2). The addition of 3 μM oligomycin to CaPi treated mitochondria maintained ATP levels similar to control values at 5 min, while ADP loss was minimal as compared to CaPi treatment alone. The addition of sulfide (0.5 mM) or antimycin (1 μM), in conjunction with CaPi, did not sustain ATP levels any differently than CaPi treatment alone; however, ADP levels were preserved as compared to CaPi treatment alone (Table 2).

The energy charge [(ATP + 0.5ADP)/(ATP + ADP + AMP)], which designates energy status, indicates that the presence of antimycin, sulfide, or oligomycin preserved energy status to varying degrees compared to CaPi treatment alone. Following treatment with CaPi, the energy charge dropped to 0.158 as compared to untreated controls (0.351). Treatment of mitochondria with either oligomycin, sulfide, or antimycin plus CaPi resulted in energy charge values of 0.409, 0.209, and 0.169 respectively, following a 5-min incubation.

Treatment with the respiratory inhibitors maintained ADP levels at least 40% greater than observed with CaPi treatment alone. Since the addition of metabolic inhibitors prevented large amplitude swelling but not inner membrane permeability (27), we tested whether ADP could prevent large amplitude swelling during CaPi treatment. Mitochondria were incubated with increasing concentrations of exogenous ADP in the presence of CaPi (Figure VI. 6). Mitochondria undergo large amplitude

swelling in the presence of CaPi (Figure VI. 6A). The addition ADP in the presence of CaPi inhibited large amplitude swelling in a dose dependent manner (Figure VI. 6B-D). At the highest dose of ADP (150 μM), large amplitude swelling was abolished during CaPi induced permeability transition (Figure VI. 6D).

Discussion

The major findings we report demonstrate that CaPi induced permeability transition is accompanied by intramitochondrial oxidation of the pyridine nucleotide pool, with the selective efflux of the NAD^+ and $NADP^+$. This contradicts the idea that CaPi induced permeability transition did not involve alterations in the pyridine or adenine nucleotide status. Although the mechanism by which CaPi treatment results in the oxidation of NADH and NADPH is not clear, apparently it does not involve the oxidation of GSH or lipid peroxidation, as no evidence of either has been detected in this system (11). In fact, under these conditions, GSH is rapidly and nearly completely released as GSH (reduced) from the matrix with a 5-min incubation (11).

Since pyridine nucleotides are of low molecular weight (<1200 Da), they presumably could be released nonspecifically through the Ca^{2+}- dependent, CsA-sensitive pore of the permeability transition (5). However, extramitochondrial NADH and NADPH levels detected during permeability transition were similar to untreated control levels. This suggests that either NADH and NADPH are not released from the mitochondrial matrix during permeability transition, or they are released and rapidly oxidized extramitochondrially. To test the latter, exogenous NADH and NADPH were added to mitochondrial suspensions following treatment with CaPi. These suspensions were monitored spectrophotometrically for redox changes in NADH and NADPH. The addition of CaPi to mitochondrial suspensions resulted in the expected oxidation of NADH and NADPH. Subsequent addition of exogenous

NADH and NADPH did not result in any further oxidation. This provides additional evidence that NADH and NADPH do not traverse the inner membrane pore.

It is unknown as to why $NAD(P)^+$, but not $NAD(P)H$, are apparently able to efflux through the inner membrane pore during CaPi induced permeability transition. One possible explanation is that NADH and NADPH are protein bound and therefore unavailable to traverse the inner membrane pore; NAD^+ and $NADP^+$ are perhaps unbound and free to efflux through the permeability transition pore. Another possible explanation centers on the issue of pore selectivity, with the pore selectively releasing NAD^+ and $NADP^+$. Although the Ca^{2+}-dependent pore has generally been regarded as being nonselective to small (<1200 Da) matrix solutes (1), other studies have suggested that inner membrane permeability may be more selective than previously thought (21).

We also measured the status of pyridine and adenine nucleotide in mitochondria treated with either oligomycin, sulfide, or antimycin in the presence of CaPi. The addition of oligomycin, sulfide, or antimycin to CaPi treated mitochondria prevented mitochondrial large amplitude swelling but not the CsA-sensitive release of GSH and Ca^{2+} (22). Treatment of mitochondria with oligomycin or sulfide in the presence of CaPi preserved the pyridine nucleotide redox couple ratios compared to CaPi alone, but less than untreated control samples. On the other hand, antimycin, in the presence of CaPi, potentiated the decrease in pyridine nucleotide redox couple ratios.

The increased oxidation of NADH and NADPH observed with antimycin treatment may be due to an increase in H_2O_2. Inhibition of

electron transport with antimycin is thought to result in an increase in H_2O_2 formation due to the electron leakage at ubiquinone (23-24). However, formation of GSSG was not detected and GSH was released to the extramitochondrial environment within 5 min. It may be possible however, that the efficiency of the GSH redox cycle is such that detection of GSSG is not possible under these circumstances; GSSG reductase activity may be reflected in the observed increase in $NADP^+$.

Inducing agents such as t-butyl hydroperoxide or hydrogen peroxide are thought to induce permeability transition due to the oxidation of pyridine nucleotides (4, 13-14). However, based on the findings we report, the redox status of pyridine nucleotides alone does not seem to regulate permeability transition. This observation is based on the finding that treatment with antimycin resulted in an increased oxidation of both NADH and NADPH compared to CaPi treatment alone; but unlike CaPi treatment, the addition of antimycin abolished large amplitude swelling and solute release was diminished. Although these findings do not dispute the significance of the pyridine nucleotide redox status in regulating permeability transition, they do seem to suggest that pyridine nucleotide redox status alone does not determine permeability transition status.

An interesting effect of the metabolic inhibitors in the presence of CaPi was the preservation of ADP. Oligomycin, sulfide, and antimycin treatment in the presence of CaPi treated mitochondria maintained ADP levels 57%, 46%, and 40%, respectively, greater than CaPi treatment alone following a 5-min incubation. Previous studies have reported that ADP can prevent permeability transition (7,25-26). Although the metabolic

inhibitors preserved ADP levels and prevented large amplitude swelling, inner membrane permeability was not inhibited (27). These findings suggest that permeability transition occurs to varying degrees in mitochondria, both with and without swelling, and varied degrees of solute release. The metabolic state of the mitochondria, as reflected by levels of ADP, ATP, pyridine nucleotides, membrane potential, and/or other parameters may determine the degree of permeability transition.

To determine if ADP could prevent large amplitude swelling of the mitochondria in the presence of CaPi, we monitored large amplitude swelling in the presence of increasing concentrations of ADP. Our findings suggest that ADP prevented large amplitude swelling in a concentration-dependent manner in the presence of CaPi. Since addition of the metabolic inhibitors prevented large amplitude swelling and preserved ADP levels compared to CaPi treatment alone, this may partially explain how the metabolic inhibitors prevented large amplitude swelling.

In conclusion, we report that during CaPi induced permeability transition, NADH, NADPH, ATP and ADP are rapidly depleted. Under these conditions, NADH and NADPH undergo extensive oxidation with the subsequent release of NAD^+ and $NADP^+$ from the mitochondrial matrix within a 5-min incubation. This release occurs through the putative Ca^{2+}-dependent inner membrane pore as CsA prevented the oxidation and release of pyridine nucleotides. ATP and ADP are also rapidly depleted with the concomitant formation of AMP during CaPi treatment. The addition of either oligomycin, sulfide, or antimycin preserved NADH, NADPH, ATP, and ADP to varying degrees.

Maintenance of ADP by the respiratory inhibitors may contribute to the partial protection observed against permeability transition.

Table VI. 1. NADH:NAD$^+$ and NADPH:NADP$^+$ Redox Ratios.
NADH:NAD$^+$ and NADPH:NADP$^+$ ratios were determined following a 5-min incubation in the absence (control) or presence of either 70 μM Ca^{2+} and 3 mM Pi (CaPi) or CaPi plus 0.5 μM CsA (+CsA), CaPi plus 3 μM oligomycin (+Oligomycin), CaPi plus 0.5 mM sulfide (+Sulfide), or CaPi plus 1μM antimycin (+Antimycin).

	NADH :NAD$^\pm$	NADPH: NADP$^\pm$
Control	4.97	13.50
CaPi	1.00	0.96
+CyA	5.81	7.88
+Oligomycin	2.56	3.25
+Sulfide	1.58	1.43
+Antimycin	.079	0.49

TABLE VI.2. Mitochondria (1mg/mL) were incubated in the presence of 70 mM Ca^{2+} and 3 mM Pi (CaPi) alone or with the addition of either 0.5 mM cyclosporin A, 3 mM oligomycin, 0.5 mM sulfide, or 1 mM antimycin. Samples were taken at the times indicated and analyzed by HPLC for ATP, ADP, and AMP as described in Experimental Procedures.
n=3-5 (± SE)

ADENINE NUCLEOTIDES EXPRESSED nmols/mg

	ATP		ADP		AMP		TOTAL
	*I	**E	I	E	I	E	
Control							
0 min	2.2 (.34)	1.7 (.18)	5.5 (.55)	.42 (.04)	7.6 (.84)	.39 (.12)	17.8
5 min	1.8 (.38)	1.5 (.16)	5.0 (.80)	.32 (.03)	8.2 (.61)	.17 (.10)	17.0
CaPi							
3 min	1.2 (.12)	2.0 (.59)	2.7 (.28)	1.4 (.20)	7.8 (1.4)	2.8 (.72)	17.9
5 min	0.9 (.09)	0.3 (.04)	1.8 (.18)	1.1 (.11)	6.6 (1.1)	6.0 (.33)	16.8
" + CsA							
3 min	2.9 (.28)	1.9 (.53)	7.6 (.51)	0.1 (.09)	4.4 (1.1)	0.03	17.0
5 min	2.7 (.13)	2.9 (.72)	7.1 (.53)	0.4 (.04)	6.3 (.82)	0.1 (.05)	19.5
" + Oligomycin							
3 min	2.3 (.20)	1.8 (.14)	4.2 (.18)	0.9 (.56)	5.2 (.85)	0.5 (.42)	15.0
5 min	2.4 (.32)	1.3 (.31)	4.2 (.80)	1.4 (.29)	5.0 (.80)	1.6 (1.1)	15.9
" + Sulfide							
3 min	1.1 (.24)	0.5 (.17)	3.6 (.44)	0.5 (.02)	6.9 (.77)	3.3 (.48)	15.9
5 min	1.0 (.22)	0.3 (.01)	3.3 (.43)	0.5 (.08)	6.0 (1.3)	4.2 (.44)	15.3
" + Antimycin							
3 min	1.0 (.22)	0.0	3.3 (.62)	0.3 (.11)	6.6 (1.3)	4.4 (.47)	15.6
5 min	1.0 (.19)	0.0	3.0 (.60)	0.4 (.03)	5.6 (.63)	5.9 (.66)	16.0

* I = Intramitochondrial   ** E = Extramitochondrial

Figure VI. 1. Intra- and extra-mitochondrial NADH and NAD$^+$. Mitochondria (1mg/ml) were incubated in the absence (control) or presence of 70 µM Ca^{2+} and 3 mM Pi (CaPi) or CaPi plus 0.5 µM cyclosporin A (CaPiCsA). Aliquots (1ml) were taken at the times indicated and analyzed for (A) intra- and extramitochondrial NADH, (B) intramitochondrial NAD$^+$ or (C) extramitochondrial NAD$^+$ as described in Experimental Procedures.
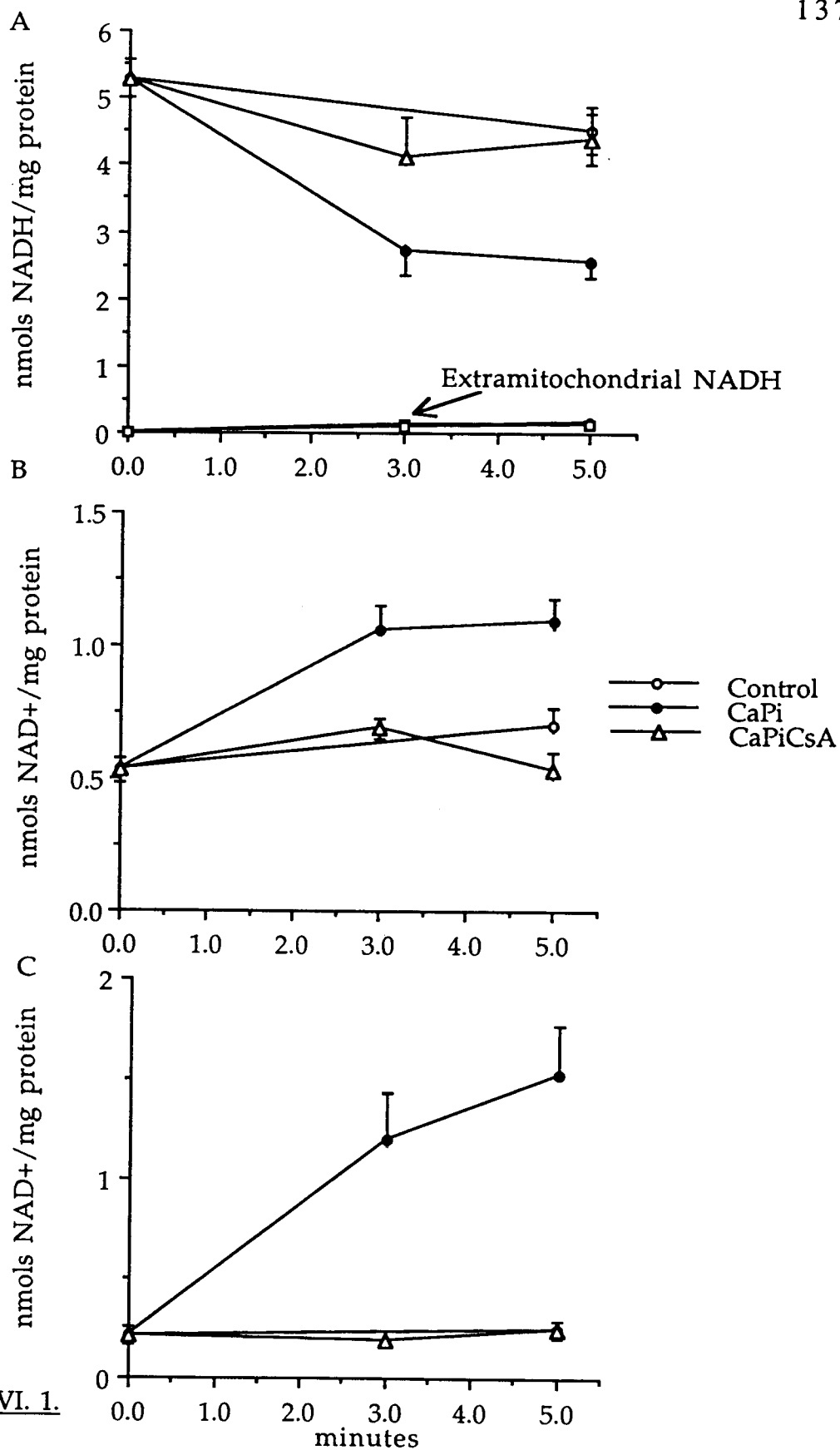
n=3-4 ± S.E.M.

Figure VI. 1.

Figure VI. 2. Intra- and extra-mitochondrial NADPH and NADP$^+$.
Mitochondria (1mg/ml) were incubated in the absence (control) or
presence of 70 μM Ca^{2+} and 3 mM Pi (CaPi) or CaPi plus 0.5 μM
cyclosporin A (CaPiCsA). Aliquots (1ml) were taken at the times
indicated and analyzed for (A) intra- and extramitochondrial NADPH,
(B) intramitochondrial NADP$^+$ or (C) extramitochondrial NADP$^+$ as
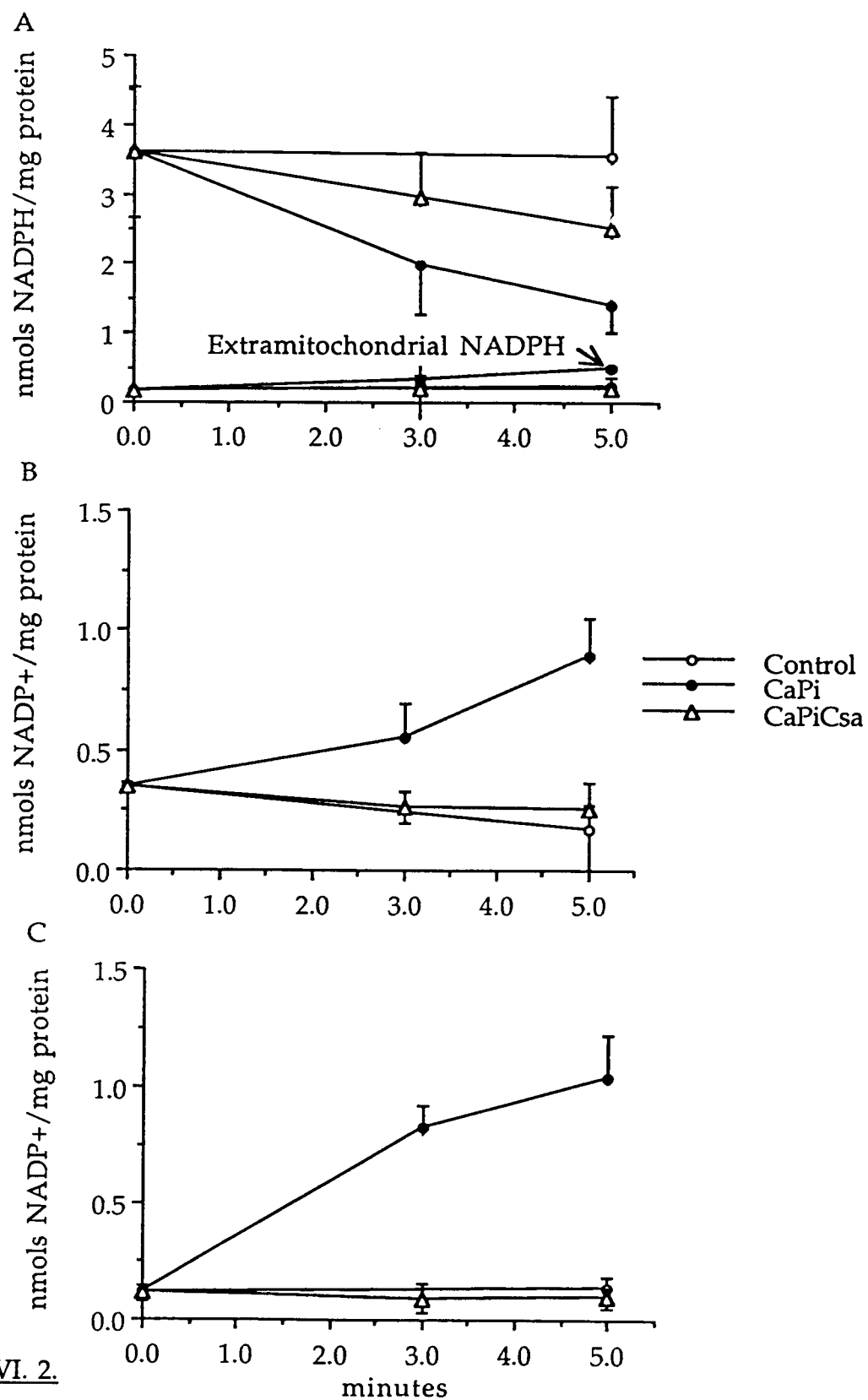described in Experimental Procedures.
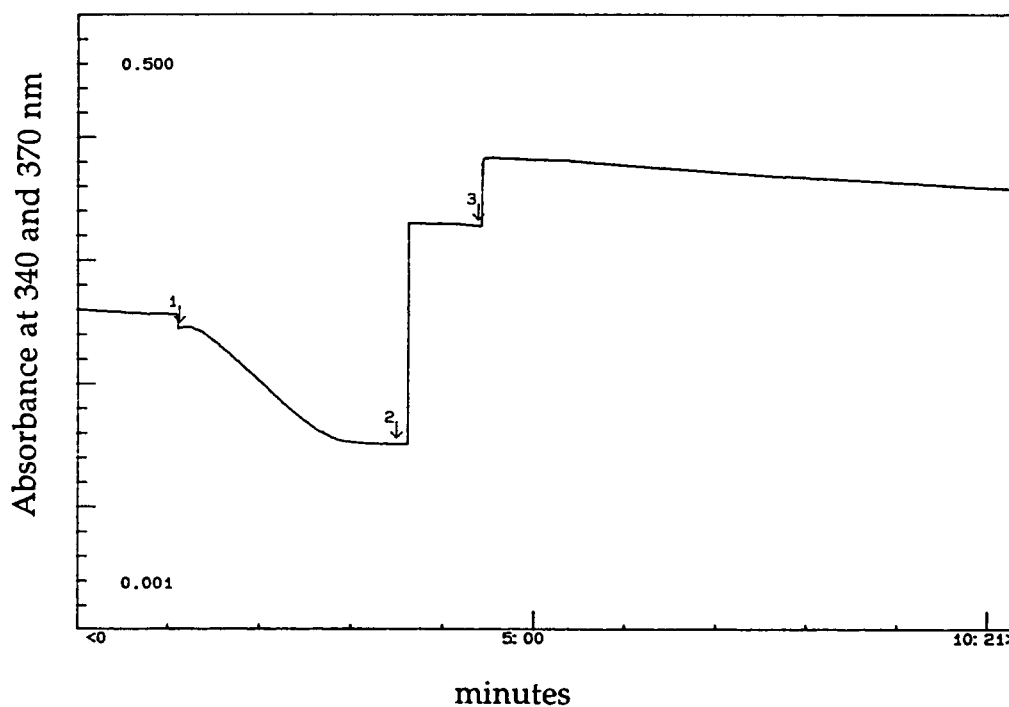n=3-4 ± S.E.M.

Figure VI. 2.

Figure VI. 3. Spectrophotometric determination of the redox state of exogenous NADPH and NADH. Pyridine nucleotide oxidation occurred in mitochondrial suspensions (1 mg/ml) treated with (1) 70 µM Ca^{2+} and 3 mM Pi. Exogenous NADH (2) and NADPH (3) were added following the oxidation of endogenous pyrdine nucleotides and monitored for redox changes.

Figure VI. 4. Effect of metabolic inhibitors on NAD(H). Mitochondria (1 mg/mL) were incubated in the presence of 70 μM Ca^{2+} and 3 mM Pi (CaPi) alone or with the addition of either 3 μM oligomycin, .5 mM sulfide, or 1μM antimycin. Aliquots (1ml) were taken at the times indicated and analyzed for (A) intra- and extramitochondrial NADH, (B) intramitochondrial NAD^+ or (C) extramitochondrial NAD^+ as described in Experimental Procedures. n=3-4 ± S.E.M.
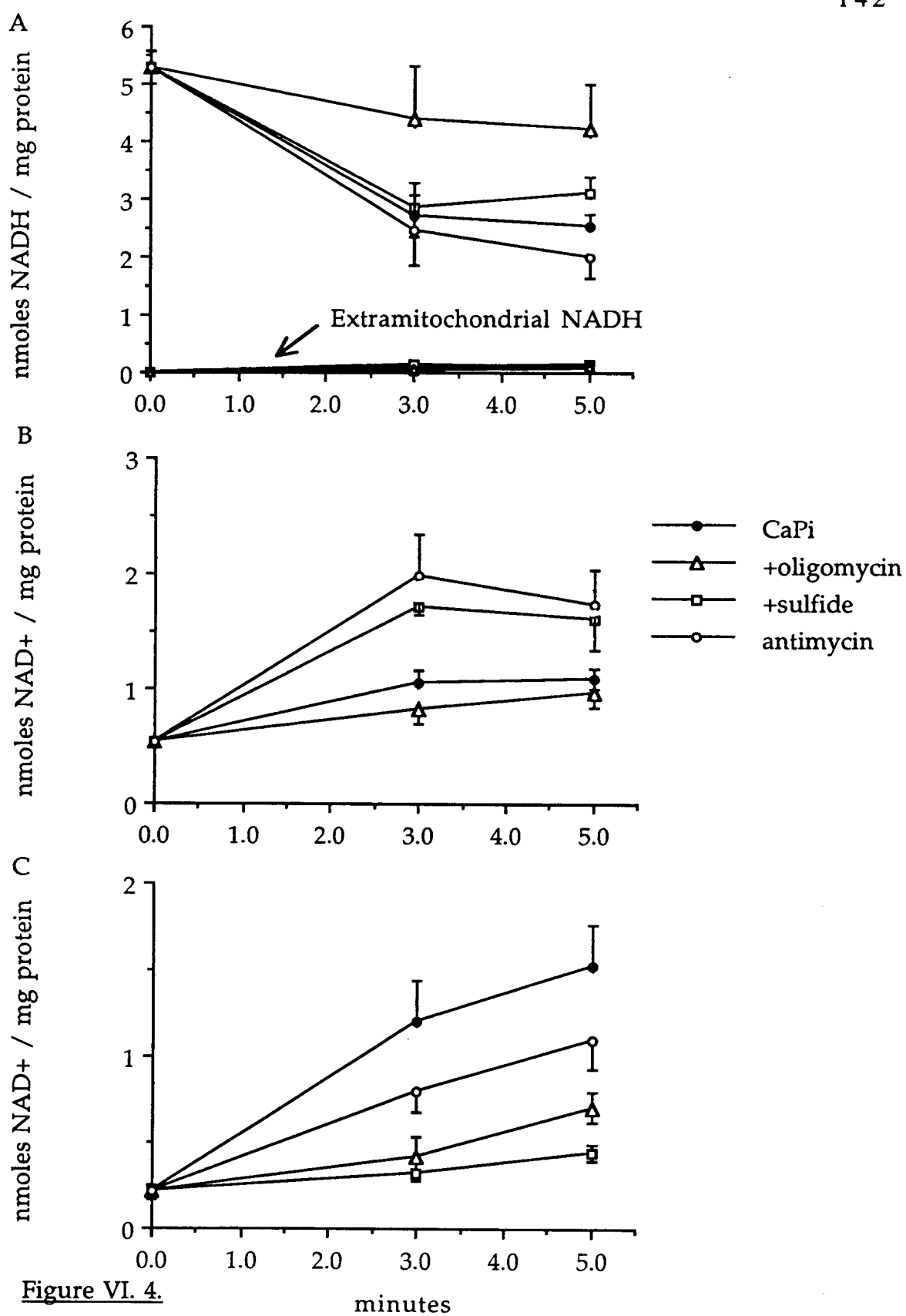
Figure VI. 4.

Figure VI. 5. Effect of metabolic inhibitors on NADP(H). Mitochondria (1 mg/mL) were incubated in the presence of 70 μM Ca^{2+} and 3 mM Pi (CaPi) alone or with the addition of either 3 μM oligomycin, .5 mM sulfide, or 1μM antimycin. Aliquots (1ml) were taken at the times indicated and analyzed for (A) intra- and extramitochondrial NADPH, (B) intramitochondrial $NADP^+$ or (C) extramitochondrial $NADP^+$ as described in Experimental Procedures.
n=3-4 ± S.E.M.

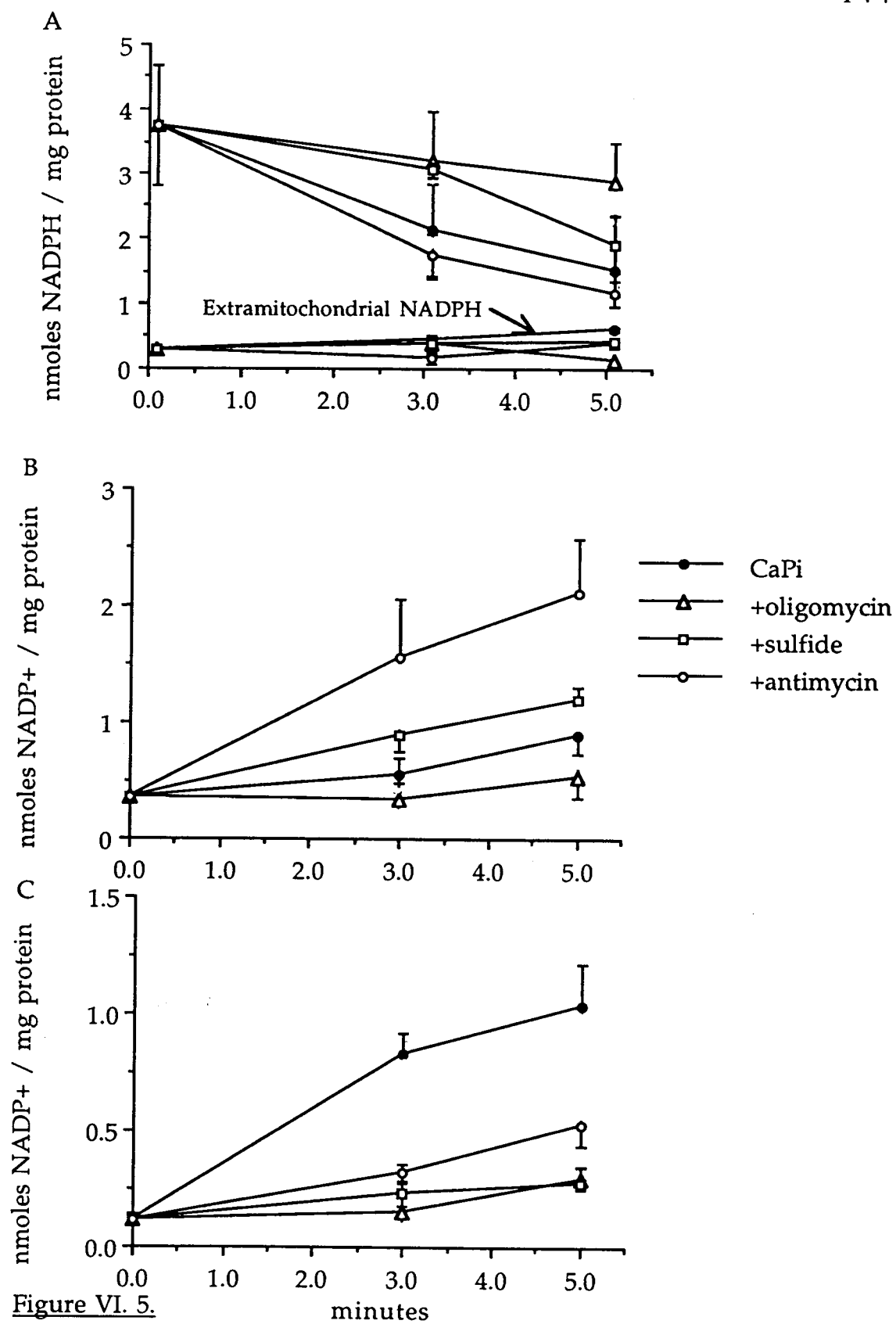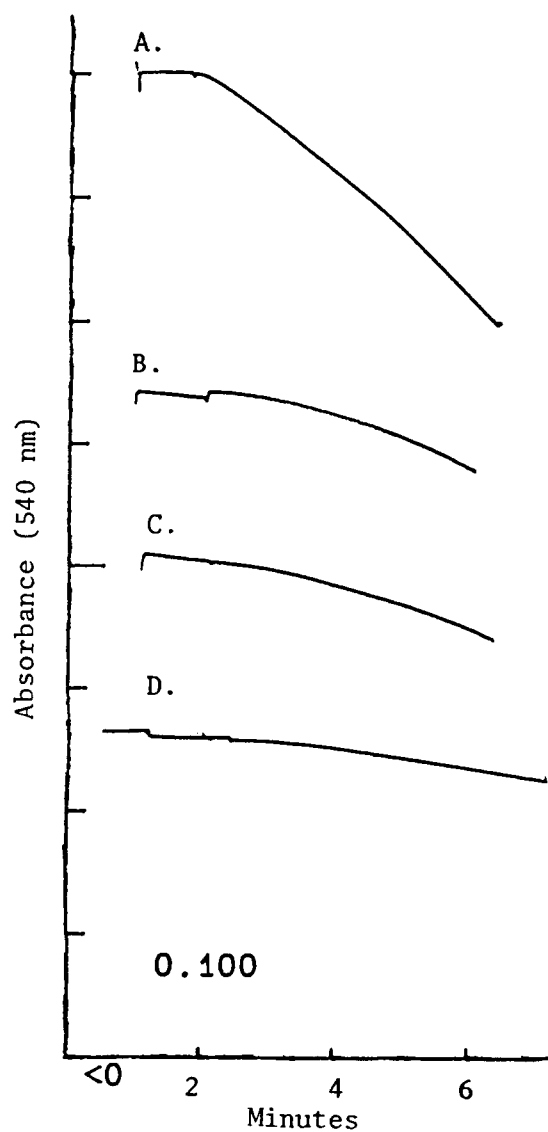Figure VI. 5.

Figure VI. 6. Dose response inhibition of large amplitude swelling by exogenous ADP. Mitochondria (1 mg/ml) were treated with: A. 70 μM Ca and 3 mM Pi (CaPi) ; B. 60 μM ADP plus CaPi; C. 80 μM ADP plus CaPi; and D. 150 μM ADP plus CaPi and monitored for large amplitude swelling.

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VII. SUMMARY, CONCLUSIONS, AND FUTURE STUDIES

The studies described in this thesis provide valuable data and observations regarding mitochondrial glutathione status, permeability transition, and energy status during Ca^{2+}- and inorganic phosphate-induced permeability transition.

In particular, this work demonstrates the rapid and nearly complete release of mitochondrial glutathione by a Ca^{2+}-dependent mechanism. The process is inhibited by CsA which suggests that release of glutathione occurs through the putative Ca^{2+}-dependent pore of the permeability transition. The significance of these findings is that glutathione efflux from mitochondria may occur under toxicological and pathological conditions in which mitochondria are exposed to elevated levels of Ca^{2+} in the presence of near physiological concentrations of inorganic phosphate.

In addition to demonstrating a mechanism of glutathione release from mitochondria during permeability transition, the study described in Chapter III also demonstrated a mechanism to load mitochondria with exogenous glutathione by the controlled opening and closing of the inner membrane pore. Exogenous glutathione could be trapped or loaded in mitochondria by the controlled opening and closing of the pore with CaPi, and CsA respectively. Opening and closing of the pore provided a mechanism for the uptake of glutathione by mitochondria that was greater than glutathione uptake by untreated control mitochondria. This observation is significant in that a proposed physiological function of the pore is in providing a mechanism for transport of mitochondrial

metabolites. Based upon this finding, cyclical opening and closing of the pore physiologically could possibly provide a mechanism to load mitochondria with glutathione.

A significant finding reported in Chapter IV demonstrates that the CsA-sensitive permeability transition, accompanied by the release of glutathione and Ca^{2+}, can occur without large amplitude swelling. The addition of either antimycin, sulfide, or oligomycin to CaPi treated mitochondria abolished large amplitude swelling, but did not prevent inner membrane permeability. Assessment of mitochondrial integrity via the light scatter assay or TEM analysis may not accurately reflect the biochemical alterations due to changes in mitochondrial inner membrane permeability. This is an important observation relevant to cellular or isolated mitochondrial studies relying on ultrastructural changes as an assessment of mitochondrial integrity. In particular, this finding is relevant for permeability transition studies which often rely on the light scatter assay as a gauge of inner membrane permeability. These findings clearly demonstrate that the absence of mitochondrial large amplitude swelling does not indicate an absence of an inner membrane permeability. Concluded from this study is that CsA-sensitive permeability transition can occur without obvious ultrastructural changes and, therefore, monitoring the CsA-sensitive release of endogenous matrix solutes, such as glutathione, may be a sensitive indicator of permeability transition.

In Chapters V and VI, the status of NAD(P)H and ATP were examined during CaPi-induced permeability transition. Several significant observations are reported. The first is that NAD(P)H is

extensively oxidized within a few minutes CaPi treatment. This is particularly important because it has been assumed heretofore that CaPi-induced permeability transition did not alter the pyridine nucleotide redox ratios. The oxidation of pyridine nucleotides occurred intramitochondrially followed by the subsequent release of oxidized pyridine nucleotides. Release of reduced pyridine nucleotides was not observed. The significance of these findings is that extensive oxidation of pyridine nucleotides, followed by their release, occurs during CaPi induced permeability transition. The redox changes in pyridine nucleotides may reflect an oxidative stress associated with this mitochondrial injury or a mechanism which depletes mitochondria of energy.

CaPi-induced permeability transition was also accompanied by the rapid depletion of ATP with the concomitant formation of AMP. The mechanism of ATP depletion is not currently understood, but is likely an important component of this mitochondrial injury. The ability of CsA to maintain NAD(P)H and ATP status during CaPi-induced permeability transition may be important in understanding the mechanism(s) by which CsA prevents permeability transition.

Treatment with metabolic inhibitors in the presence of CaPi prevented large amplitude swelling but did not prevent inner membrane permeability, although solute release was diminished. Under these conditions, ADP levels were preserved. Since ADP is an inhibitor of permeability transition, the mechanism by which metabolic inhibitors prevented large amplitude swelling may be reflected in the maintenance of ADP. Concluded from this study is that permeability transition could

occur to varying degrees, both with and without swelling and varied rates of solute release. Maintenance of mitochondrial energy status in the form of NAD(P)H, ATP, and ADP may play an important regulatory role in permeability transition.

Directions of future studies include the need to address the mechanism by which CaPi induced permeability transition oxidizes pyridine nucleotides as well as the mechanism of ATP depletion. Future studies might examine superoxide dismutase activity, the effect of antioxidants such as vitamin E and BHT, and the measurement of reactive oxygen species such as hydrogen peroxide.

In addition, it would be interesting to investigate whether reduced pyridine nucleotides can be loaded into mitochondria. This could be accomplished with the use of radiolabelled reduced pyridine nucleotides incubated with CaPi treated mitochondria followed by the addition of CsA. The effect would be to open and close the pore following a short incubation with radiolabelled reduced pyridine nucleotides. This experiment would address whether the permeability transition pore is selective for oxidized pyridine nucleotides.

In the experiments described, succinate was the substrate for mitochondria. Another set of experiments could involve the use of NAD^+-linked substrates. This may be revealing of the mechanism by which pyridine nucleotide redox status is controlled.

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