A CYCLOSPORIN A-SENSITIVE

MITOCHONDRIAL PORE

Thesis submitted in accordance with the requirements of University College London, University of London, for the degree of Doctor in Philosophy, by,

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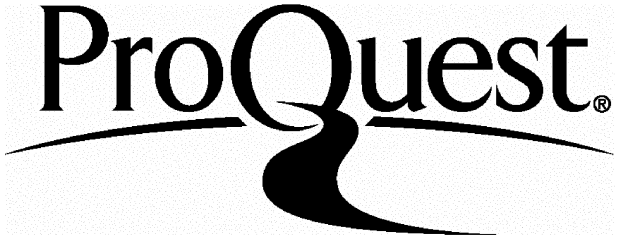

ABSTRACT

Cyclosporin-A is a potent immunosuppressant used to prevent graft rejection. Work in this laboratory has suggested a novel action of cyclosporin, namely protection against the cell injury caused by energy deprivation e.g. during ischaemia by blocking a non-selective, Ca^{2+}-sensitive mitochondrial pore. The long-term aim of this investigation is to reconstitute the Ca^{2+}-sensitive pore and determine which parameters are important in its control. Initially, previous studies were repeated in liver mitochondria mimicking more closely pathophysiological conditions. Isolated rat liver mitochondria exposed to buffered Ca^{2+} concentrations above 1-2μM, low levels of ATP and either a supraphysiological Pi concentration, or a high oxygen tension cannot maintain a high inner membrane potential on addition of succinate as substrate. [U-^{14}C]-Sucrose entrapment studies confirm that this is due to the reversible opening of a Ca^{2+}-sensitive pore in the inner mitochondrial membrane. Pore closure is induced immediately on chelation of Ca^{2+} with EGTA. Pore opening, again assayed by the degree of entry and entrapment of [U-^{14}C]-sucrose in the matrix is inhibited by cyclosporin A at the extremely low concentration of 25nM and by physiological concentrations of ATP. However 600nM cyclosporin is needed to prevent proton permeability and allow the maintenance of a high inner membrane potential. [^{3}H]-Cyclosporin binding studies revealed that cyclosporin A binds to two components in rat liver mitochondria, a soluble matrix component with high affinity and a membrane component with low affinity as well as non-specific binding to mitochondrial phospholipids. There is a good correlation between the total amount of bound cyclosporin yielding maximal pore inhibition and the amount of the soluble matrix component estimated in the binding studies. The soluble matrix component was identified as cyclophilin, a ubiquitous protein known to mediate cyclosporin's immunosuppressive effects and which has previously been shown to be a peptidyl-prolyl-*cis-trans*-isomerase. Soluble mitochondrial cyclophilin was purified $\simeq$1000x using cation exchange and gel filtration FPLC . Cyclophilin was found to uncouple respiring sub-mitochondrial particles and this effect was inhibited by cyclosporin . These results strongly suggest that cyclosporin interacts with mitochondrial cyclophilin to induce pore blockage.

A subsidiary aim of this study was to establish whether or not ADP-ribosylation was involved in modulating pore activity. ADP-ribosylated mitochondrial membrane and soluble proteins were identified using ^{32} P-NAD and gradient SDS-PAGE.

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LIST OF ABBREVIATIONS:

ANS^-;	1-Anilino-8-naphthalene sulphonate anion
BSA;	Bovine serum albumin
CCCP;	Carbonyl cyanide-*m*-chlorophenylhydrazone
CP;	Creatine phosphate
CPK;	Creatine phosphokinase
cpm;	counts per minute
CsA;	Cyclosporin A
c-state;	state of adenine nucleotide translocator in which binding site is accessible from the cytosol
diS-C_3-5^+;	3,3'-dipropylthiodicarbocyanine iodide anion
DDA^+;	Didecyldimethylammonium
EGTA;	Ethyleneglycol-*bis*-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
FCCP;	Carbonyl cyanide-*p*-triflouromethoxyphenylhydrazone
GLC;	Gas liquid chromatography
GSH;	Reduced glutathione
GSSG;	Oxidised glutathione
HEDTA;	*N*-Hydroxyethylethylenediaminetriacetic acid
Hepes;	*N*-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IMM;	Inner mitochondrial membrane
LDS;	Lithium dodecyl sulphate
MCC;	Multiconductance channel
MeOH;	Methanol
MIBG;	*m*-iodobenzylguanidine
MOPS;	Morpholino propone sulphonate
MST;	Buffer for mitochondrial preparation containing: 210mM mannitol; 70mM sucrose; 10mM tris-HCl at pH 7.2
m-state;	state of adenine nucleotide translocator in which the binding site is accessible from the matrix
MW;	Molecular weight
NF-AT;	Nuclear factor from activated T-cells

PMSF;	Phenyl-methyl-sulphonyl fluoride
PPIase;	Peptidylprolyl-*cis-trans*-isomerase
PVC;	Polyvinylchloride
RLM;	Rat liver mitochondria
SDS;	Sodium dodecyl sulphate
SN;	Supernatant
state IV respiration;	State in which the respiratory rate of mitochondria is low due to depletion of ADP even though there is still substrate available
TEMED;	*N,N,N',N'*-Tetramethlethylenediamine
THF;	Tetrahydrofuran
TPB^-;	Tetraphenylboron anion
TPP^+;	Tetraphenylphosphonium cation
Tris;	Tris[Hydroxymethyl]aminomethane
Xaa-proline;	Xaa is any amino acid adjacent to a proline residue
Δ;	Dehydrogenase
$\Delta\Psi$;	Mitochondrial membrane potential

CHAPTER 1: A CYCLOSPORIN A-SENSITIVE MITOCHONDRIAL PORE.

[1.1] *History.*

The deleterious effects of Ca^{2+} on the permeability properties of the inner mitochondrial membrane have been known for many years. In the mid 60's it was shown that Ca^{2+} and Pi can cause permanent uncoupling of respiration [Chappell *et al.*, (1963); Lehninger *et al.*, (1967)]. This impairment of mitochondrial function was prevented by adenine nucleotides and later shown to be promoted by agents which oxidise pyridine nucleotides (e.g. oxaloacetate [Lehninger *et al.*, (1978)]). The possibility that the increase in permeability observed under these conditions might reflect the existence of a transmembrane hydrophilic channel or pore was proposed by Haworth and Hunter in the late 70's [Haworth and Hunter (1976)]. They demonstrated that this pore is non-specific insofar as both neutral and charged molecules, sucrose and any salt can pass through the membrane, the exclusion limit to passage being determined by molecular weight rather that molecular type. The studies of Crompton and his colleagues in the mid 80's using a novel ^{14}C-sucrose entrapment procedure for studying the IMM permeability [Al Nasser and Crompton (1986a/b)] tended to support the proposal that the Ca^{2+} induced increase in permeability reflected the presence of a protein pore in the inner membrane. This group went on to develop a rapid pulse flow solute entrapment technique for studying the kinetics of pore activity [Crompton and Costi (1988); Crompton and Costi (1990)]. It was shown that pores in any single mitochondrion open simultaneously [Crompton and Costi (1988)] in response to Ca^{2+} and Pi, a process termed "permeabilization".

In 1987 Fournier *et al.* published data showing that cyclosporin A (CsA), a cyclic peptide with potent immunosuppressive properties, promoted retention of Ca^{2+} by isolated mitochondria. However, this was interpreted to reflect inhibition of a physiological Ca^{2+} release mechanism. Crompton and his colleagues showed that [Crompton et al., (1988)] CsA potently inhibited the non-specific pore in rat heart mitochondria. Pore activity was assayed directly using the ^{14}C-sucrose entrapment procedure. A CsA concentration of 60pmol/mg of mitochondrial protein completely inhibited Ca^{2+} and Pi induced "permeabilization" of mitochondria. Clearly such a tight binding inhibitor would be useful in identifying one molecular component of the pore.

Prior to the discovery that CsA potently inhibited the non-specific pore it was unclear if the Ca^{2+} and Pi induced increase in IMM permeability reflected the presence of a single pore. This was because, as will be discussed below (section [1.3.4]), a bewildering array of agents can substitute for Pi in promoting the deleterious effects of Ca^{2+}. It has since been shown that CsA blocks the increase in IMM permeability when it is induced by a diverse range of these "promoters" plus Ca^{2+} [Broekemeier *et al.*, (1989); Sokolove, (1990)]. So, it may be reasonable to assume that these "promoters" of the Ca^{2+}-induced permeability increase, despite their diversity, exert their effects at a common site.

Just recently, electrophysiological patch clamp studies of giant mitochondria have revealed the existence of a high conductance channel (1.3nS) in the inner mitochondrial membrane which is inhibited by CsA [Szabo and Zoratti, (1991)]. The high conductance channel has been shown to exist in a number of sub-states and is thus referred to as the Multi Conductance Channel (MCC). The fact that CsA inhibits the MCC together with evidence showing that the MCC is activated by Ca^{2+} and inhibited by Mg^{2+} and ADP [reviewed in Szabo and Zoratti, (1992)] suggest that the MCC and the non-specific inner membrane pore are the same species.

Thus, the discovery that the immunosuppressant CsA is a potent inhibitor of the Ca^{2+}-induced increase in IMM permeability, has revealed a useful tool for the resolution of the speculation and debate which has surrounded this topic for the last twenty years.

[1.2] ***Pore size.***

Early observations of the osmotic behaviour of permeabilized mitochondria suspended in a range of poly(ethylene glycol) species with a range of increasing M_r suggested a sharp cut-off in the permeability of the pore at a M_r of 1500 [Haworth and Hunter (1980)]. By directly measuring the relative rates of permeation of the solutes sucrose, mannitol and Arsenazo III into "permeabilized" mitochondria, Crompton and Costi (1990) calculated that the putative pore has a diameter of 2.3 nm. This correlates well with the early determinations of pore size by Haworth and Hunter given that the hydrodynamic radius of poly(ethylene glycol) 1500 is about 1.2 nm [Ginsburg and Stein, (1987)]. The high conductivity of the MCC observed in patch clamp studies and which has been identified with the Ca^{2+}-activated non-

specific pore (see section [1.1] is consistent with a pore of this rather large diameter [Kinally *et al.*, (1989); Petronilli *et al.*, (1989); Szabo and Zoratti, (1990); Kinally *et al.*, (1991)]. Other aqueous pores of comparable size are porin isolated from mitochondrial outer membranes with a pore diameter of about 1.7nm [Ludwig *et al.*, (1986)] and gap junctions with pore diameters of 1.6-2nm [Lowenstein *et al.*, (1985)].

[1.3] ***Pore effectors.***

[1.3.1] *Ca^{2+}.*

A high matrix free Ca^{2+} is an absolute requirement for pore opening. Sr^{2+} will not substitute for Ca^{2+} in mediating its effects [Hunter *et al.*, (1976)]. Using direct measurements of permeation via the pore, Al Nasser and Crompton (1986a) showed that the amounts of Ca^{2+} required are relatively high, the rate of permeation being far from saturated by 25μM matrix free Ca^{2+}. Although pore opening requires neither electron flow nor energy [Hunter and Haworth (1979a)], energization facilitates Ca^{2+} entry into the mitochondrial matrix. Ca^{2+} enters energized mitochondria passively down the electrochemical gradient through a transport system termed the *uniporter* [for a review, see Crompton, (1985)]. This description was coined as it has been shown that Ca^{2+} transport is not coupled to that of any other ion or molecule. Inhibition of the *uniporter* by the polycation ruthenium red prevents mitochondria from being "permeabilized" by a rise in extramitochondrial free Ca^{2+} [for a review, see Crompton *et al.*, (1988)]. The inhibitory effect of ruthenium red can be overcome using the Ca^{2+} ionophores A23187 or ionomycin [Haworth and Hunter (1979); Vercesi *et al.*, (1988)]. Thus, it was concluded that the Ca^{2+}-induced increase in permeability must be due to Ca^{2+} binding to some internal site.

Patch clamp studies of the MCC reveal a similar Ca^{2+} dependance [Kinally *et al.*, (1991)]. In these studies, if the free Ca^{2+} was kept at 10^{-7}M or lower during mitochondrial isolation, no channel activity was observed. On raising the free Ca^{2+}, MCC activity was observed in 96% of the patches.

[1.3.2] *Redox state.*

The accumulation of >50 nmoles of Ca^{2+}/mg of protein by liver mitochondria in the presence of inorganic phosphate (0.5-2.0 mM) is followed by time-dependent swelling, uncoupling of succinate oxidation, dissipation of $\Delta\Psi$, oxidation of pyridine nucleotides, and losses of intramitochondrial K^{+}, Mg^{2+}, adenine nucleotides and preaccumulated Ca^{2+} presumably due to pore opening [Chappell *et al.*, (1963); Siliprandi *et al.*, (1975); Nicholls and Brand, (1980); [illegible] Harris and Heffron, (1982)]. It appears that swelling, loss of $\Delta\Psi$, and the inability to retain Ca^{2+} are promoted by substances which increase the degree of oxidation of pyridine nucleotides e.g. acetoacetate and oxaloacetate. Also, rereduction of pyridine nuceotides by β-hydroxy butyrate reverses these effects [Lehninger *et al.*, (1978), Nicholls and Brand, (1980), Beatrice *et al.*, (1980)]. Although their primary action is assumed to be the enzymatic oxidation of NADH, acetoacetate via β-hydroxybutyrate dehydrogenase and oxaloacetate via malate dehydrogenase (shown schematically in Fig. 1.1), both these metabolites have been shown to inhibit succinate dehydrogenase and thus respiration [Singer *et al.*, (1973); Lehninger *et al.*, (1978)]. That the effect of acetoacetate is mediated either directly or indirectly by the oxidation of pyridine nucleotides is demonstrated by the fact that, AS-30D hepatoma mitochondria which cannot reduce acetoacetate to β-hydroxybutyrate do not undergo an increase in permeability when exposed to this agent [Vercesi *et al.*, (1988)].

[1.3.3] *Inorganic phosphate.*

The effects of Ca^{2+} are promoted by inorganic phosphate. Thus replacement of phosphate with lactate prevents the swelling and loss of preaccumulated Ca^{2+} from liver mitochondria on pyridine nucleotide oxidation by acetoacetate [Wolkowicz and McMillin-Wood, (1980)]. Phosphate appears to act intramitochondrially since it is ineffective when its entry is blocked by *N*-ethylmaleimide (Beatrice *et al.*, (1980)]. Heart mitochondria are much more resilient than liver mitochondria. The Pi concentration must be increased from 2 to 20mM before accumulation of up to 100nmoles Ca^{2+}/mg of protein has any deleterious effects [Palmer and Pfeiffer, (1981).

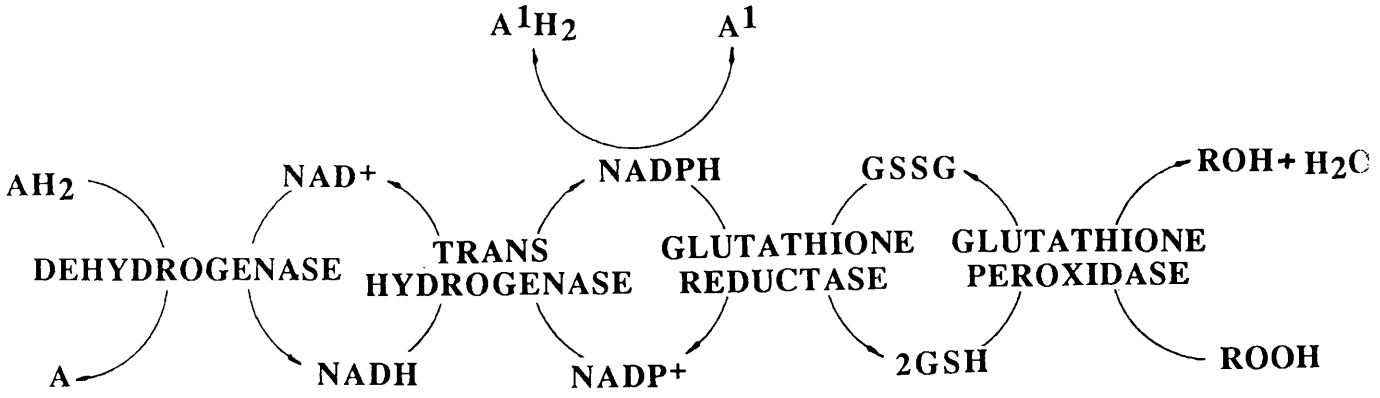


Fig. 1.1

Acetoacetate and oxaloacetate (AH_2) and tert-*butyl hydroperoxide and hydrogen peroxide (ROOH) induce oxidation of mitochondrial pyridine nucleotides through the activity of an enzyme cascade consisting of glutathione peroxidase, glutathione reductase and the energy linked transhydrogenase.*

[1.3.4] *Other "promoters".*

A bewildering array of agents can substitute for Pi in acting synergistically with Ca^{2+} to promote swelling of mitochondria, uncoupling of succinate oxidation, dissipation of $\Delta\Psi$, oxidation of pyridine nucleotides, and subsequent losses of intramitochondrial K^{+}, Mg^{2+}, adenine nucleotides and preaccumulated Ca^{2+}. These include oxidising agents, sulphydryl agents, fatty acids, inhibitors of acyl-CoA: lysophospholipid acyltransferase and effectors of the adenine nucleotide translocator (for a comprehensive list, see Gunter and Pfeiffer, (1990)).

(1.) Oxidising agents.

(a.) *Tert*-butyl hydroperoxide and hydrogen peroxide [Lötscher *et al.*, (1979); Bellomo *et al.*, (1982a,b); Jewell *et al.*, (1982)]. These oxidants induce oxidation of mitochondrial pyridine nucleotides due to the activity of an enzyme cascade consisting of glutathione peroxidase, glutathione reductase and the energy linked transhydrogenase [Oshino and Chance, (1977); Sies and Moss, (1978)] as shown in Fig. 1.1. By directly assaying sucrose permeation via the pore Crompton and Costi (1988) showed that in respiring heart mitochondria, Ca^{2+} and *tert*-butylhydroperoxide act synergistically to induce pore opening. Recent evidence from the group of Richter *et al.* suggests that under certain conditions *tert*-butyl hydroperoxide can induce Ca^{2+} release from mitochondria, in the absence of pore activity [Schlegel *et al.*, (1992)]. This group has always interpreted *tert*-butyl hydroperoxide's effects in terms of a physiological Ca^{2+} release mechanism. However previous studies did not distinguish between this specific Ca^{2+} release mechanism and possible activation of the non-specific pore. It is assumed that *tert*-butyl hydroperoxide promotes Ca^{2+} release by the oxidation of redox couples as it is ineffective with Se-deficient mitochondria when glutathione peroxidase is depressed [Lötscher *et al.*, (1979)]. In addition, *tert*-butyl hydroperoxide is without effect if glutathione reductase is selectively inactivated [Richter and Frei, (1985)]. This data implicates both oxidised NAD and GSSG in mediating *tert*-butyl hydroperoxide's effects.

Quinones, including the antibiotic adriamycin or doxorubicin [Sokolove and Shinaberry (1988); Sokolove, [redacted] (1990); Chacon and Acosta (1991)], its major

metabolite, 7-deoxy aglycone and menandione (2-methyl-1,4-napthoquinone) [Beatrice *et al.*, (1984); Broekemeier *et al.*, (1989)]. The metabolism of quinones, such as menadione in liver can occur by one-electron and two-electron reduction [Orrenius, (1985)]. While the latter seems to be catalysed primarily by NADH:(quinone-acceptor) oxidoreductase, one electron reduction resulting in the formation of semiquinone radicals, which can enter redox cycles with molecular oxygen, appears to be catalysed by several other flavoprotein reductases. In both cases, quinone metabolism is associated with pyridine nucleotide oxidation. Quinones may also modify mitochondrial sulphydryl groups [Sokolove, (1988)].

(2.)Sulphydryl reagents.

These include diamide [Vercesi *et al.*, (1988); Rizzuto *et al.*, (1987); Carbonera and Azzone, (1988);]; mersalyl [Harris *et al.*, (1979)]; phenylarsine oxide [Novgorodov *et al.*, (1987)] and *N*-ethyl maleimide [Le Quôc and Le Quôc (1985,1988)]. These agents result in the accumulation of glutathione disulphide and presumably in the cross-linking of membrane bound protein sulphydryl groups. Nitrofurantoin and rhein [Beatrice *et al.*, (1980); Carbonera *et al.*, (1988)] are also thought to promote the deleterious effects of Ca^{2+} by causing glutathione disulphide accumulation in the matrix space, secondary to the inhibition of glutathione reductase.

(3.) Fatty acids [Harris *et al.*, (1979); Harris and Chen., (1982)]. In addition to possible direct effects on the mitochondrial membrane fatty acids can act as natural ionophores or uncouplers [Pressman and Lardy, (1956); Rottenberg and Hashimoto (1986); Dedukhova *et al.*, (1991); Skulachev, (1991)].

(4.) Inhibitors of acyl-CoA: lysophospholipid acyltransferase [Riley and Pfeiffer (1986)]. The hypolipidemic drugs, WY-14643 and clofibric acid prevent mitochondrial acyl-CoA: lysophospholipid acyltransferase from utilizing acyl-CoA to regenerate diacyl phospholipid.

(5.) Impermeant inhibitors of the adenine nucleotide translocator which stabilise it in the *c*-state conformation [Lê Quôc and Lê Quôc (1988); Novgorodov *et al.*, (1990); Novgorodov *et al.*, (1991)]. These include carboxyatractylate, atractylate and palmitoyl coenzyme A (in the absence of carnitine). Atractylate is only effective when ADP is substantially depleted. Also, evidence has been presented that when

the NAD/NADH and NADP/NADPH couples are substantially oxidised the adenine nucleotide translocator is in the *c*-state [[Lê Quôc and Lê Quôc (1988)] suggesting that changes in the conformation of the the ADP/ATP carrier may play a role in NAD(P) dependent modifications of membrane permeability. Preincubation of mitochondria with bongkrekic acid and ADP (+oligomycin) both of which which stabilise the adenine nucleotide translocator in the *m*-configuration oppose the effects of carboxyatractylate and palmitoyl coenzyme A. Pi was necessary for these effects.

In all these studies "permeabilization" was followed by monitoring the absorbance changes due to the associated swelling. The ADP/ATP carrier is present in a fairly large amount in rat liver mitochondria and is the most abundant protein of the beef heart mitochondrial inner membrane [Krämer and Klingenberg (1977)]. Thus changes in its conformation may have a gross effect on mitochondrial morphology. Indeed, it has been shown that ATP, ADP, carboxyatractyloside and bongkrekic acid produce substantial light scattering in heart mitochondria, which are associated with changes in the conformation of the adenine nucleotide translocase [Packer *et al.*, (1966); Klingenberg *et al.*, (1971); Stoner and Sirak, (1973a,b); Scherer and Klingenberg, (1974)]. These changes often reflect matrix expansion and contraction without an accompanying change in permeability. However Lê Quôc and Lê Quôc (1988) also monitored the dissipation of $\Delta\Psi$, and release of Ca^{2+} associated with "permeabilization" which would imply that a conformational change in the adenine nuleotide translocator is at least involved in an increase in proton and Ca^{2+} permeability.

[1.3.5] *Adenine nucleotides and* Mg^{2+}.

Mitochondria which have been "permeabilized" and subsequently resealed are much more sensitive to Ca^{2+} than normal mitochondria [Al Nasser and Crompton (1986a)]. This may be because of the loss of endogenous factors which normally protect mitochondria against the adverse effects of Ca^{2+} during prolonged "permeabilization". Indeed it has been shown that addition of phosphoenol pyruvate, which exchanges with internal adenine nucleotides, stimulates swelling of mitochondria and loss of preaccumulated Ca^{2+} [Hunter and Haworth, (1979a)]. Adenine nucleotides and Mg^{2+} have long been known to protect mitochondria from the adverse effects of high Ca^{2+} [Chappell *et al.*,, (1963); Carafoli *et al.*, (1965); Rossi and Lehninger, (1964); Manery, (1966); Lehninger *et al.*, (1967);Reynolds,

(1972); Binet and Volfin, (1975); Asimakis and Sordahl, (1977); Harris, (1979); Zoccaroto *et al.*, (1981) ; Lötscher *et al.*, (1980); Nicholls and Scott, (1980); Coelho and Vercesi, (1980), Zoccarato *et al.*, (1981)]. Data indicate that Mg^{2+} can act synergistically with ADP in mediating its protective effect [Hunter and Haworth, (1979b); Azzi and Azzone, (1966)]. If Ca^{2+} loaded mitochondria are uncoupled , the subsequent depletion of ATP results in a stimulation of rate of "permeabilization" [Igabova and Pfeiffer [redacted] (1988)].

In addition, as mentioned in section [1.1] the MCC detected in electrophysiological patch-clamp experiments is inhibited by Mg^{2+} and ADP.

[1.3.6] *Inhibitors of the non-specific pore.*

As a high matrix free Ca^{2+} is an absolute requirement for "permeabilization" it can be prevented by buffering rises in matrix Ca^{2+} with EGTA or inhibiting mitochondrial accumulation of Ca^{2+} via the uniporter with Ruthenium Red. Although the number of positive effectors which in addition to Ca^{2+} promote pore opening is bewildering, the use of pore inhibitors indicates that these synergists act by a common mechanism.

(1.) The free radical scavenger butylated hydroxytoluene at concentrations of $<50\mu M$ is an effective inhibitor of "permeabilization" when it is induced by Ca^{2+} acting synergistically with either: Pi [Novgorodov *et al.*, (1989)]; the oxidant *tert*-butyl hydroperoxide [Carbonera and Azzone (1988)]; Fe^{2+} and ascorbate (which produce oxygen radicals by a Fenton type reaction) [Novgorodov *et al.*, (1989)]; adriamycin (which produces oxygen radicals due to redox cycling) [Sokolove, (1990); Powis (1989)]; the sulphydryl reagents phenylarsine oxide and diamide [[Novgorodov *et al.*, (1987); Carbonera and Azzone (1988)]; or nitrofurantoin [[Carbonera *et al.*, (1988)].

(3.) Agents which lock the adenine nucleotide translocator in the [redacted]conformation. It has been shown that the massive swelling associated with the Ca^{2+}- induced increase in IMM permeability, can be blocked by the addition of bongkrekate and ADP [Chappel and Crofts, (1965); Haworth and Hunter (1979); Harris *et al.*, (1979); Lê Quôc and Lê Quôc, (1988)]. These results are ambiguous however, because, as stated in section [1.3.4(5)] it has been shown that bongkrekate alone

causes mitochondria to contract [Packer *et al.*, (1966)] and swelling measurements are not a reliable indication of its possible effects on "permeabilization".

(4.) Inhibitors of phospholipase A_2. The calmodulin antagonists, W7 and trifluoperazine and the Ca^{2+} channel blockers nifedipin, verapamil and diltiazem are pharmacological compounds of dissimilar structure which have been shown to inhibit "permeabilization" with a potency equal to that with which they inhibit phospholipase A_2 (range 50-200μM) [Broekemeier *et al.*, (1985)].

(5.) pH. The effect of Ca^{2+} is sharply inhibited by a drop in pH [Haworth and Hunter (1979); Al Nasser and Crompton, (1986); Halestrap, [redacted] (1991)]. At pH 7.2 the apparent k_m for Ca^{2+} (in the absence of ADP and NADH) is $< 10\mu$M and is still dropping with increasing pH [Haworth and Haworth (1979)]. When sucrose permeation via the pore is measured directly, the rate constant for "permeabilization" to sucrose decreases slightly when the pH is decreased from 7.5 to 6.5., whereas the rate of dissipation of $\Delta\Psi$ was markedly increased. This was taken to indicate H^+ permeation precedes sucrose permeation via the pore. [Al Nasser & Crompton, (1986)]

(6.) CsA. As already mentioned in section [1.1], in 1987 Fournier *et al.*, published data showing that the immunosuppressant CsA, promoted retention of Ca^{2+} by isolated mitochondria. However, this was interpreted to reflect inhibition of a physiological Ca^{2+} release mechanism. Crompton and his colleagues showed that in [Crompton et al., (1988)] rat heart mitochondria CsA potently inhibited the non-specific pore. "Permeabilization" of heart mitochondria, (as assayed directly by entry and entrapment of radiolabelled solute), induced by Ca^{2+} plus Pi or Ca^{2+} plus *tert*-butyl hydroperoxide was almost completely inhibited by 60pmol of CsA/mg of mitochondrial protein. Rather more CsA was required to prevent the dissipation of $\Delta\Psi$ which occurred under these circumstances.

Broekemeier *et al* (1989) showed that CsA potently inhibited "permeabilization" (assayed indirectly by monitoring the swelling induced by Ca^{2+} uptake) whether it was induced by N-ethyl maleimide, Hg^{2+} plus dithiothreitol, WY-14643, oxalacetate, rhein or ruthenium red plus uncoupler in addition to Ca^{2+}. It was found that 20-50 pmol of CsA/mg of protein reduced the swelling response by 50% with complete inhibition obtained with about 150 pmol/mg of protein. CsA also

potently inhibits the "permeabilzation" induced by 7-deoxyglycone a major metabolite of adriamycin or doxorubicin [Sokolove, (1990)].

The inhibition by CsA of the dissipation of $\Delta\Psi$ and concomitant high amplitude swelling induced by a high Pi and Ca^{2+}, is reversed by carboxyatractyloside and the bifunctional sulphydryl agent, phenylarsine oxide [Novgorodov *et al.*, (1990).

CsA is the most potent inhibitor of "permeabilization" found to date and is discussed in more detail at the end of this chapter.

[1.4] ***"Permeabilization" reflects the operation of a cycle of phospholipid deacylation and reacylation.***

Despite the strong evidence that "permeabilization" reflected the presence of a protein pore in the IMM a rather different concept has been proposed by Pfeiffer and colleagues [Broekemeier *et al.*, (1985); Riley *et al.*, (1986); and Beatrice *et al.*, (1980)]. These workers proposed that "permeabilization" involves a cycle of phospholipid acylation/deacylation. The cycle begins with the action of inner membrane phospholipase A_2 which requires Ca^{2+} for activity [Waite, [redacted] (1969)], displays a pH optimum 8.5 [Zurini *et al.*, (1981)] and which has a preference for phosphatidyl ethanolamine as substrate [Natori *et al.*, (1983)]. Fatty acids released by phospholipase can be activated to acyl-CoA derivatives by either of two acyl-CoA synthetases. One of these requires ATP and generates AMP plus PPi [Groot *et al.*, (1974)] while the other utilizes GTP specifically and generates GDP plus Pi [Galzigna *et al.*, (1967)]. An inner membrane lysophospholipid acyl transferase selective for lysophospholipid ethanolamine utilizes acyl-CoA to generate the initial diacyl-phospholipid [Nachbaur *et al.*, (1969)]. It is proposed that agents which elevate the intramitochondrial level of glutathione disulphide [Beatrice *et al.*, (1984)] or otherwise alter sulphydryl groups essential for the maintenance of acyltransferase activity [Pfeiffer *et al.*, (1979)] aggravate the process, making it essentially irreversible by interfering with reacylation [Broekemeier *et al.*, (1985)].

Support for this mechanism comes from several observations. Before the discovery of CsA, it was not clear how the diverse range of agents which promoted the deleterious effects of Ca^{2+} could exert their effects at a common site. When it has been investigated phospholipase A_2 reaction products have always been found to

accumulate as the transition proceeds and these compounds, when added exogenously, can provoke the effect [Harris and Cooper, (1981); Dalton *et al.*, (1984)]. Pharmacological agents including: the Ca^{2+} channel blockers, verapamil, nifedipine, diltiazem and prenylamine; and the calmodulin antagonists trifluoperazine, dibucaine, and quinacrine inhibit permeabilization with an equal potency as they inhibit phospholipase A_2 [Broekemeier *et al.*, (1985); Vercesi *et al.*, (1988)]. Direct inhibitors of acyl-CoA:lysophospholipid acyltransferase activity WY-14643 and clofibrate act synergistically with Ca^{2+} to induce "permeabilization" [Riley and Pfeiffer, (1986)]. However, in all the above studies "permeabilization" was not measured directly in these experiments but by monitoring the swelling associated with the process, the results of these experiments are ambiguous.

This interpretation of "permeabilization" has also been criticised [Crompton and Costi (1988) on the basis that as "permeabilized" mitochondria are not permeable to sucrose [Hunter and Haworth (1976); Al Nasser and Crompton (1986a)] and arsenazo III (molecular mass 774 Da [Al Nasser and Crompton (1986b); Hayat and Crompton (1987)]) small, essential substrates for reacylation would presumably be lost from the matrix, e.g. ATP, and possibly CoA, required by matrix CoA synthetase [Groot *et al.*, (1974)]. In addition Crompton and Costi (1988) demonstrated that ATP/Mg^{2+} and CoA have no stimulatory effect on the rate of resealing of "permeabilized" mitochondria triggered by chelation of Ca^{2+} with EGTA. The inhibitors of permeabilization described above are of relatively low potency and have many other non-specific effects. In addition, *N*-acylethanolamine, a lipid class which accumulates to high levels in ischaemic heart and brain, inhibits "permeabilization" but is not active against phospholipase A_2 [Broekemeier *et al.*, (1985)].

[1.5] ***Possible involvement of ADP-ribosylation in the modulation of pore activity.***

As mentioned in section [1.3.4(1)] Richter and his coworkers have shown *tert*-butyl hydroperoxide leads to Ca^{2+} release from mitochondria. Ca^{2+} release is also associated with the hydrolysis of oxidised pyridine nucleotides to ADP-ribose and nicotinamide most likely by an NAD^+ glycohydrolase localised on the inner side of the inner mitochondrial membrane [Lotscher *et al.*, (1980); Moser *et al.*, (1983); Hilz *et al.*, (1984); and Masmoudi and Mandel, (1987)]. They propose that there is a functional link between Ca^{2+}-release and the ADP-ribosylation of a single inner

membrane mitochondrial protein of ≃ 30, 000 Da. This proposal is based on several lines of evidence. Firstly, the activity of the NAD^+ glycohydrolase, like Ca^{2+}-release is inhibited by ATP [Hofstetter *et al.*, (1981)]. Secondly, both pyridine nucleotide hydrolysis and Ca^{2+} release show the same sigmoidal dependence on the mitochondrial Ca^{2+} load [Frei *et al.*, (1985)]. Finally, *m*-iodobenzylguanidine (MIBG), a substrate for mono-ADP-ribosylation reactions and an inhibitor of ADP-ribosyltransferase, does not affect pyridine nucleotide oxidation, but prevents Ca^{2+} release [Richter, (1990)], presumably by competing for ADP-ribose with the mitochondrial acceptor proteins.

There is no firm evidence that the Ca^{2+} release induced by *tert*-butyl hydroperoxide does not proceed via the non-selective pore. The fact that Richter found that CsA inhibits both Ca^{2+} and nicotinamide release [Richter *et al.*,(1991)] suggests the non-selective pore may be implicated in this process.

[1.6] ***The pore may reflect a distortion of the adenine nucleotide translocator:***

Le Quôc and Le Quôc (1988) have proposed that the pore reflects a distortion of the adenine nucleotide translocator resulting in a loss of specificity of the carrier for the molecules which it normally transports. This proposal is supported by the observation that reorientation of the carrier from the *m*- to the *c*- or conversely from the *c*- to the *m*- state causes pore opening and closure respectively (see sections [1.3.4] and [1.3.6]. In addition, Dierks and Kramer (1989) have shown that the adenine nucleotide carrier is able under certain conditions to form a hydrophillic pore. These coworkers showed that the modification of two SH groups of the adenine nucleotide translocator, reconstituted into liposomes, results in an increase in the permeability of the liposomal membranes.

[1.7] ***Dynamic features of "permeabilization".***.

[1.7.1] *The transitional nature of "permeabilization": There is a dynamic distribution between the open and closed states of the pore.*

Evidence that pores in any single mitochondrion flicker open and closed comes from the observation that at intermediate concentrations of matrix free Ca^{2+}, when permeabilization is far from maximal, labelled sucrose eventually enters the whole

mitochondrial population despite maintainance of the inner-membrane potential at a constant value with time (Al Nasser and Crompton, 1986a). These observations have led to the proposal that the permeable fraction at any point in time represents that fraction in continual steady-state interconversion with the impermeable fraction and that an increase in free [Ca^{2+}] displaces this steady state to the permeable form:

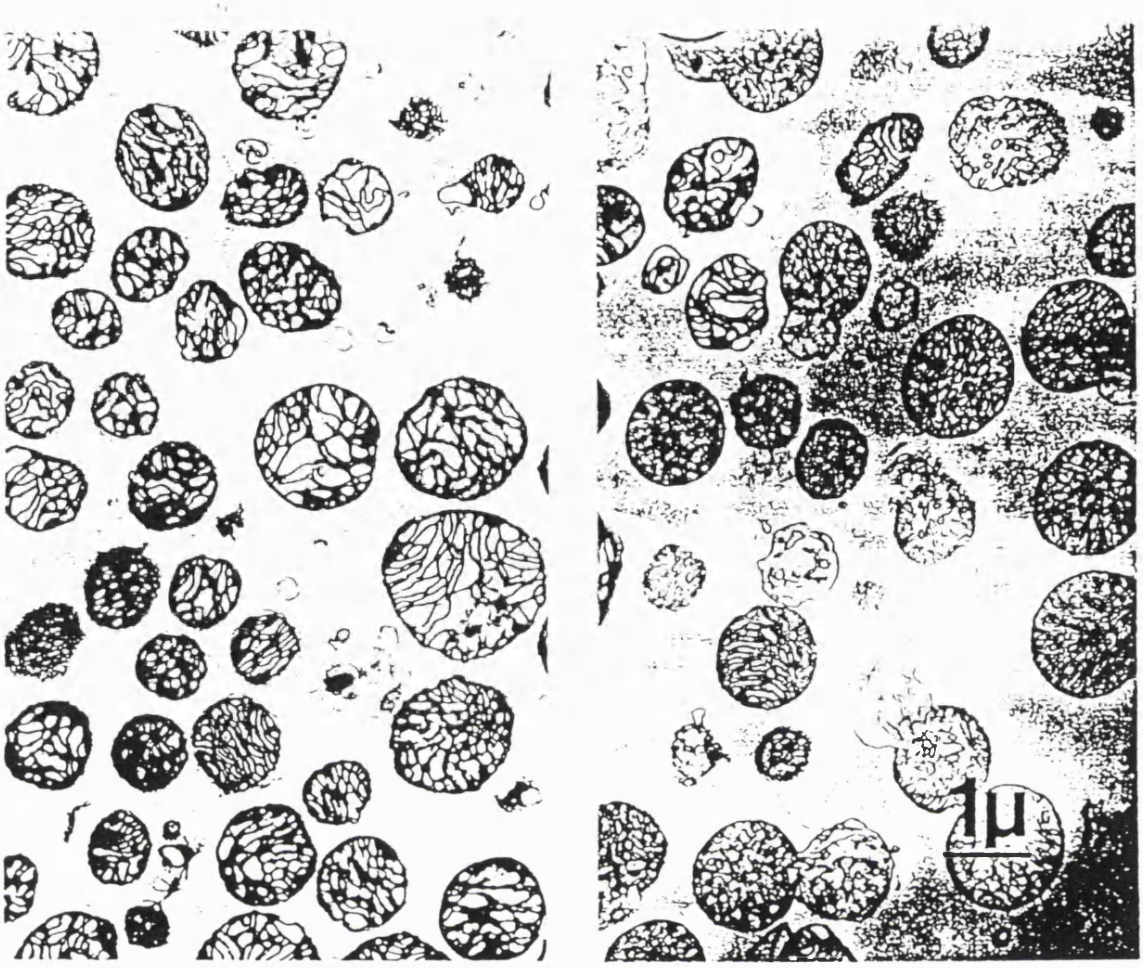
$$\text{Impermeable} \underset{k_r}{\overset{k_p \text{ (increased by } Ca^{2+})}{\rightleftharpoons}} \text{Permeable}$$

In this model, as long as extramitochondrial Ca^{2+} does not rise unduly, pore opening would allow release of accumulated matrix Ca^{2+} facilitating pore closure. Those agents that protect mitochondria against the adverse effects of Ca^{2+} shift the distribution to the left.

Patch clamp studies of the MCC which has been identified with the non-specific Ca^{2+}-activated pore have revealed a flickering current which may indicate rapid opening and closing of channels, but could be due to the superimposition of several channels or alternatively noise due to an unstable seal [Antonenko *et al.*, (1991)].

At higher concentrations of matrix free Ca^{2+}, complete "permeabilization" proceeds across the mitochondrial population exponentially with time. Solute entrapment studies indicate that at any point in time, that fraction of the mitochondrial population which is permeable to sucrose is maximally permeable to sucrose. Thus, the same degree of sucrose entry into the population occurred irrespective of whether [^{14}C]-sucrose was present throughout "permeabilization" or added 4s before resealing mitochondria by chelation of Ca^{2+} with EGTA [Al Nasser and Crompton (1988)]. This indicates that as each mitochondrion became permeable it quickly attained a high degree of permeability and that pores in any single mitochondrion opened with some degree of synchrony, a feature that has its counterpart in resealing.

By monitoring the configurational status of mitochondria during "permeabilization" using electron microscopy it has been shown that this sudden increase in permeability is associated with a conformational change from aggregated to orthodox as shown in Fig. 1.2 [Haworth and Hunter, (1979)].



A. Aggregated B. Orthodox

Fig. 1.2

Electron micrograph of mitochondria showing the Ca^{2+}*-induced configurational transition.* Mitochondria fixed 15s (A) or 7min (B) after addition of $CaCl_2$. As can be seen, the aggregated conformation is characterised by a shrunken darkly staining matrix space and a large intracristal space. The orthodox configuration is characterised by an expanded matrix space which stains much less intensely (from Hunter *et al.*, (1976).

Thus, a time dependent decrease in light scattering exactly parallels the Ca^{2+} efflux which occurs as a result of permeabilization. The orthodox configuration can be induced by suspending the mitochondria in hypotonic medium, but is only associated with "permeabilization" when accompanied by Ca^{2+} accumulation. The aggregated conformation (Fig. 1.2A) is characterized by a shrunken, darkly staining matrix space and a large intracristal space. The orthodox configuration (Fig. 1.2B) is characterized by an expanded matrix space which stains much less intensely.

[1.7.2] ***Reversibility of "permeabilization"/stimulation of pore closure.***

As "permeabilization" requires the synergistic action of Ca^{2+} and some other agent chelation of Ca^{2+} alone with EGTA suffices to induce pore closure [Al Nasser and Crompton (1986a)]. The reversibility of the process has allowed the entrapment of markers of matrix activity i.e. NAD which restores the capacity (lost on "permeabilization") to oxidise NAD linked substrates; and the Ca^{2+} indicator arsenazo III, the absorbance of which is then governed entirely by factors that determine matrix Ca^{2+}, i.e. $\Delta\Psi$ (Ca^{2+} influx), ruthenium red (inhibition of Ca^{2+} influx), Na^{+} (Ca^{2+} efflux), and may be used to monitor the Ca^{2+} fluxes into and out of the matrix [Al Nasser and Crompton (1986b); Hayat and Crompton (1987)]. A kinetic analysis of EGTA-induced pore closure reveals that pores in any single mitochondria close simultaneously while the mitochondrial population reseals exponentially with time [Crompton and Costi (1988; 1990)]. Crompton has developed a rapid pulsed flow solute entrapment technique for investigating the kinetics of resealing/pore closure using low MW radiolabelled solutes such as ^{14}C-sucrose and ^{14}C-mannitol. This technique has allowed the rate of resealing and the rate of permeation of solute through open pores during resealing to be calculated. Resealing occurs relatively quickly even in the absence of added nucleotides ($t_{1/2}$=8s) and the maximal rate of resealing ($t_{1/2}$=1s) occurs in the presence of ADP ($K_m \approx$ 30μM). ADP is not an absolute requirement for resealing, as if trace amounts of ADP are removed (prior to resealing) by the addition of Mg^{2+}, pyruvate kinase and phospho*enol*pyruvate the rate of EGTA-induced pore closure is unaffected [Crompton and Costi (1988)]. Paradoxically, in addition to stimulating the rate of resealing, 200μM ADP increased the permeability of unresealed mitochondria during resealing [Crompton and Costi (1990)]. Further investigation revealed that the relative permeabilities to solutes of different sizes were stimulated equally, indicating an increase in open pore number, rather than an increase in pore dimensions. In

these experiments ADP had no effect on mitochondrial permeability in the absence of EGTA. It was found that the Ca^{2+} ionophore A23187 also stimulated the rate of EGTA induced resealing, indicating that the rate of removal of matrix free Ca^{2+} is limiting for pore closure. It is suggested that by increasing the number of open pores ADP would actually facilitate EGTA entry, Ca^{2+} removal and pore closure. The proposed model of pore closure when EGTA is added to a population of "permeabilized" mitochondria is illustrated schematically below:

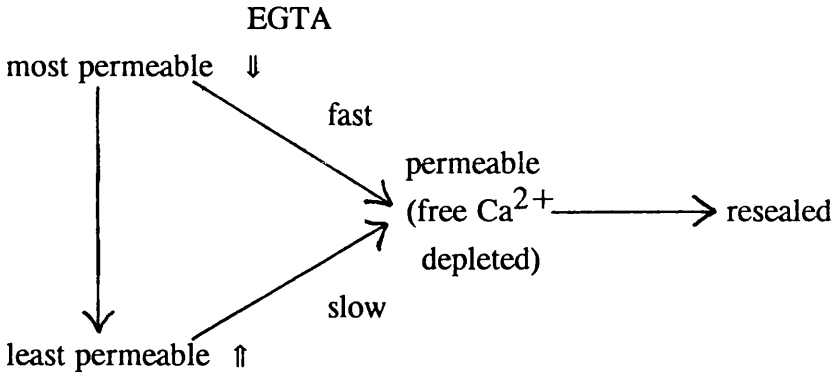


Fig. 1.3 EGTA-induced pore closure. The proposed model of pore closure when EGTA is added to a population of permeabilized mitochondria containing Ca^{2+}, (from Mc Guinness *et al.*, (1990)).

However, the work of Haworth and Hunter (1980) indicates that if ADP is given time to equilibrate with the matrix space ~ 20μM ADP is sufficient to reseal mitochondria. It was suggested that ADP competes with Ca^{2+} for some internal site. NADH is also effective although 200 times less effective than ADP. ADP and NADH act synergistically to inhibit "permeabilization" being effective at much lower concentrations than either are alone [Haworth and Hunter (1980)]. In this instance pore activity was assayed by monitoring the shrinkage induced on addition of impermeable polyethylene glycol to permeabilized mitochondria by following changes in light scattering. These results are ambiguous, as it has been shown that ADP alone causes mitochondria to contract [Packer *et al.*, (1966)] in the absence of any changes in permeability. Clearly the effects of ADP are not fully understood and further work is necessary in order to clarify its role.

[1.8] ***Roles of the pore.***

[1.8.1] *Possible physiological roles of the pore.*

In all tissues examined mitochondrial Ca^{2+} efflux occurs either in exchange for Na^+ or via a Na^+ independent route whose mechanism is not fully understood. Of the two, the latter is the main route for efflux in liver. The mechanism of efflux is not understood. Clearly the Ca^{2+}-induced increase in IMM permeability does not reflect the operation of a physiological Ca^{2+} release mechanism as it is unlikely that such a non-specfic pore would have evolved to mediate Ca^{2+} release. Indeed, Al Nasser and Crompton have resolved this issue and shown that there is a Na^+-independent Ca^{2+} release carrier distinct from Ca^{2+} release due to the non-specific pore [Al Nasser and Crompton (1986b)]. In addition, unlike passive Ca^{2+} release via the non-specific Ca^{2+} activated pore, the more specific Na^+-independent Ca^{2+} release route is promoted by increased $\Delta\Psi$ indicating active Ca^{2+} transport [Bernardi, P and Azzone, G.F. (1983)].

Indeed it is not clear that the pore has a physiological role as it is activated under pathological conditions. It could concievably be involved in protein import. Another alternative, is that it is involved in intermitochondrial communication. It has been shown that groups of mitochondria in human fibroblasts and rat cardiomyocytes can behave as united electrical systems (Amchenkova *et al.*, (1988)]. Intermitochondrial junctions allow $\Delta\Psi$ to be transmitted from one mitochondrion to another. Finally, one might postulate that as in thermogenic brown adipose tissue, the pore acts as an uncoupler stimulating respiration to meet increased energy demands.

[1.8.2] *Consequences of pore opening under pathological conditions.*

The group of Crompton *et al.*, (1988) were the first to point out that the conditions which prevail in the cell on reperfusion after an ischaemic period might activate the non-specific pore and that pore opening under these conditions would have drastic consequences. Exposure of the F_1 ATPase to the cytoplasm would result in reverse hydrolysis of ATP. The resulting rapid depletion of ATP would further compromise the cells ability to maintain Ca^{2+} homeostasis with cell death ensuing. It has often been suggested that high [Ca^{2+}] may be detrimental to mitochondrial energy transduction in those forms of injury associated with excess cellular Ca^{2+}, and that mitochondrial

dysfunction might contribute to the progression of the injury [Lochner *et al.*, (1975); Henry *et al.*, (1977); Chien *et al.*, (1977); Peng *et al.*, (1980); see also review by Crompton (1988)]. If, as is suspected activation of the non-specific pore contributes to mitochondrial dysfunction during tissue anoxia/reoxygenation, the discovery that CsA potently inhibits the pore should help elucidate one molecular component of the pore and pin-point a site for pharmacological intervention.

[1.9] *Cyclosporin A (CsA)*.

[1.9.1] *CsA inhibits Ca^{2+} associated intracellular signalling events.*

CsA, a cyclic undecapeptide was originally isolated from the fungus *Tolypocladium inflatum*. [Borel (1986); Stähelin, (1986)]. It is a highly lipophilic molecule and its structure is shown in Fig. 1.4. Initially found to possess weak antifungal properties it has since been widely used as an immunosuppressive agent to prevent rejection following organ transplantation [Kahan, (1989)]. The immunosupressive effects of CsA are considered to result primarily from an interference with normal T-cell function [Herold *et al.*, (1986); Havele *et al.*, (1988)]. CsA inhibits these responses with an ED_{50} of 10-100nM. The molecular mechanism of action of CsA is thought to be the blocking of production of interleukin-2, which is required for the differentiation and proliferation of cytolytic T-cells [Hess *et al.*, (1983); Kronke *et al.*, (1984). T-cell activation requires increased Ca^{2+} and there is evidence that CsA may inhibit Ca^{2+}-dependent events that follow this increase (Metcalfe, 1984). More specifically, cyclosporin may prevent the Ca^{2+}-dependent translocation of a cytoplasmic subunit of NF-AT (nuclear factor from activated T-cells), to the nucleus of T-cells [Flanagan *et al.*, (1991)]. NF-AT binds to a regulatory region of interleukin-2 stimulating its transcription. In addition to effects on lymphokine gene expression, CsA can interfere with Ca^{2+}-dependent exocytotic events in lymphokine cells and in many cells of the hematopoietic lineage [Lancki *et al.*, (1989); Trenn *et al.*, (1989); Marone *et al.*, (1988)]. CsA has also been shown to prevent Ca^{2+}-dependent programmed cell death of T-cells [Mercep *et al.*, (1989); Shi *et al.*, (1989)]. It is clear that CsA interferes with a set of Ca^{2+}-associated signal transduction events.

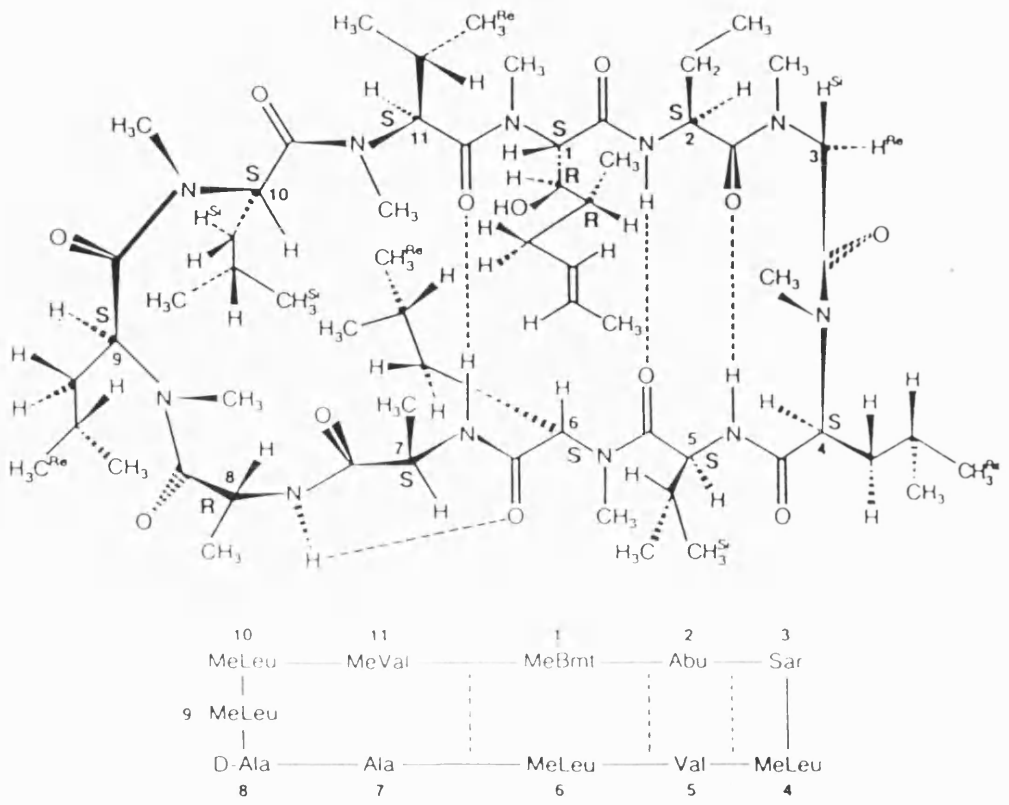


Fig. 1.4

Schematic representation of the structure and conformation of CsA in the solid state (from Wenger, R, (1986)).

[1.9.2] *CsA binds to and potently inhibits the activity of cyclophilin, a peptidyl prolyl* cis-trans-*isomerase.*

The major intracellular receptor of CsA is cyclophilin, an 18,000 Da cytosolic protein originally isolated from bovine thymus [Handschumacher *et al.*, (1984)]. It constitutes 0.1-0.4% of total cytosolic protein. Cyclophilin is responsible for the concentration of CsA by lymphoid and nonlymphoid mammalian cells [Merker *et al.*, (1983); Merker and Handschumacher, (1984)]. The K_d for the interaction of cyclosporin A with cyclophilin has varied between 0.2 and $20x10^{-8}$ [Handschumacher *et al.*, (1984); Harrison and Stein (1990)]. The cyclophilin gene has been cloned from a diverse group of organisms and there is surprising sequence homology across species [Haendler *et al.*, (1987); Haendler *et al.*, (1989); Tropschug *et al.*, (1988)].

Although the majority of cyclophilin is located in the cytosol in *Neurospora Crassa* cyclophilin has been shown to have a dual localisation in the cytosol and in the mitochondrial matrix [Tropschug *et al.*, (1988)]. One gene codes for both the cytosolic and mitochondrial forms. The mitochondrial cyclophilin is translated in the cytosol as a precursor having an amino terminal presequence of 44 amino acids that is cleaved in two steps upon entry into the mitochondrial matrix. A novel 20,000 Da protein which shares 64% sequence homology with cyclophilin and which is exclusively located in the endoplasmic reticulum has recently been identified [Hasel *et al.*, (1991)].

Cyclophilin is in fact identical to peptidyl-prolyl *cis-trans* isomerase (PPIase) [Takahaishi, *et al.* (1989), Fischer *et al.*, (1989)]. PPIase catalyses the slow *cis-trans* isomerization by Xaa-proline peptide bonds in short synthetic peptides. The reaction is shown in Fig. 1.5. The mechanism of this reaction is thought to involve catalysis by distortion in which PPIase binds and stabilises a transition state that is characterised by partial rotation about the C-N bond [Harrison and Stein, (1990)]. The enzyme may be involved in the correct folding of proteins [Fischer, G. and Bang, H. (1985); Bachinger, (1987)]. In this context it is worth considering a peculiar member of the cyclophilin family, namely the product of the *nina*A gene in Drosophila [Stamnes *et al.*, (1991)]. This membrane bound protein is essential for proper phototransduction. More specifically, it is thought to be involved in the

proper synthesis, folding or transport of rhodopsin by isomerization about an Xaa-Pro bond.

CsA abolishes the activity of PPIase by binding to the active site [Fischer and Schmid, (1990)]. Indeed CsA may serve as a transition state analog of a bound peptide substrate as it has been demonstrated that the 9,10 peptide bond of CsA is in the *trans* conformation when bound to cyclophilin, in contrast to the *cis* 9,10 peptide bond found in the crystalline and solution conformations of CsA [Fesik *et al.*, (1990). The fact that while certain analogs of CsA are potent inhibitors of cyclophilin's PPIase activity but have no immunosuppressive activity [Sigal et al., (1991)] indicates that inhibition of this activity alone is not sufficient to cause immunosuppression.

There is mounting evidence that it is actually the CsA/cyclophilin complex which mediates CsA's immunosuppressive effects. It is envisaged that cyclosporine A acts as an allosteric effector of cyclophilin the complex then modulating the activity of other enzymes. The complex of CsA-cyclophilin has been shown to interact with calcineurin, a calcium-calmodulin dependent protein phosphatase [Friedman and Weissman, (1991); Liu et al., (1991)]. Neither the drug nor the receptor alone can bind to calcineurin, but the drug receptor complex binds to and modulates the activity of the phosphatase. CsA causes growth inhibition of *Neurospora crassa* and *Saccharomyces cerevisiae*. In cyclosporin A resistant mutants of these organisms, cyclophilin, is either lost, or has lost its ability to bind to cyclosporin A [Tropschug et al., (1988)]. Absence of the cyclophilin gene, however, is not detrimental to the organism's viability. This again indicates that drug and receptor act as a complex to mediate growth inhibition.

[1.9.3] *CsA binds to the Ca^{2+} binding protein calmodulin.*

CsA has also been shown to bind to calmodulin [Colombani *et al.*, (1985)]. This interaction involves the binding of CsA to the hydrophobic pocket of calmodulin, a property it shares with other calmodulin antagonists [Le Grue *et al.*, (1986)].

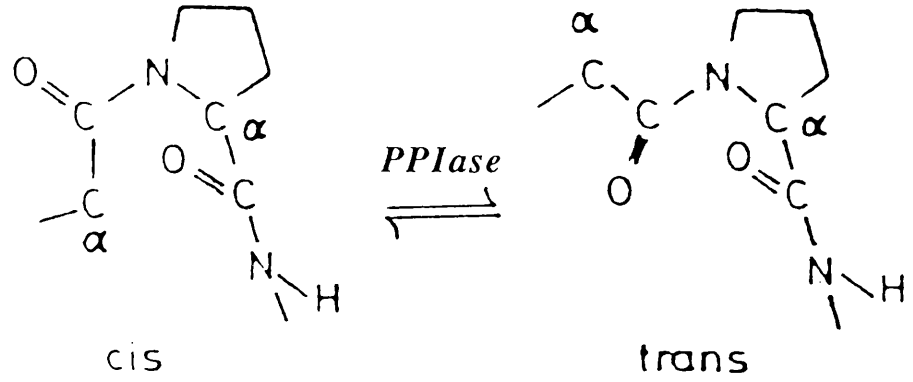


Fig. 1.5

Diagram showing the rotation about the CN bond in adjacent peptidyl-proline residues of a protein catalysed by peptidyl-prolyl-cis-trans-isomerase (PPIase; from Fischer *et al.*, (1984).

[1.10] ***Principal aims of this investigation.***

(1.) To establish conditions which minimise Ca^{2+}-induced phospholipase A_2 activity and thus exclude increases in permeability due to membrane degradation creating non-specific leakiness. This should provide optimal conditions for studying pore behaviour without interference from other factors.

(2.) To determine the nature and number of CsA binding components in mitochondria. It will be necessary to establish the location of the CsA binding component(s) and to discover whether or not it is a mitochondrial isoform of cyclophilin as found in *Neurospora Crassa* [Tropschug *et al.*, (1988)]. If this is the case, is this isoform membrane bound like the novel protein product of the *nina*A gene in *Drosophila Melanogaster* Stamnes *et al.*, (1990)] or is it soluble like the yeast mitochondrial isoform. Alternatively, are CsA's effects on "permeabilization" mediated by a completely different protein from that known to mediate its immunosuppressive effects.

(3.) To try and reconstitute pore activity in sub-mitochondrial particles (inside-out membranes), by selectively adding back agents thought to be involved in "permeabilization" - Ca^{2+}, Pi, NAD, NADP, GSSG -at what would normally be the inner face of the inner membrane.

(4.) Finally, to use this system to confirm which CsA binding component is involved in its inhibition of "permeabilization".

Reconstitution of pore activity will help establish which parameters are important in its control. An elucidation of the individual components involved will clarify how they might contribute to mitochondrial dysfunction on reperfusion after an ischaemic period and reveal potential targets for pharmacological intervention. CsA should facilitate the isolation of one component of the system.

CHAPTER 2: METHODS AND MATERIALS.

[2.1] *Mitochondrial Preparation:*

Mitochondria were isolated from the livers of male Sprague-Dawley rats (180-280g body weight) using a standard method as follows. The rats were killed by cervical dislocation and their livers were removed to an ice-cold solution containing 210mM mannitol, 70 mM sucrose, 10mM HCl (Tris salt), 1mM EGTA and BSA (60mg/100ml) at pH 7.2. The tissue was then chopped finely into small pieces, transferred to 30ml centrifuge tubes (2 tubes per liver) and homogenised for $\simeq$5s using a Polytron homogeniser with the rheostat at position 4. The Polytron probe was precooled to 4°C before use. The resulting suspensions were spun at 2250rpm for 7min in a Sorvall RC5B Refrigerated Superspeed Centrifuge (rotor; SS34) at 4°C to sediment cell debris. The supernatants were then decanted into new 30ml centrifuge tubes and spun at 8000rpm for 8min to pellet the mitochondria. To wash the mitochondria the pellets were initially resuspended in a small volume of 210mM mannitol, 70mM sucrose, 10mM tris-HCl at pH 7.2 (MST) using a glass test tube filled with crushed ice (a "cold-finger"). The volumes were then made up to 30ml with ice-cold MST and the mitochondria pelleted as before by centrifuging at 8,000rpm for 8min. The mitochondria were washed once more in MST and were finally resuspended in MST at a concentration of 40-50mg of mitochondrial protein/ml. Throughout the preparation the mitochondria were kept on ice and all centrifugations were carried out at 4°C.

[2.2] *Quantitation of mitochondrial protein:*

Mitochondrial protein was routinely estimated by a modified biuret method [Kroger, [redacted] and Klingenberg, [redacted] (1966)] against Bovine Serum Albumin as a standard. 50μl of the final mitochondrial suspension (*test*) or 50μl H_2O (*blank*) was mixed with 450μl of H_2O, 200μl of sodium cholate (4%) and 2mlNaOH (10%) in a test tube. The suspension was mixed well. 300μl of $CuSO_4$ (1%) was added and the suspension was again mixed well. After 10min the absorbance of the *test* was read against the *blank* at 540nm using a Perkin Elmer Spectrophotometer.

[2.3] *Preparation of sub-mitochondrial particles*:

[2.3.1] *Preparation of sub-mitochondrial particles for the cyclosporine-A binding studies:*

Rat liver mitochondria were prepared as described in section [2.1] The final pellet ($\simeq$ 120mg of mitochondrial protein) was resuspended in 8ml of 5mM Pi (Tris salt) at pH 7.0. Aliquots of the above suspension were sonicated (MSE Soniprep F150 sonifier) in 6ml glass tubes, for a total of 2min (10s bursts with an interval of 20s), at 10-14 amp. Remaining mitochondria were sedimented by spinning at 10,000g in an Eppendorf bench centrifuge for 2min. The supernatant was spun in a Beckman ultracentrifuge (rotor; 70.1 Ti) at 40,000g_{av} for 50min. The supernatant (soluble matrix fraction) was removed into a separate test tube and the sub-mitochondrial particles were resuspended in 5mM Pi with the aid of a Potter-Elvehjem glass-Teflon homogeniser.

[2.3.2] *Preparation of sub-mitochondrial particles competent for energy transduction:*

Rat liver mitochondria were prepared as described in section [2.1] with the following alterations. After discarding cell debris the supernatant was centrifuged at 9,000rpm for 9min. The pellets were resuspended in MST using a "cold-finger" as before and then washed four more times by spinning at sequentially lower speeds i.e. 8,750, 8,250, 7,750 and 7,250rpm for 9min. This method [Blume, (1979)] removes lysosomes and adsorbed microsomes (after each wash an increase in activity of the microsomal marker, glucose-6-phosphatase and the lysosomal marker acid phosphatase was detected in the supernatant). Without the introduction of this step it was impossible to get coupled particles competent for energy transduction. It has been shown that the free fatty acid content of rat liver microsomes (with myristate being the most potent), uncouples mitochondria and stimulates mitochondrial ATPase [Pressman and Lardy, (1956)]. This may be the explanation for the lack of demonstrable coupling of particles seen before removal of adsorbed microsomes. The final mitochondrial pellet ($\simeq$ 120mg of mitochondrial protein) was resuspended in 8ml of *sonication buffer* (see below). Aliquots of the above suspension were sonicated (MSE Soniprep F150 sonifier) in 6ml glass tubes, for a total of 2min (10s bursts with an interval of 20s), at 10-14 amp. Remaining mitochondria were sedimented by spinning at 10,000g in an Eppendorf bench centrifuge for 2min. The supernatant was spun in a Beckman ultracentrifuge (rotor; 70.1 Ti) at 100,000g_{av} for 50min. The supernatant (soluble matrix fraction) was removed into a

separate test tube and the sub-mitochondrial particles were resuspended in *resuspension buffer* (see below) with the aid of a Potter-Elvehjem glass-Teflon homogeniser. When the method of Schatz and Racker (1966) was employed, the *sonication buffer* was 10mM H_2SO_4 (Tris salt), pH 7.4 and the *resuspension* buffer was 2mM Hepes (Tris salt), pH 7.8. When the method of Azzi *et al.*, (1971) was employed the *sonication buffer* was 2mM EDTA (Tris salt), pH 8.4 and the *resuspension buffer* was 10mM HCl (Tris salt), pH 7.4. The relative merits of the different preparations are discussed in Chapter 5. In the final method chosen for routine preparation the final suspension of rat liver mitochondria was sonicated in 2mM EDTA (Tris salt) pH 8.4 and the final sub-mitochondrial pellet was resuspended in 10mM HCl (Tris salt) pH 7.4 containing 250mM sucrose [Azzi *et al.*, (1971)].

[2.4] *Ca^{2+} Buffers:*

[2.4.1] *Preparation of Ca^{2+} Buffers:*

In order to buffer Ca^{2+} in the 1-10μM range, a series of 20mM HEDTA/ 20mM EGTA/ Ca^{2+}/Mg^{2+} buffers were made up. These buffers contained 20mM HEDTA, 20mM EGTA, 40mM $MgCl_2$ and sufficient $CaCl_2$ to provide a range of buffers giving finally 0.8μM, 2.5μM and 5.6μM free Ca^{2+} when diluted 1:20. The amount of Ca^{2+} required was determined by titrating small aliquots of a 1mM stock solution of $CaCl_2$ into a 1:20 dilution of the stock buffer containing HEDTA,EGTA and $MgCl_2$ at the concentrations indicated above in a final volume of 3ml. During this procedure the free [Ca^{2+}] established by the buffers was measured as described below (section 2.4.2). The final free Mg^{2+} established by each buffer was 1.2mM and was determined as described in section 2.4.3.

[2.4.2] *Measurement of [Ca^{2+}] established by buffers:*

The [Ca^{2+}] established by each 1mM HEDTA/ 1mM EGTA/ Ca^{2+}/Mg^{2+} buffer was measured using a Philips 1556 Ca^{2+}-selective electrode assembled as shown in Fig. 2.1. With this commercial electrode Ca^{2+}-selectivity is achieved by incorporating a Ca^{2+} ionophore within the PVC membrane. The output from the Ca^{2+} electrode was processed through a PW9409 digital pH meter and displayed on a Bryans 2800 chart recorder. The electrode was calibrated by measuring the shift E

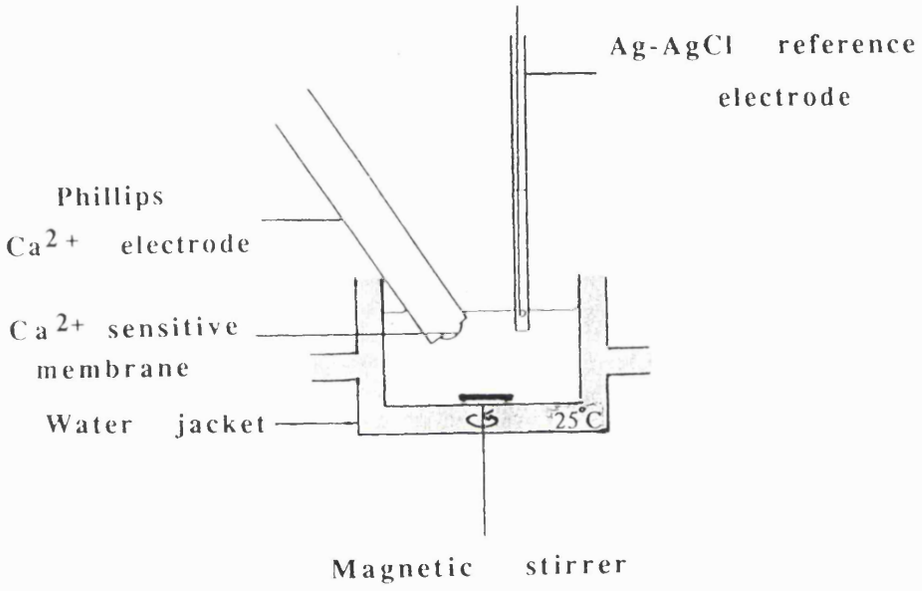


Fig. 2.1

Ca^{2+}-electrode.

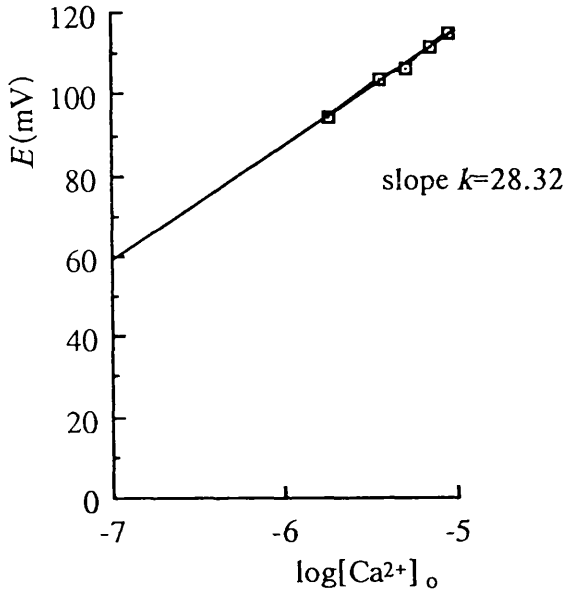


Fig. 2.2

Calibration curve showing [Ca^{2+}] versus the shift (mV).

in mV caused by the addition of Ca^{2+} in the 20μM-640μM range and then extrapolating back to the mV range required. This range is chosen because in the μM range of the buffers the response of the electrode is non-linear due to interference by other cations. From the Nernst equation the electromotive force:

$$E=2.3RT/nF.\log[Ca^{2+}]_o/[Ca^{2+}]_i$$

where R,T and F have their usual thermodynamic significance, $[Ca^{2+}]_o$ is the concentration of Ca^{2+} in the standard incubation ; and $[Ca^{2+}]_i$ is the concentration of Ca^{2+} in the Ca^{2+}-electrode which is constant. Thus the above equation can be written:

$$E=2.3RT/2F.\{\log[Ca^{2+}]_o-\log[Ca^{2+}]_i\}$$

$$=29 \times \log[Ca^{2+}]_o - C$$

where C is a constant. Therefore at 37°C a graph of E against $\log[Ca^{2+}]_o$ should have a slope of 29. The electrode was calibrated by adding sequentially 60μl aliquots of a stock solution of $CaCl_2$ (1mM) to 3ml of a standard medium containing 120mM KCl and 10mM Hepes (K^+ salt), pH 7.0. The calibration curve is shown in Fig. 2.2. As predicted by the above equation the slope of the calibration curve was 28.32 ($\simeq$ 29) at 37°C.

This enabled the free $[Ca^{2+}]$ established by the buffers to be measured.

[2.4.3] *Measurement of the final $[Mg^{2+}]$ attained by Ca^{2+} buffers:*

The final free $[Mg^{2+}]$ established by these buffers was measured using the metallochromic indicator Eriochrome Blue SE in conjunction with dual-wavelength spectroscopy as described by Scarpa *et al.* (1974). Metallochromes are small molecules whose absorption spectra changes on binding metal ions. The chemical structure of Eriochrome Blue is shown in Fig. 2.3(A). As shown in Fig. 2.3(B) the difference spectrum of Eriochrome Blue versus Eriochrome Blue plus Mg^{2+} exhibits a positive absorption maximum at 551nm and a negative absorption maximum at 572nm, with an isobestic point at 563nm. Ca^{2+} in contrast produces a much broader absorbance from 500-650nm. This allows for a selection of wavelength pairs (i.e.

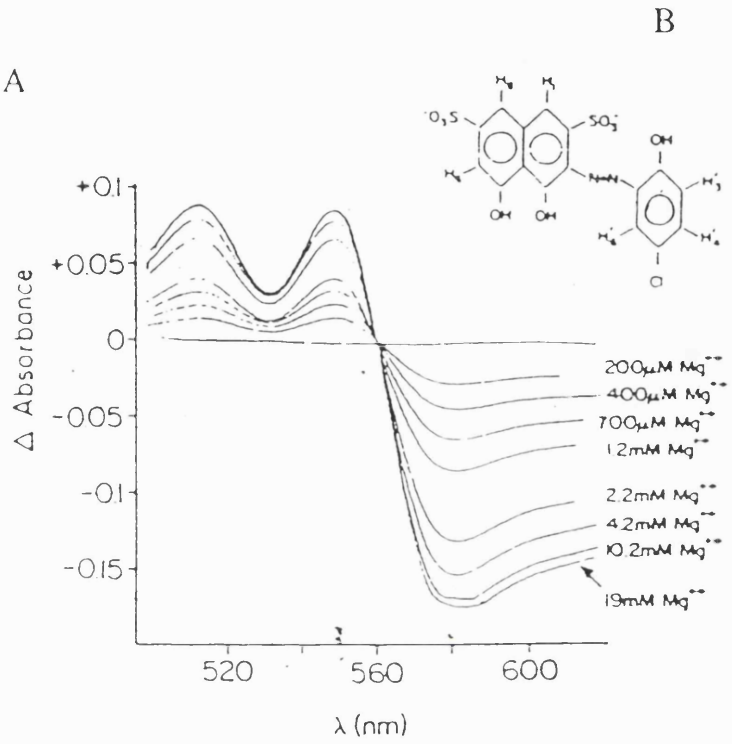


Fig. 2.3

Difference spectra of Eriochrome Blue SE versus Eriochrome Blue SE plus Mg^{2+}. (A.) Structure of Eriochrome Blue SE(from Scarpa, (1974)) (B)

592-554nm) at which [Mg^{2+}] can be measured without interference by Ca^{2+}. Eriochrome Blue also has the advantage that its complex with Mg^{2+} has a high dissociation constant (k_D=1.6mM at pH 7.1). There is thus minimum perturbation of the free [Mg^{2+}] in the dye by the buffer.

A calibration curve was constructed by measuring the increase in absorbence on the dual wavelength spectrophotometer (Perkin Elmer Model 356) using the wavelength pair; λ_1=592nm, λ_2=554nm over a range of Mg^{2+} concentrations. The final [Mg^{2+}] was increased in 0.5mM increments by adding sequentially 17μl aliquots of $MgCl_2$ (100mM) to 3.4ml of standard incubation medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K^+ salt) and 30μM Eriochrome Blue in a 4ml cuvette. The calibration curve is shown in Fig. 2.4.

This enabled the final free [Mg^{2+}] established by the Ca^{2+} buffers to be estimated. 170μl of each buffer was added to 3.4ml of standard incubation medium (i.e. a 1:20 dilution) in a 4ml cuvette. 30 μM Eriochrome Blue was added as indicator and the increase in absorbance measured as above. In all instances, the final free [Mg^{2+}] established by the buffers was 1.2mM which is close to the physiological [Mg^{2+}] found in the cytosol.

In some experiments, an ATP regenerating system, comprising 40mM creatine phosphate, creatine phosphokinase (20 units), 2.5mM KH_2PO_4 and ATP (variable concentrations) was added to the incubation. Since creatine phosphate, creatine phosphokinase and ATP all bind Mg^{2+}, it was important to determine how much Mg^{2+} to add to reestablish a free concentration of 1.2mM in these experiments. To do this, 170μl of the EGTA, HEDTA Ca^{2+}/Mg^{2+} buffer yielding 1.2mM free Mg^{2+} and 5.6μM free Ca^{2+} was added to standard medium (pH7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), together with 40mM creatine phosphate, creatine phosphokinase (20 units), 2.5mM KH_2PO_4 and ATP (variable concentrations) in a 4ml cuvette. 30 μM Eriochrome Blue was added as indicator and the increase in absorbance measured as above. From the calibration curve, the extent to which the free Mg^{2+} was reduced under these conditions was established. The incubation mixture was then titrated with aliquots of 100mM $MgCl_2$ to restore the free Mg^{2+} to 1.2mM.

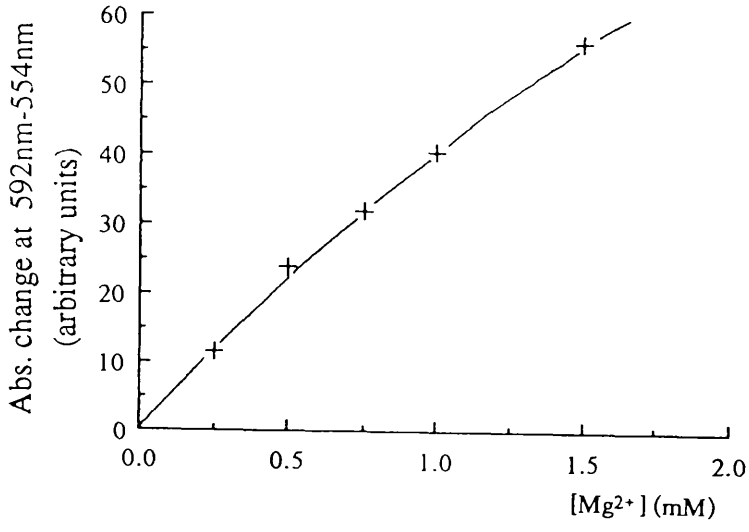


Fig. 2.4

Changes of Eriochrome SE absorbance versus $[Mg^{2+}]$.

[2.5] ***ΔΨ Measurements:***

Pore state in mitochondria and sub-mitochondrial particles was assessed by monitoring the membrane potential as pore opening allows equilibration of protons across the mitochondrial membrane causing a dissipation of $\Delta\Psi$. Early studies by Haworth and Hunter (1976) indicated that $\Delta\Psi$ dependent parameters are closely coupled to non-specific inner membrane permeability and can be used as an assay of it. The membrane potential was monitored: by the use of ion selective electrodes; and by the use of potential sensitive dyes as optical probes in conjunction with fluorescence spectroscopy.

[2.5.1] *ΔΨ measurements using ion selective electrodes:*

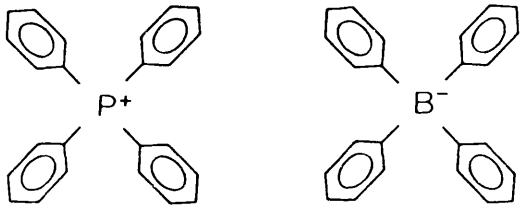
There are a number of cations and anions with delocalised charge due to the possession of π-orbital systems with lipid solubility. Energized mitochondria accumulate lipophilic cations but not anions, while sub-mitochondrial particles with inverted polarity accumulate lipophilic anions but not cations. Two such lipophilic ions, the tetraphenylphosphonium cation (TPP^+) and the tetraphenylboron anion (TPB^-) are shown in Fig. 2.5. These ions accumulate electrophoretically and distribute across the membrane according to the Nernst relation.

$$\Delta\Psi = 2.3RT/zF.\log[C]_i/[C]_o$$

where R,T and F have their usual thermodynamic significance, z stands for the valency of the lipid soluble ions, $[C]_o$=the concentration of ion in the medium and $[C]_i$=the concentration of the ion inside the mitochondrion or sub-mitochondrial particle. The use of ion selective electrodes allows continuous monitoring of changes in TPP^+ and TPB^- in the medium and has the obvious advantage over other techniques that involve the use of radioactive isotopes, in giving a continuous readout.

[2.5.1.1] *Construction and use of the TPP^+ selective electrode:*

The IMM potential ($\Delta\Psi$) developed in mitochondria on addition of succinate as substrate was estimated from the accumulation of tetraphenylphosphonium cation (TPP^+) measured with a TPP^+ sensitive electrode which was constructed as



A B

Fig. 2.5

The lipophilic cation TPP^+ (A) and the lipophilic anion TPB^+ (B) used to monitor membrane potential.

described by Kamo (1979). This involved the synthesis of a polyvinylchloride-based membrane containing tetraphenylboron anion (TPB^-) as an ion exchanger. To prepare the membrane, 3ml of 10^{-2}M sodium tetraphenylboron in tetrahydrofuran (THF) was added to 10ml of THF solution containing 0.5g of polyvinylchloride (PVC). 1.5ml of dioctylphthalate was added as plasticizer. This solution was poured into a glass petri dish (60cm^2 in area) covered with a beaker and left for $\simeq$24hr to allow the THF to evaporate leaving the membrane. The membrane was transparent and 0.15-0.2mm thick. A disc of membrane 2mm in diameter was cut with a punch iron and placed on a smooth glass surface. 2μl exactly of THF was placed at the centre of the membrane disc. Immediately a piece of PVC tubing 15cm in length (cut from a length of PVC tubing with a 2mm internal diameter was placed over the disc). The THF glues the membrane to the tube forming an airtight seal. This tube was then filled with an internal reference solution containing 5mM TPP^+ and half-saturated KCl (to form a bridge between the reference solution and the Ag-AgCl electrode). The electrode assembly is shown in Fig. 2.6. Both the TPP^+ electrode and the reference electrode were connected to a 610C electrometer (Keithly instruments) and the output was displayed on a Perkin Elmer 56 chart recorder.

The instrument was calibrated by adding sequentially small known amounts of TPP^+ to 1ml of standard medium containing 120mM KCl, 10mM Hepes (K^+ salt) at pH7.0. A typical calibration curve is shown in Fig. 2.7. The shift in potential was recorded on increasing the [TPP^+] from 0-5μM in 1μM increments. This was done by adding sequentially 10μl aliquots of a stock solution of TPP^+ (1mM) to 1ml of medium containing 120mM KCl, 10mM Hepes (K^+ salt), pH7.O.

$\Delta\Psi$ can be calculated from the Nernst relation as described above. In practice, because of its lipophilic nature, TPP^+ dissolves in the inner mitochondrial membrane, leading to an overestimation of the values of $\Delta\Psi$ calculated from the distribution of TPP^+. This was shown by Rottenberg (1984) who compared the values of $\Delta\Psi$ calculated by distribution of lipophilic cations with those calculated by measuring the distribution of ^{86}Rb in the presence of valinomycin. ^{86}Rb is an inorganic cation which does not bind extensively to the inner and outer membrane. Values were thus corrected by assuming that only 1/8 of the TPP^+ accumulated is free in the matrix space, the rest partitioning into the membrane. Strictly this correction is not ideal as the extent of partitioning into the membrane may not be

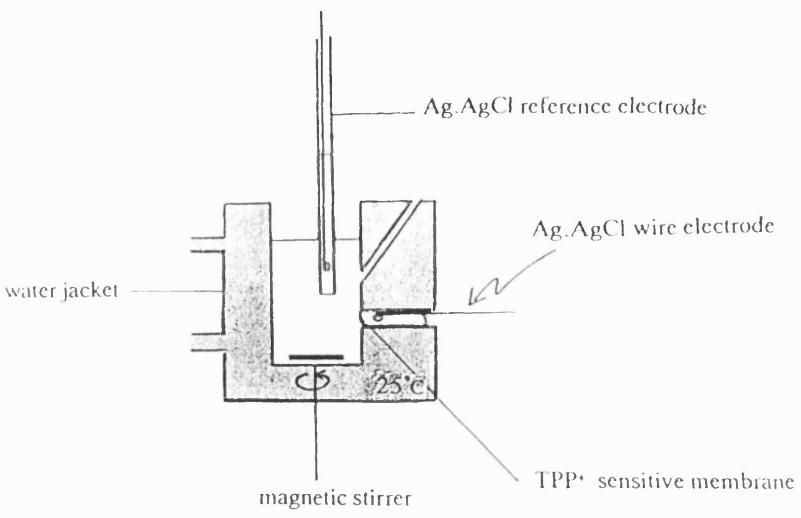


Fig. 2.6

TPP^+ electrode.

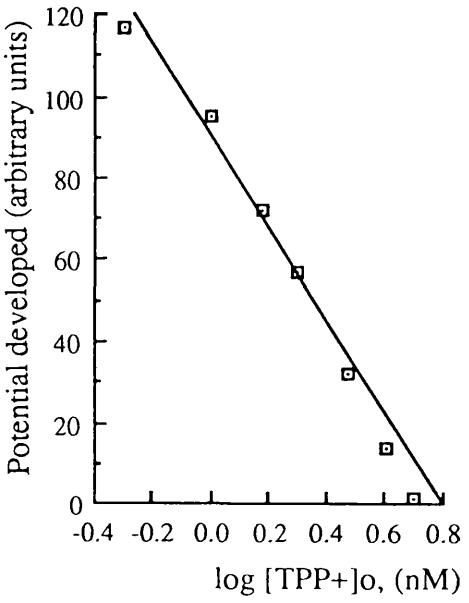


Fig. 2.7

Calibration curve showing the relationship betweem log[TPP^{+}]$_o$ versus the potential developed (arbitrary units).

constant over a range of $\Delta\Psi$ [Rottenberg, (1984)]. However, it gives a good approximation especially at high values of $\Delta\Psi$. Thus using the Nernst relation:

$$\Delta\Psi = 2.3RT/F.\log[TPP^+]_i/[TPP^+]_o$$

Say the potential developed by respiring mitochondria (0.5mg/ml) reduced extramitochondrial [TPP^+] from 5μM to 1.17μM.
Then the $[TPP^+]_i$ is 5-1.17 = 3.83nmol/ 0.5mg mitochondrial protein).
Assuming 1mg of mitochondrial protein contains 1μl of matrix space then 3.83nmol TPP^+/0.5mg is equivalent to 7.66nmol TPP^+/μl or 7.66mM TPP^+. However, we assume that only 1/8 of this value or 0.997mM is free in the matrix space.
Then from the Nernst equation above:

$$\Delta\Psi = 60\log 997/1.17 = 175\text{mV}.$$

At high concentrations lipophilic cations cause $\Delta\Psi$ to collapse [Rottenberg (1984)] and have also been reported to inhibit oxidative phosphorylation [Higuti *et al.* (1979;1980)]. The concentration of TPP^+ was therefore maintained below 5nmol/mg of protein in all experiments as recommended by Rottenberg (1974).

[2.5.1.2] *Construction of the TPB^- selective electrode:*

Initially the IMM potential developed ($\Delta\Psi$) on addition of succinate to sub-mitochondrial particles was estimated from the accumulation of tetraphenylboron ion (TPB^-) measured with a TPB^- selective electrode which was constructed and assembled as described by Shinbo, (1978) except TPB^- was used as internal reference solution instead of DDA^+. As for the TPP+ selective electrode this involved the synthesis of a PVC membrane containing TPB^- as ion exchanger. This initially involved formation of dibenzyldimethyl ammonium-tetraphenylboron salt (DDA-TPB). To do this 0.066g of TPB.Na and DDA.Cl were each dissolved in 19ml of H_2O. The solutions were mixed in a flask forming a white precipitate. 50ml of dichloroethane was added and the flask was shaken vigorously for 24hrs. The DDA-TPB partitions into the organic/dichloroethane phase. 6ml of the dichloroethane phase was evaporated to dryness and the residue was dissolved in 2.32ml of tetrahydrofuran (solution A). 0.31g of PVC was dissolved in 7.75ml of

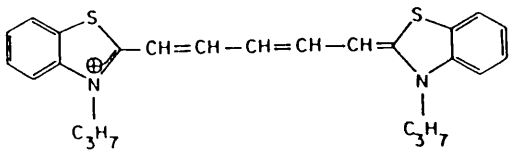
tetrahydrofuran and 1.16ml of dioctylphthalate was added as plasticiser (solution B). Solutions A and B were mixed and poured into a glass petri dish ($60cm^2$), covered with a beaker and left for $\simeq$24 hr to allow the tetrahydrofuran to evaporate leaving the membrane. A disc of membrane was punched out as described above and fused to a short length of PVC tubing. This tube was filled with a reference solution containing 2.5mM TPB and 5% saturated NaCl. The final electrode assembly was identical to that shown for the TPP^+ selective electrode.

[2.5.2] *$\Delta\Psi$ measurements using potential sensitive dyes:*

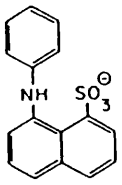
Potential measurements in sub-mitochondrial particles using a TPB^--selective electrode were very insensitive. Thus, these experiments were repeated using potential sensitive dyes as indicators of $\Delta\Psi$. Potential sensitive dyes are molecules whose fluorescence changes on moving from an aqueous to a lipid environment. These dyes fall into a few major categories: catacondensed aromatic sulphonic acids; and polyene dyes of the oxonol, merocyanine and cyanine classes. These dyes are ions at neutral pH. The charge on the dye may be either localised as in the case of sulphonic acids or delocalised as in the case of most of the polyene dyes. The cation 3,3'-dipropylthiodicarbocyanine iodide, (di-S-C_3-5^+) as described by Laris and Hoffman (1974) and the anion, anilinonapthalene sulphonate, (ANS^-) as described by Azzi *et al.*, (1974) were used to monitor $\Delta\Psi$ in sub-mitochondrial particles using fluorescence spectroscopy. The structure of these dyes is shown in Fig. 2.8. For di-S-C_3-5 excitation was at 622nm and emission was at 670nm. For ANS excitation was at 366nm and emission was at 470nm. Incubation conditions together with the concentrations used varied and this information together with the problems these techniques posed are described in more detail in Chapter 5.

[2.6] ***Measurement of passive permeability in sub-mitochondrial particles using the K^+ ionophore valinomycin:***

Pore opening was assessed in deenergized sub-mitochondrial particles by measurement of passive permeability using potassium and valinomycin. Valinomycin is a mobile carrier ionophore which catalyses the electrical uniport of Cs^+, Rb^+, K^+ or NH_4^+. Its structure is shown in Fig. 2.9. It consists of alternating hydroxy- and -amino acids. Addition of potassium and valinomycin to sub-mitochondrial particles induces a transient diffusion potential which rapidly



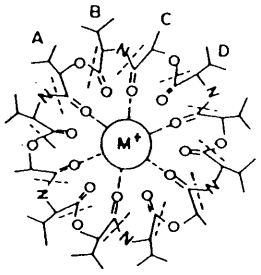
Cyanine, di-C_3-(5)



1-Anilino-8-napthalene sulphonate

Fig. 2.8

Structure of two extrinsic probes used to monitor membrane potential: (A) cyanine di-S-C_3-(5) ; and (B) 1-anilino-8-napthalene sulphonate.

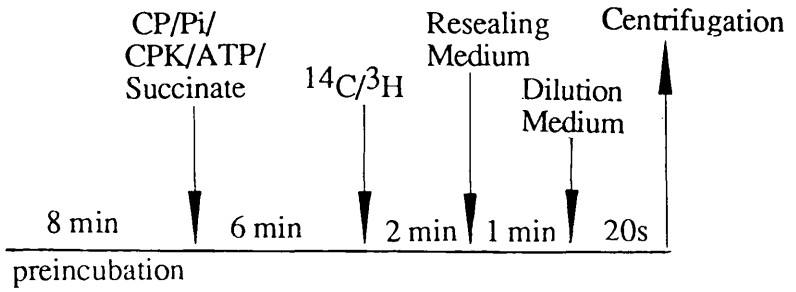


Valinomycin

Fig. 2.9

Valinomycin. The residues between the dashed lines are (A) D-α-hydroxy-isovaleric acid, (B) L-valine, (C) L-lactic acid and (D) D-valine.

A



B

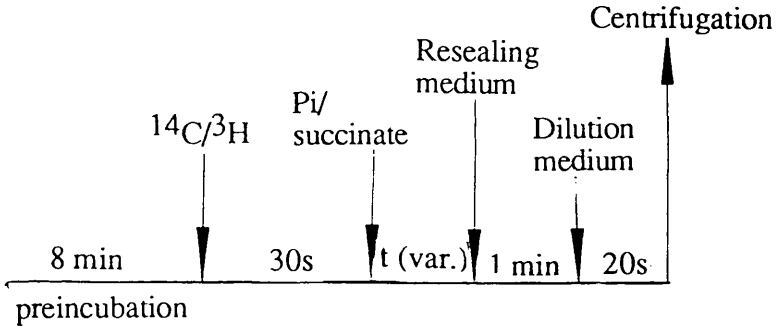


Fig. 2.10

Schematic scheme of the sequence of events in ^{14}C-sucrose entrapment experiments.
(A) Absolute entrapment after 6 min ; (B) rate of "permeabilization" .

decays following redistribution of permeant ions. The rate of decay depends on the integrity of the particles. Thus a faster rate of decay would be indicative of pore opening. The fluorescent indicator ANS was added to monitor the diffusion potential. Experimental conditions were as stated in the figure legends in Chapter V.

[2.7] ***Measurements of rates of oxygen consumption in sub-mitochondrial particles:***

Pore opening uncouples oxidative phosphorylation resulting in a faster rate of oxygen consumption. Rates of oxygen consumption were therefore measured using a Clark-type oxygen electrode. It was used only to measure steady state rates. For more sensitive measurements in sub-mitochondrial particles, two oxygen electrodes were set up in parallel. Identical incubations were set up and additions were made to one incubation. The difference in response of the two electrodes was monitored, amplified and displayed on a Perkin Elmer 56 Chart Recorder.

[2.8] ***Matrix [^{14}C]-Sucrose Entrapment Experiments:***

This is the most direct and unambiguous way of assessing pore opening. It involves monitoring the entry and entrapment of [U-^{14}C]-sucrose in the matrix space of mitochondria after induction of pore opening. [U-^{14}C]-sucrose permeates open pores rapidly ($t_{½}$ = 800ms [Crompton and Costi (1988)]). The procedure exploits the fact that while Ca^{2+} and Pi or a pro-oxidant synergistically act to promote pore opening, removal of Ca^{2+} alone, by chelation with EGTA is sufficient to induce rapid pore closure. Such entrapment studies are superior to other assays of pore opening for the following reasons:

(1.) Although they allow continuous monitoring of mitochondrial state, assays which use $\Delta\Psi$ as an index of permeability give no indication of permeability to species other than H^+.

(2.) Some investigators assess pore opening by following mitochondrial matrix shrinkage and the associated decrease in light scattering. This method gives ambiguous results because matrix expansion and contraction might occur without accompanying changes in permeability. For example, ADP causes mitochondria to contract [Packer *et al.*, (1966)].

(3.) Pore opening is often assessed by measuring mitochondrial Ca^{2+} release. Such measurements can be misleading as unless the uniporter is blocked using ruthenium red, measuring Ca^{2+} release does not distinguish non-specific permeability changes from reverse uniport activity due to the depolarization of the mitochondrial membrane.

[2.8.1] *Experimental Procedure for Matrix [^{14}C]-Sucrose Entrapment Experiments:*

To investigate the effect of free Ca^{2+} on the degree of [U-^{14}C]-sucrose entrapment, mitochondria (10mg protein/ml), were preincubated for 8 min at 25°C in medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), rotenone 1μg/mg protein, 12-13mM sucrose and a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer yielding varying concentrations of free Ca^{2+} and 1.2mM free Mg^{2+} in a final volume of 0.9ml. After preincubation, an ATP regenerating system comprising 0.5mM ATP, 40mM creatine phosphate, 20 units of creatine phosphokinase and 2mM KH_2PO_4 was added, followed immediately by 5mM succinate to initiate respiration. Six min later (the time taken for the complete dissipation of $\Delta\Psi$ under the same experimental conditions) 0.6μCi of [U-^{14}C]-sucrose and 1μCi of [^{3}H]-H_2O were added to the medium. 2 min later any accumulated [U-^{14}C]-sucrose was entrapped in the mitochondrial matrix by adding 87μl of resealing medium containing (100mM EGTA and 1mM ADP). EGTA chelates any Ca^{2+} causing pore closure (Al Nasser and Crompton (1986)). ADP was added to stimulate the process as it was shown by Crompton and Costi (1988) to increase the rate of pore closure eight-fold. 1 min later, sufficient time to allow complete resealing (this was checked as described by Al Nasser and Crompton (1986)), the mitochondrial suspension was diluted five-fold with a dilution medium containing 120mM KCl, 10mM Hepes (K^+ salt) at pH 7.0. 3 x 1ml aliquots of the suspension were transferred to Eppendorf tubes and mitochondria were immediately sedimented by centrifugation in an Eppendorf bench centrifuge for 2min. The procedure is schematically illustrated in Fig. 2.10(A).

To measure the time course of "permeabilization" in the presence and absence of CsA and the effect of varying concentrations of CsA on this time course, the procedure was as follows. Mitochondria (10mg protein/ml) were preincubated for 8 min in medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K+ salt), rotenone (1μg/mg of protein), 12-13mM sucrose, varying concentrations of CsA (where

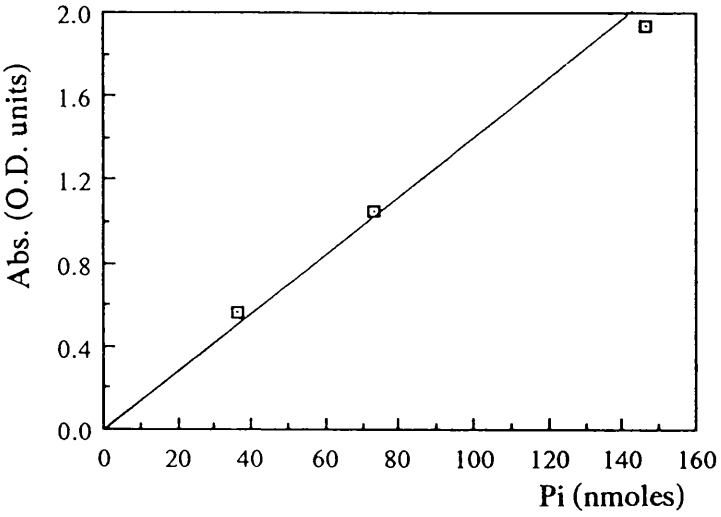


Fig. 2.11

Pi calibration curve for determination of mitochondrial phospholipid content.

added) and a 1mM EGTA, 1mM HEDTA Ca^{2+}/Mg^{2+} buffer yielding 5.6μM free Ca^{2+}. Following preincubation, $^{3}H_20$ (3μCi) and [U-^{14}C]-sucrose (1μCi) were added and 30s later "permeabilization" was initiated by addition of 2mM KH_2PO_4 and 5mM succinate to trigger Ca^{2+} uptake. [U-^{14}C]-sucrose entry into the matrix space was measured at intervals thereafter by transferring 0.3ml portions of the incubate to tubes containing 50mM EGTA and 5mM ADP to induce resealing. As before, after 1min, when resealing was complete, the samples were diluted with a ten-fold volume of dilution medium containing 120mM KCl, 10mM Hepes (K^+ salt), pH7.0. Again, 3 x 1ml aliquots of each suspension were transferred to Eppendorf tubes and mitochondria were immediately sedimented by centrifugation in an Eppendorf bench centrifuge. The procedure is illustrated schematically in Fig. 2.10(B).

In all [U-^{14}C]-sucrose entrapment experiments, after sedimentation, a 10μl aliquot of the supernatant was withdrawn into 4ml of scintillation fluid for counting. The remainder of the supernatant was sucked off, the inside of the tube was carefully dried and the pellet was resuspended in 300mM $HClO_4$ to denature any protein. Denatured protein was sedimented by spinning in an Eppendorf bench cemtrifuge at 10,000g for 2 min. 80μl of the supernatant was then withdrawn into 4ml of scintillation fluid for counting.

[2.8.2] *Rationale of Matrix [^{14}C]-Sucrose Entrapment Procedure :*

The rationale of the procedure is as follows. On "resealing" matrix [U-^{14}C]-sucrose becomes entrapped and is unaffected by subsequent dilution. In contrast, $^{3}H_2O$ is still free to equilibrate across the inner mitochondrial membrane and is diluted five-fold. Thus, the ratio of ^{14}C to ^{3}H in the pellet is much greater than that in the supernatant. From:

$$(^{14}C\text{-}^{3}H)_{pellet}\text{-}(^{14}C\text{-}^{3}H)_{supernatant}$$

the excess ^{14}C of the pellet i.e. ^{14}C entrapped can be calculated. From the activity of the supernatant sample the concentration of sucrose can be expressed in cpm/μl. Knowing the amount of protein spun down in each sample, the activity of the pellet in cpm/mg can then be converted to μl/mg to give the volume of sucrose entrapped or the "sucrose accessible space".

In terms of the choice of the permeating solute for the above studies, it should be noted that Crompton and Costi (1988) have considered radiolabelled mannitol as an alternative solute for such entrapment studies. Mannitol permeates mitochondria about three-fold faster than sucrose. However, in these studies, whereas sucrose losses from resealed mitochondria after dilution were negligible (3%/min), those of mannitol (30%/min) necessitated the introduction of a correction. [U-^{14}C]-sucrose has thus been employed routinely for these studies.

[2.9] ***Estimation of [^{3}H]-cyclosporin A binding to mitochondria :***

A series of 500μl incubations (at pH7.0) containing mitochondria (1mg protein/ml) or sub-mitochondrial particles (2mg protein/ml), 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/ml, 2mM KH_2PO_4, 5mM succinate, [^{3}H]-cyclosporin A(0.1μCi; 2nM) and [U-^{14}C]-sucrose (0.1μCi) and varying concentrations of "cold" CsA (0-1000nM) were set up at 25°C. 8 min later (the standard preincubation time in TPP^+-electrode and sucrose entrapment studies) 150μl of the mitochondrial/sub-mitochondrial suspension was removed into 4ml of Ecoscint for liquid scintillation counting. The remaining suspension was centrifuged in an Eppendorf bench centrifuge to sediment the mitochondria. The sub-mitochondrial particles were sedimented by spinning in a Beckman ultracentrifuge at 40,000rpm for 30 min. In both instances 150μl of the supernatant was then removed into 4ml of Ecoscint for counting and used to calculate CsA_{free}. The CsA_{bound} was obtained from the difference in counts before and after centrifugation.

(i) To correct for ^{14}C spillover into the ^{3}H window, [U-^{14}C]-sucrose (0.1μCi) was added to 500μl of 120mM KCl, 10mM Hepes (K^+ salt) pH 7.0. 150μl was withdrawn into 4ml of Ecoscint for counting. The measured spillover was then used to correct ^{14}C spillover in the samples.

(ii) CsA is highly lipophilic and was dispersed routinely from ethanolic solution: the final [ethanol] of the incubate was maintained at 0.5%. CsA bound variably (20-40%) to the inside of the incubation tubes and to pipette tips. Thus, to ensure complete transfer of CsA in aqueous solution it was necessary to rinse all pipettes in ethanol.

(iii) In addition, since CsA bound to the inside of the centrifuge tube, it was necessary to know how much bound in order to calculate precisely the free CsA and the bound CsA. This was achieved by determining the total ^{3}-H-CsA recovered from each tube, the difference being the amount that adhered to the tube.

[2.10] ***To examine the effect of Ca^{2+} on [^{3}H]cyclosporin A binding to mitochondria:***

[^{3}H]-Cyclosporin binding studies were performed as described above except a 1mM HEDTA/ 1mM EGTA Ca^{2+}/Mg^{2+} buffer yielding 1.2mM free Mg^{2+} and either 0.1μM free Ca^{2+} or 5.6μM free Ca^{2+} was included in the preincubation.

[2.11] ***Measurement of [^{3}H]cyclosporin A binding to phospholipid vesicles:***

[2.11.1] *Extraction of total mitochondrial phospholipid:*

Mitochondrial phospholipids were extracted in chloroform/methanol (1.5:1) as follows: 48ml of MeOH was added to 20 ml of medium containing 120mM KCl, 10mM Hepes (K^{+} salt), pH 7.0 and mitochondria 5mg/ml in a flask. The suspension was shaken for 1 min and left for 20 min. 24ml of $CHCl_3$ was then added and the suspension was again shaken for 1 min and left for 20 min. A further 24ml of $CHCl_3$ was then added and the suspension was shaken for 30s and left for 10 min. 24ml of H_2O was then added and the suspension was shaken for 30s and left for 10 min. Solids were then filtered through Whatman (type 1) filter paper, the filtrate being collected in a filter funnel. The filtrate was then washed in 24ml of $CHCl_3$. The filtrate was left to separate into two layers and the lower layer was collected in a 100ml round bottomed flask. The suspension was then evaporated to dryness in a rotary evaporator (Buchi Rotavapor-R) at 40°C. The phospholipids were resuspended in 2.4ml of 120mM KCl, 10mM Hepes (K^{+} salt), pH 7.0 using a marble. The suspension was sonicated under N_2 until clear (30 min) to obtain phospholipid vesicles.

[2.11.2] *Determination of mitochondrial phospholipids:*

In order to allow a comparison of cyclosporin partitioning into phospholipids with the total cyclosporin binding to mitochondria, the phospolipid content of

mitochondria was determined after extraction of total phosholipid from mitochondria. The method used was that of Ames (1966). The reagents used for the assay were as follows:

(1.) 10% $Mg(NO_3)_2$;
(2.) 0.5N HCl;
(3.) 10% ascorbic acid (which when kept capped in the fridge was stable for about a month);
(4.) 0.42% ammonium molybdate.$4H_2O$ in 1N H_2SO_4 (28.6ml of concentrated H_2SO_4 and 4.2g of ammonium molybdate.$4H_2O$ made up to 1L with H_2O). This solution was stable at room temperature.
Mix: To 1 part of (3.) was added 6 parts of (4.). This solution could be kept on ice for a day.

The procedure was as follows:

0.03ml of the magnesium nitrate solution was added to 20-30μl of a 1:5 dilution of the phospholipid stock obtained after extraction of total phospholipid from mitochondria in a Pyrex tube. This suspension was dried and ashed by shaking the tube over a strong flame until the brown fumes disappeared. The tube was allowed to cool and 0.3ml of 0.5N HCl was added. The tube was capped with a tear drop condenser and heated in a boiling water bath for 45min to hydrolyse to phosphate any pyrophosphate formed in the ashing procedure. The tops of the tubes were cooled with a stream of air to minimise evaporation. 0.7ml of the *mix* was then added and the resulting solution was incubated for 20min at 45°C. The absorbance was read on a Perkin-Elmer Spectrophotometer at 820nM. The assay was performed on KH_2PO_4 standards yielding finally 36, 73 and 146nM Pi to allow the reagent to be calibrated. The colour change was stable for several hours. The calibration curve is shown in Fig. 2.11. The phospholipid content of mitochondria was 140μg per mg of mitochondrial protein.

[2.11.3] *Experimental procedure for measuring [^{3}H]-Cyclosporin A binding to phospholipid vesicles :*

A series of 50μl incubations (at pH7.0) containing phospholipid vesicles (the amount of phospholipid contained in 2mg mitochondrial protein; 280μg of phospholipid),

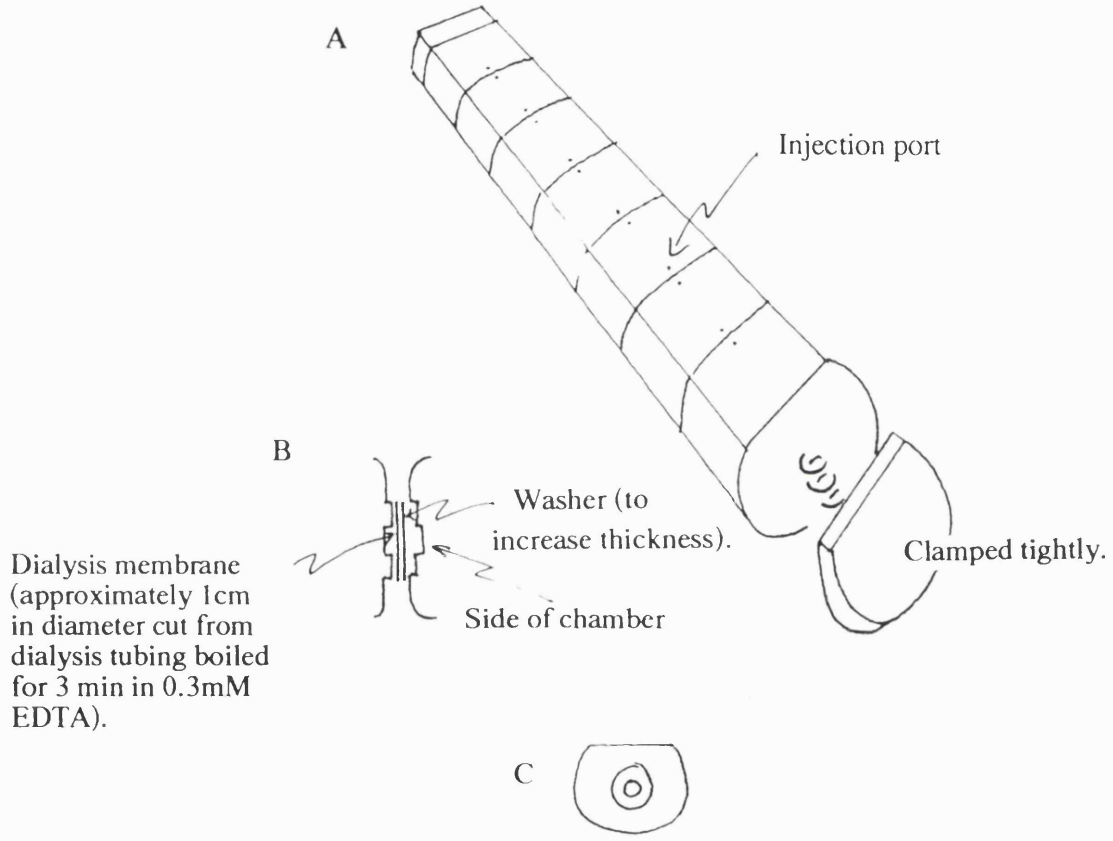


Fig. 2.12

Equilibrium dialysis apparatus. (A) Full view ; (B) longitudinal section ; and (C) transverse section.

120mM KCl, 10mM Hepes (K^+ salt) and 5mM succinate, [^{3}H]-cyclosporin A (0.01μCi; 5nM) and [U-^{14}C]-sucrose (0.01μCi) and varying amounts of "cold" cyclosporine A were set up at 25°C. 8 min later 10μl of each incubate was removed into 4ml of Ecoscint for liquid scintillation counting. The phospholipid vesicles in the remainder of the incubate were immediately sedimented by centrifugation in a Beckman Airfuge at 100,000g for 30 min. 10μl of the supernatant was then removed into 4ml of Ecoscint for counting. Bound CsA was obtained by difference as in the case of CsA binding to mitochondria.

[2.12] ***Estimation of [^{3}H]cyclosporine A binding to mitochondria, sub-mitochondrial particles and to the soluble matrix fraction using equilibrium dialysis:***

A multiwell micro-dialysis apparatus was constructed from Plexiglass as shown in Fig. 2.12. It accommodated six incubations. Each half-well as shown in longitudinal section held a volume of 40μl and was separated from the other half-well by a circular dialysis membrane 1cm in diameter. 35μl of medium containing 120mM KCl, 10mM Hepes (K^+ salt), 2.5mM Pi, 1mM EGTA, 21mM mannitol, 7mM sucrose, 10mM tris, [U-^{14}C]-sucrose ($2x10^4$ cpm/ml), [^{3}H]-cyclosporin ($2x10^6$ cpm/ml) and "cold" cyclosporin at various concentrations was added to each half-well. [U-^{14}C]-sucrose was added to correct for pipetting inaccuracies. Finally 2μl of a suspension of either mitochondria (42mg/ml), a suspension of sub-mitochondrial particles (10.5mg/ml) or the soluble mitochondrial matrix fraction (31.5mg/ml) was added to one half-well to give final protein concentrations of 2.5 mg/ml, 0.625 mg/ml and 1.875mg/ml respectively. The contents of each half-well were allowed to equilibrate over 40-70hr and 20μl aliquots were removed from each half-well into 4ml of Ecoscint, at time intervals and counted for ^{14}C and ^{3}H.

[2.13] ***Measurement of peptidyl-prolyl-cis-trans-isomerase activity:***

[2.13.1] *Rationale of assay:*

PPIase activity was routinely assayed by the procedure of Fischer *et al.* (1989). Briefly the *cis-trans* isomerization of the ala-pro peptide bond in the test-peptide *N*-succinyl-ala-ala-pro-phe-4-nitroanilide was measured in a coupled assay with chymotrypsin. This is based on the ability of chymotrypsin to cleave the test peptide

only when the ala-pro is *trans*. The reaction can be illustrated schematically as follows:

chymotrypsin

N-succ-ala-ala-pro-phe-4-nitroanilide (*trans*) → N-succ-ala-ala-pro-phe

FAST +

SLOW ⇑ PPIase nitroanilide

(yellow chromophore)

N-succ-ala-ala-pro-phe-4-nitroanilide (*cis*)

85% of the substrate commercially obtained is already in the *trans* isomeric form. In the assay the preexisting *trans isomer* was hydrolysed within the mixing time (3-5s) to release the yellow chromophore. Hydrolysis of the 4-nitroanilide in the *cis* isomeric form (the remaining 25%) is limited by the *cis-trans* isomerization of the ala-pro bond. This slower reaction was monitored by following the increase in absorbance due to release of the yellow chromophore as described in section [2.13.3].

[2.13.2] *Derivation of rate equation:*

The rate of disappearance of the *cis* isomer is given by the relation:

$$dcis/dt = -kcis$$

where *cis* is the concentration of the *cis* isomer at any time t and *k* is the rate constant for the reaction.

$$cis_t = cis_0 e^{-kt} \quad (1)$$

where cis_t is the concentration of the *cis* isomer remaining at any time t and cis_0 is the initial concentration of the *cis* isomer.

Now:

$$cis_0 = cis_t + trans_t$$

Therefore:

$$cis_t = cis_0 - trans_t$$

where $trans_t$ is the concentration of *trans* isomer which has been formed at any time t.

Substituting this into equation (1) gives:

$$(cis_0 - trans_t)/cis_0 = e^{-kt}$$

Now:

$$cis_0 = trans_\infty$$

where $trans_\infty$ is the concentration of *trans* isomer formed on complete isomerization of the *cis* isomer.

Therefore:

$$trans_\infty - trans_t / trans_\infty = e^{-kt}$$

In practice:

$$trans_t \propto A$$

where A is the absorbance change that has occurred at any time t due to hydrolysis of the *trans* isomer.

So:

$$A_\infty - A_t / A_\infty = e^{-kt}$$

or:

$$Ln(A_\infty - A_t / A_\infty) = -kt$$

or for brevity:

$$Ln(A/A_\infty) = -kt$$

where A is the absorbance change which has occurred subsequent to any time t, and A_∞ is the total absorbance change which has occurred.

Thus the rate constant of isomerization k is given by the slope of a graph of $Ln(A_t/A_\infty)$ versus time (t). Examples of experimentally derived graphs are presented in Chapter 4, section C4.4].

[2.13.3] *Experimental protocol for assay of PPIase activity:*

A 1.2mM stock solution of the 4-nitroanilide was made up in 50% fresh EtOH and sonicated in a bath sonicator to aid dispersion. All assays were carried out in medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), a small aliquot of the protein sample to be assayed and CsA (when added) at 25°C in a 4ml glass cuvette. After preincubation for 8 min 50μM chymotrypsin was added. 1 min later 15μM *N*-succinyl-ala-ala-pro-phe-4-nitroanilide was added to start the reaction. Release of the chromophore was monitored by following the increase in absorbance on a Perkin Elmer dual beam spectrophotometer using the wavelength pair 390nm-480nm. When CsA was added, the cuvette was thoroughly washed out with EtOH after each assay.

[2.14] ***ADP-ribosylation of sub-mitochondrial particles:***

On day 1, the soluble matrix fraction obtained after preparation of sub-mitochondrial particles (section 2.3.2) was dialysed for 18hr in medium (pH7.4) containing 10mM HCl (Tris salt) and 250mM sucrose with two buffer changes. On day 2, sub-mitochondrial particles were again prepared fresh as described previously. Fresh sub-mitochondrial particles (0.4mg protein/ml) $\pm$ the dialysed soluble matrix fraction were incubated with 30μM [^{32}P]-(adenylate)NAD (17μCi) in medium (pH7.4) containing 10mM tris/HCl, 250mM sucrose and 1.2mM $MgSO_4$ at 37°C. At 2, 10 and 20min time intervals, the reaction was stopped by transferring aliquots (containing $\simeq$ 0.1mg of protein) into an equal volume of cold sample buffer (0.25M citric acid, 0.031M H_3PO_4 brought to pH 4.0 with Tris salt) containing 0.8M urea, 5% w/v lithium dodecyl sulphate and 4% w/v mercaptoethanol.

Initial attempts to remove unincorporated [^{32}P]-(adenylate)NAD using NAP 5 columns (Sephadex G25; Sigma) were unsuccessful. This was probably because the [^{32}P]-(adenylate)NAD diffused through the column as fast as the sample proteins were eluted. To overcome this problem, each denatured sample was diluted 1:4 with H_2O (final volume 2ml) and then concentrated overnight to 40-70μl by centrifugal dialysis using Centricon 3 microconcentrators (3000 MW cut-off; Amicon). The sample was washed three more times by making up the volume in the microconcentrator to 2ml in 0.25% lithium dodecyl sulphate and concentrating to 40-70μl by centrifugal dialysis for 3hr. This procedure successfully removed any unincorporated [^{32}P]-NAD.

[2.15] ***Analysis of labelled proteins by low pH lithium dodecyl sulphate polyacrylamide-gradient-gel electrophoresis:***

The electrophoretic method employed was that described by Jones *et al* (1981). The gel was polymerised between two glass plates 18cm x 20cm, separated by two 1mm thick Perspex strips and held together with gel sealing tape. The bottom of the plates was sealed by clamping them vertically in a perspex trough and pouring in a 16% polyacrylamide plug. $FeSO_4.7H_2O$, ascorbic acid and H_2O_2 were added just before the gel was poured to catalyse polymerisation of the gel. At room temperature, the gel formed within 30-45min. A linear gradient of gel was formed on top of this plug, with a high concentration of acrylamide at the bottom of the plate and a low

concentration at the top. Solutions containing 18% and 10% acrylamide were mixed to form the gradient using a gradient mixer. The gel formed within 20 min at room temperature. The stacking gel was dispensed with as it did not polymerise under several conditions tried and anyway a good resolution of proteins was obtained without it. The protein wells were thus formed at the top of the gradient gel by insertion of a 1mm thick perspex comb. The unpolymerised components of the gel were made up as follows:

Plug:

10.60ml 30% acrylamide (stock)
1.60ml resolving buffer (pH4.0) (see below)
0.20ml 10% lithium dodecyl sulphate (LDS)
3.70ml saturated sucrose solution
3.33ml $FeSO_4.7H_2O$ (0.025% w/v))
0.17ml ascorbic acid (8.33% w/v)) *FENTON REAGENT*
0.20ml H_2O_2 (2.5% w/v)) (made up fresh)

Resolving gel:

18% Acrylamide solution	*10% Acrylamide solution*
0.76ml H_2O	8.87ml H_2O
10.60ml 30% acrylamide	5.04ml 30% acrylamide
1.36ml resolving buffer	1.36ml resolving buffer
0.17ml 10% LDS	0.17ml LDS
3.30ml saturated sucrose	
0.40ml $FeSO_4.7H_2O$*	0.40ml $FeSO_4.7H_2O$*
0.17ml ascorbic acid*	0.17ml ascorbic acid*
0.20ml H_2O_2*	0.20ml H_2O_2*

*The concentrations of stock solutions of the components of the *Fenton Reagent* were as tabulated above and as indicated, made up fresh.

Resolving buffer:

The resolving buffer contained 1M citric acid and 0.125M phosphoric acid brought to pH 4.0 with Tris salt.

Electrophoresis buffer:

The electrophoresis buffer used was that described by Lichter and Wolf (1979). It contained 93.8mM citric acid, 12.4mM phosphoric acid, and 0.1% lithium dodecyl sulphate(LDS) brought to pH 4.0 with Tris salt. Jones *et al* recommended an electrophoresis buffer containing 0.51 M-L-glutamic acid and 0.1% LDS brought to pH 4.0 with Tris salt. However, it was found that glutamic acid was not soluble in water at this concentration and at this pH.

Preparation of protein samples for loading:

The protein samples (already denatured) were mixed with an equal volume of cold sample buffer (0.25M citric acid, 0.031M H_3PO_4 brought to pH 4.0 with Tris salt) containing 0.8M urea, 5% w/v LDS, 4% w/v 2-mercaptoethanol, 0.15% glycerol and 0.1% w/v basic Fuschin as tracking dye.

Electrophoresis:

Electrophoresis was performed at 4°C in the cold room. The electrophoresis buffer was slowly circulated between the anode and the cathode resevoirs in order to prevent pH changes which occur as a consequence of electrophoresis. A constant voltage of 150mV moved the tracker dye basic Fuschin, to the bottom of the gel in 20hr.

Handling of gels:

Gels were immediately dried at 80°C under vacuum and were autoradiographed for one week using Kodak XAR-5 films. Labelled bands were excised and immersed in 4ml of Ecoscint for scintillation counting.

[2.16] ***Assay of protein yield during chromatographic purification of mitochondrial cyclophilin:***

During chromatographic purification of cyclophilin, protein was routinely measured using the Bradford (1976) reagent which has a protein sensitivity in the range 0.2-20μg. The dye reagent for the assay was prepared by dissolving 100mg of Coommassie Blue G in a mixture of 100ml of 85% phosphoric acid and 50ml of 95% ethanol. The mixture was then sonicated to aid dissolution of the dye. Finally the volume was made up to 1L with H_2O. 50μl of NaOH (1M) and 1ml of dye reagent was added to 5-20μl protein samples in 1ml polystyrene cuvettes. After 5 min the absorbance was measured at 590nm using a Perkin Elmer spectrophotometer.

[2.17] ***Resolution of mitochondrial proteins using sodium dodecyl sulphate(SDS)-polyacrylamide gel electrophoresis:***

Polyacrylamide gel electrophoresis was a modification of that described by Laemmli (1970). The gel was polymerised between two glass plates 18cm x 20cm, separated by two 1mm thick Perspex strips and held together with gel sealing tape. The bottom of the plates was sealed by clamping them vertically in a perspex trough and pouring in a 16% polyacrylamide plug. Ammonium persulphate and *N, N, N',N'*-tetramethylethylenediamine (TEMED) were added just before the gel was poured to catalyse polymerisation of the gel. At room temperature the gel formed within 15-30min. A 16% resolving gel was poured on top of the plug. 1ml of a 50% solution of MeOH was poured slowly onto the unpolymerised gel in order to form a sharp interface on polymerisation. The MeOH was then poured off and the top of the gel was washed with H_2O. The gel polymerised in 15-20min. A 3.5% polyacrylamide stacking gel in which the protein wells were formed by the insertion of a 1mm thick Perspex comb, was polymerised on top of the resolving gel. The unpolymerised components of the gel were made up as follows:

Plug/16% Resolving gel:

9.85ml H_2O
16.00ml 30% (w/v) acrylamide (stock)
3.57ml resolving buffer (pH8.8)
0.30ml 10% (w/v) SDS
0.10ml 10% (w/v) ammonium persulphate (made up fresh)
8.75μl TEMED

3.5% Stacking gel:

6.30ml H_2O
1.25ml 30% (w/v) acrylamide
2.50ml stacking buffer (pH 6.8)
0.10ml 10% (w/v) SDS
0.10ml 10% (w/v) ammonium persulphate (made up fresh)
7.50μl TEMED

Resolving gel:

The resolving buffer contained 1.5M Tris salt and was brought to pH8.8 using HCl.

Stacking buffer:

The stacking buffer contained 0.5M Tris salt and was brought to pH 6.8 using HCl.

Preparation of protein samples for loading:

Protein samples were prepared for electrophoresis by boiling for 5 min in an equal volume of stock sample buffer containing:

30.0ml 10% (w/v) SDS
12.5ml stacking buffer

15.0ml glycerol
37.5ml H_2O

Electrophoresis buffer:

This was a 0.25M Tris salt, 1.92M glycine, 1% SDS buffer, brought to pH 8.3 with HCl.

Electrophoresis:

The gel was electrophoresed at 20mA (current limiting) until the dye front moved into the resolving gel ($\simeq$ 2hr). The current was then raised to 45mA until the dye front had reached the bottom of the gel (2-3hr).

Staining of the gel:

The gel was fixed by washing in 10% MeOH, 10% acetic acid (3 x 180ml for 20min). The gel was then washed in 25% MeOH (3 x 180ml for 10min). The gel was then stained by immersing it in Sigma's Colloidal Coomassie Blue for 1½hr. The dye was discarded and the gel was initially destained in 10% acetic acid, 25% MeOH for 60s. This solution was discarded and the gel was then destained in 25% MeOH for 2hr.

MATERIALS.

The rats used for mitochondrial preparation were fed male Sprague-Dawley rats (180-280g) body weight. They were bred in University College's central animal house. Cyclosporin A was supplied by Sandoz Pharmaceuticals. The chemicals for gel electrophoresis were a special electrophoretic grade supplied by BDH. The DDACl and the THF were also supplied by BDH. The FPLC chromatographic columns were prepacked and supplied by Pharmacia. A stirred ultrafiltration cell and Centricon-3 microconcentrators were supplied by Amicon. Radiolabelled [^{14}C]-sucrose and [^{3}H]-H_2O were obtained from Amersham. NAP gel filtration columns (G25) and all other reagents were supplied by Sigma.

CHAPTER 3: ADVERSE EFFECTS OF: A HIGH CA^{2+} CONCENTRATION; HIGH PI CONCENTRATION; LOW ADENINE NUCLEOTIDE CONCENTRATION AND A HIGH OXYGEN TENSION ON RAT LIVER MITOCHONDRIAL ENERGY TRANSDUCTION AND PROTECTION AGAINST SUCH DELETERIOUS EFFECTS BY PHYSIOLOGICAL CONCENTRATIONS OF ATP AND μM AMOUNTS OF THE IMMUNOSUPPRESSANT CYCLOSPORIN A (CsA).

[3.1] *Background*.

As shown previously in rat heart mitochondria [Crompton and Costi (1988)] pores in any single mitochondrion open simultaneously in response to Ca^{2+} and an inducer, a process termed "permeabilization". Initial studies were carried out in order to establish suitable conditions for studying pore activity in rat liver mitochondria. In particular it seemed necessary, firstly to establish the minimal extramitochondrial [Ca^{2+}] required for "permeabilization" of mitochondria and secondly to ascertain whether factors (Pi, oxidative stress and adenine nucleotides), which are considered to be effectors of pore opening showed significant stimulatory or inhibitory activity in the experimental system adopted.

In the past, "permeabilization" has generally been triggered by exposure of mitochondria to >100nmol Ca^{2+}/mg mitochondrial protein. There is evidence (Chapter 1; section 1.4) that exposure of mitochondria to such high Ca^{2+} concentrations leads to activation of phospholipase A_2 whose activity may cause disruption of the membrane lipid phase [e.g. Beatrice *et al* (1984)]. In addition, an increase in H^+ permeability may occur due to the associated production of free fatty acids. Fatty acids have been shown to act as uncouplers [Pressman and Lardy (1956)], but whether this is due to an inherent protonophoric action or due to free fatty acid action on inner membrane proteins is still a matter of debate [Pressman and Lardy (1956); Rottenberg and Hashimoto (1986); Dedukhova *et al.*, (1991); Skulachev, (1991)]. There are strong indications that any phospholipid hydrolysis is unconnected with "permeabilization". Nevertheless, if "permeabilization" is to be interpreted as reflecting the activity of a protein pore, it was desirable to obtain experimental conditions under which detectable free fatty acid release, at least, did not occur. This was achieved by using the minimal extramitochondrial free [Ca^{2+}] required for "permeabilization" of respiring mitochondria. For this purpose, a series

of HEDTA/ EGTA/ Ca^{2+}/Mg^{2+} buffers were made up (Chapter 2; section 2.4) to allow the extramitochondrial free $[Ca^{2+}]$ to be manipulated appropriately. Once the minimal level of Ca^{2+} required for "permeabilization" had been established, samples of permeabilized mitochondria were assayed for free fatty acids by Dr. N. Yafei (this group) in collaboration with Dr. A. Smith and J. Belin (Dept. Chem. Pathol., UCL).

Regarding the effects of adenine nucleotides on "permeabilization", all studies reported to date (Chapter 1; section 1.3.5) have simply involved the addition of adenine nucleotides to incubation mixtures. This is obviously unsatisfactory, since added nucleotides may be metabolized quickly (by oxidative phosphorylation, uncoupled F_1 ATPase and contaminating ATPases and adenylate kinase). In fully "permeabilized" rat heart mitochondria, for example, added ATP is 50% hydrolysed in 1s [(Crompton and Costi (1988)]. In this study the level of ATP, when present, was maintained constant by the introduction of an ATP regenerating system containing creatine phosphate (CP), creatine phosphokinase (CPK) and ATP. As ATP is being continuously hydrolysed to produce Pi, an inducer of "permeabilization", in this system conditions were chosen so that the [Pi] did not increase above 20mM during the course of the experimental incubation reported. To establish these conditions, control experiments were performed in which aliquots of the incubate were removed at intervals and assayed for Pi. The other nucleotide investigated, ADP, was clamped by a system comprising glucose and hexokinase. For both regenerating systems, control experiments were performed in which the incubation mixtures were deproteinised (0.6N $HClO_4$; centrifugation), neutralised and then enzymatically assayed for the appropriate nucleotide. ATP was assayed using hexokinase and glucose-6-phosphate dehydrogenase and ADP was assayed using pyruvate kinase and lactate dehydrogenase (Lestaborsk et al. [5]). These control experiments confirmed that the initial added ATP or ADP concentration was maintained (± 5%) throughout the experimental incubation reported.

[3.2] ***The sensitivity of "permeabilization" to buffered extramitochondrial free Ca^{2+}.***

Preliminary experiments were conducted with Ca^{2+} buffers to assess the minimal Ca^{2+} concentration required for pore opening. Since H^+ ions would be expected to permeate the open pore rapidly, causing uncoupling in energized mitochondria, pore

state was assessed initially by monitoring changes in the mitochondrial potential ($\Delta\Psi$) developed on addition of succinate as respiring substrate. $\Delta\Psi$ was measured with a TPP^+(tetraphenylphosphonium ion)-sensitive electrode (Chapter 2; section 2.5).

Fig. 3.1 illustrates the sensitivity of $\Delta\Psi$ to varied external $[Ca^{2+}]$ in the presence of 10mM Pi and 0.5mM ATP (maintained by an ATP regenerating system). On exposure to a low buffered Ca^{2+} concentration of 0.3μM the mitochondria develop and maintain a high potential ($\Delta\Psi$; 145-155mV) as assessed from the accumulation of the TPP^+ ion. $\Delta\Psi$ was dissipated slightly at a free Ca^{2+} concentration of 2.5μM. In this case oscillations in $\Delta\Psi$ were observed. Such oscillations have been reported previously (Al Nasser and Crompton (1986a)] and, it was suggested, may be due to cyclic phases of "permeabilization", Ca^{2+} release, resealing (pore closure), Ca^{2+} uptake, "permeabilization" *etc.* by mitochondria that are initially largely in phase. At a free Ca^{2+} concentration of 5.6μM, however, $\Delta\Psi$ was progressively dissipated.

As $\Delta\Psi$ is only an index of permeability to H^+ ions , the state of the inner mitochondrial membrane (IMM) pore was also assayed more directly under the above conditions by measuring entry of [U-^{14}C]-sucrose into the mitochondrial matrix. Since pore opening is fully and rapidly reversed by Ca^{2+} chelation (EGTA), ^{14}C-sucrose entry is most conveniently measured from its entrapment in the matrix space on Ca^{2+} chelation. In addition, during "permeabilization" the pores in any particular mitochondrion appear to open simultaneously, so that each mitochondrion undergoes a transition between (extremely) low and high permeability to sucrose. This means that "permeabilization" may be appropriately expressed in units of μl matrix space accessible to sucrose. The sucrose entrapment measurements were done in parallel with $\Delta\Psi$ measurements, by splitting the incubate after preincubation, prior to addition of succinate as substrate.

Preliminary investigation revealed that [U-^{14}C]-sucrose was accumulated even before initiation of respiration by addition of succinate as substrate i.e. during the preincubation. The extent of entrapment was independent of the extramitochondrial buffered free $[Ca^{2+}]$ and amounted to 0.3 ± 0.09 μl/mg mitochondrial protein. It is not clear whether this entrapment is mitochondrial or not. Whatever its nature, this initial entrapment was always measured and termed "residual entrapment" This

Fig. 3.1: *The Ca^{2+} sensitivity of respiring rat liver mitochondria exposed to a high [Pi].* Mitochondria (1mg protein/ml) were preincubated for 6 min at 25°C in medium (pH 7.0) containing, 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 3μM tetraphenyl-phosphonium ion (TPP^+), and a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer giving finally 1.2mM free Mg^{2+} and the [Ca^{2+}] (μM) indicated in parentheses. After preincubation 40mM creatine phosphate plus 20 units of creatine phosphokinase were added. At the time indicated in the figure 3mM succinate was introduced simultaneously with 0.5mM ATP and 10mM KH_2PO_4.

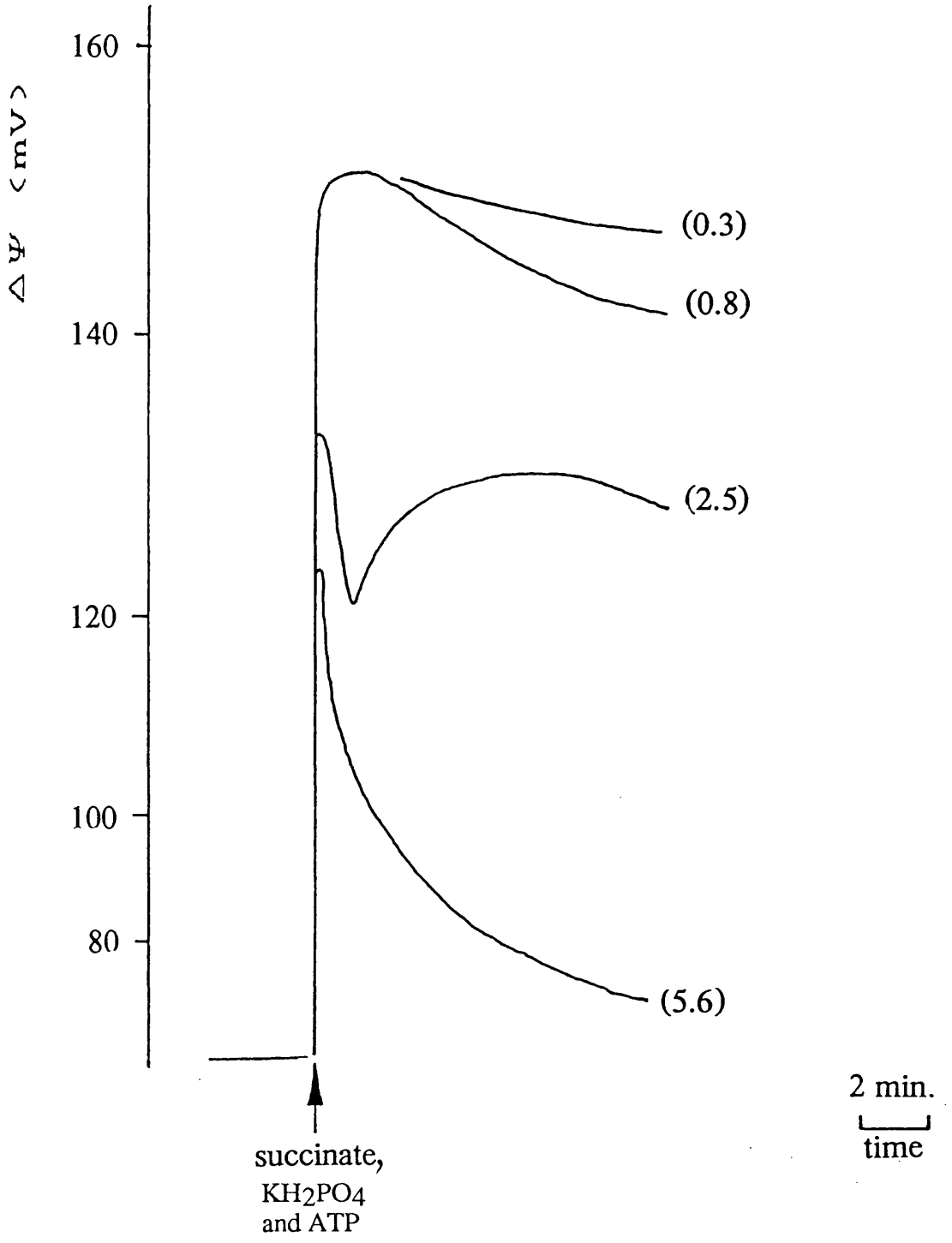

160
140
120
100
80
Δψ (mV)
(0.3)
(0.8)
(2.5)
(5.6)
2 min.
time
succinate,
KH2PO4
and ATP

Fig. 3.1

residual entrapment was subtracted from the entrapment which occurred subsequent to addition of succinate and presumably in response to the electrophoretic accumulation of Ca^{2+} to give the values shown in Fig. 3.2. This figure shows the extent of sucrose entrapment as a function of the external free [Ca^{2+}]. Thus, at a free Ca^{2+} concentration of 0.3μM there is no entrapment of [U-^{14}C]-sucrose. In contrast, at free Ca^{2+} concentrations of 2.5μM and 5.6μM the IMM pore opens allowing entry and entrapment of [U-^{14}C]-sucrose. [illegible]. The entrapment which occurs at a buffered free Ca^{2+} concentration of 5.6μM is similar to the degree of sucrose entrapment which occursat 50μM external free Ca^{2+} showing that 5.6μM external free Ca^{2+} is sufficient for maximal "permeabilization" within 6 min.

From experiments in which matrix free Ca^{2+} was clamped over a constant range of values by means of matrix entrapment of Ca^{2+} buffers it is clear that "permeabilization" is a first order process in which the apparent rate constant is increased in a linear fashion by increasing the matrix free Ca^{2+} to >20μM [Al Nasser and Crompton (1986a)]. On these grounds, the submaximal entrapment obtained with 2.5μM external Ca^{2+} would represent a submaximal rate of "pemeabilization". Also, although the rate of "permeabilization" does not show any threshold dependence on matrix free Ca^{2+} [Al Nasser and Crompton (1986a)], the present experiments clearly show a threshold dependence on external free Ca^{2+} (Figs. 3.1, 3.2). The threshold relationship presumably reflects the dependence of matrix free Ca^{2+} on external free Ca^{2+}, i.e. that the increase in matrix free Ca^{2+} with increasing external Ca^{2+} becomes progressively steeper as external Ca^{2+} is raised. In practical terms it means that whereas exposure of respiring rat liver mitochondria to <1μM external Ca^{2+} would induce little activation of the pore, exposure to >5.6μM Ca^{2+} would induce maximal pore activation within 6 min. These conditions were therefore adopted when investigating the possible contributation of free fatty acids to Ca^{2+}-induced $\Delta\Psi$ dissipation.

[3.3] ***The free fatty acid content of mitochondria during and without permeabilization.***

The possible activation of phospholipase A_2 by the minimal [Ca^{2+}] of 5.6μM found to trigger pore opening was investigated. About 42% of the fatty acids esterified at

Fig. 3.2: *The Ca^{2+} sensitivity of pore opening as assessed by [U-^{14}C]-sucrose entrapment.* Incubation conditions were as described in the legend to Fig. 3.1. IMM pore opening was assayed as described in the Chapter 2; section 2.8 by measuring [U-^{14}C]-sucrose entry and entrapment in the matrix space (here indicated as sucrose accessible matrix space). Respiration was started by the simultaneous addition of 0.5mM ATP, 10mM KH_2PO_4 and 3mM succinate. Pore closure was induced 6 min after addition of succinate by chelation of Ca^{2+} with EGTA. The values are given as means $\pm$ S.E.M. (4 experiments).

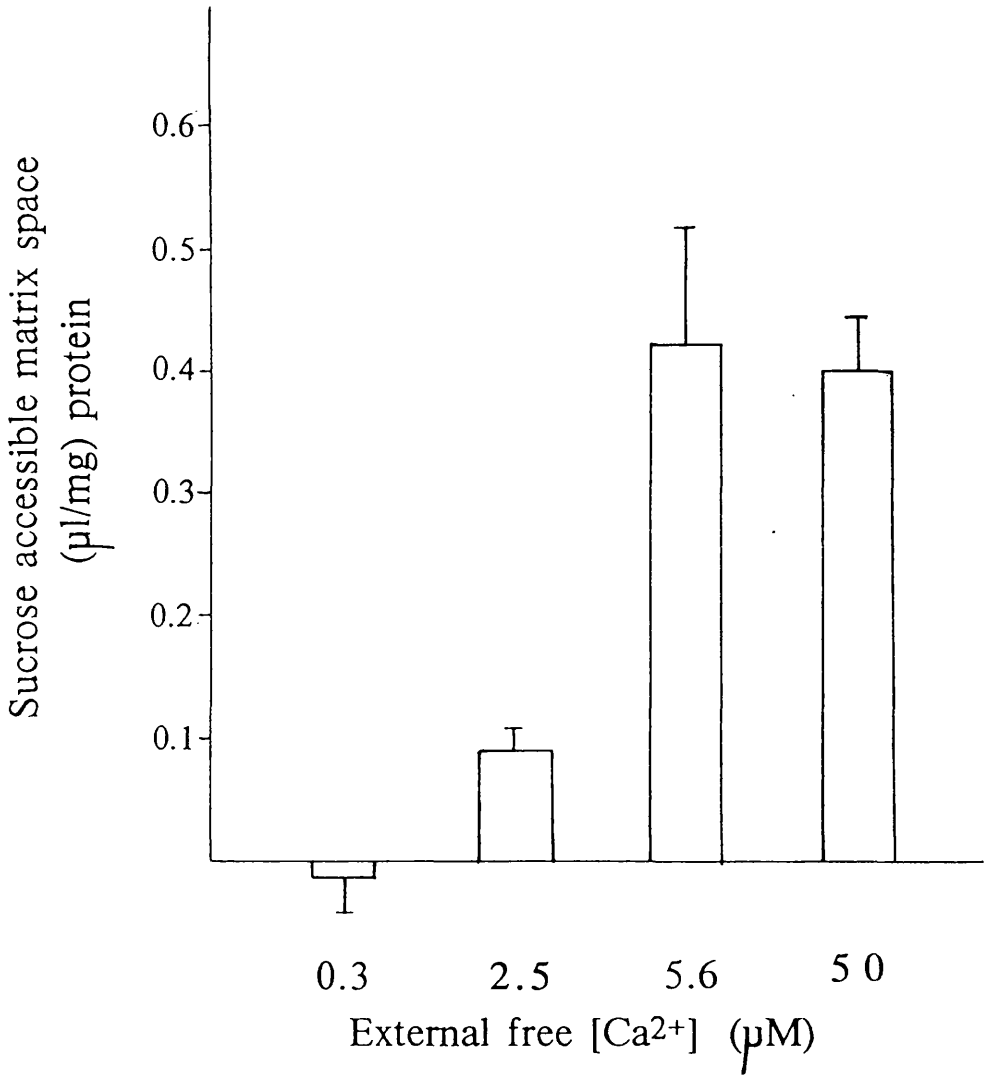

Sucrose accessible matrix space
(µl/mg) protein
0.6
0.5
0.4
0.3
0.2
0.1
0.3
2.5
5.6
5 0
External free [Ca2+] (µM)

Fig. 3.2

carbon-2 of the glycerol of the mitochondrial phospholipids are polyunsaturated [Pfeiffer *et al* (1979)], and phospholipase A_2 would be expected to liberate free and in particular polyunsaturated fatty acids. Mitochondria were preincubated under the conditions used for the sucrose entrapment and TPP^+ electrode experiments described above. Six minutes after energization with succinate (the time taken for the complete collapse of $\Delta\Psi$, free fatty acids were extracted from incubates by the procedure of Dole and Meinertz (1960). The extracts were analysed by GLC using a Chrompak SIL5 CB column. A known amount of C_{17} fatty acid was added to the extraction medium as internal standard. Table 3.1 illustrates the % change in free fatty acids induced by 5.6μM Ca^{2+} free as opposed to 0.3μM free Ca^{2+}.

Free fatty acid	Amount with 0.3μM free Ca^{2+} (μg/mg protein)	Change induced by 5.6μM Ca^{2+} (%)
16:0	10.6±1.8	+1.2±4.7
18:0	13.6±1.2	-1.0±5.0
18:1	3.8±0.8	+1.0±4.9
18:2	18.4±3.9	+3.1±3.6
20:4	8.1±0.9	-0.8±0.8
22:6	4.3±0.4	-5.7±5.8

Table 3.1: *The free fatty acid content of "permeabilized" and non-"permeabilized" mitochondria.* Rat liver mitochondria (1mg/ml) were preincubated for 6 min at 25°C in medium (pH7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 3μM TPP^+, $MgCl_2$ (0.5mM) and a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer giving finally 1.2mM free Mg^{2+} and Ca^{2+} sufficient (5.6μM) and Ca^{2+} insufficient (0.3μM) to induce "permeabilization". After preincubation 40mM creatine phosphate plus 20 units of creatine phosphokinase were added. At the time indicated in the figure, 3mM succinate was introduced simultaneously with 0.5mM ATP and 10mM KH_2PO_4. Free fatty acids were extracted 6 min after respiration was started.

These results show that there was no significant difference between the free fatty acid

content of mitochondria exposed to [Ca^{2+}] sufficient (5.6μM) and insufficient (0.3μM) to induce pore opening. This reinforces the idea that "permeabilization" is due to the presence of a protein pore in the inner membrane as initially proposed by Haworth and Hunter (1976) and more recently by Crompton and his colleagues [Al Nasser and Crompton (1986ab); Crompton and Costi (1988); Crompton and Costi (1990)], rather than reflecting perturbation of the membrane bilayer by phospholipid deacylation. It also seems unlikely that "permeabilization" and uncoupling is due to fatty acid action on the inner membrane unless the amounts involved are very small.

[3.4] ***Mg^{2+} is required to induce pore opening and uncoupling in response to a buffered extramitochondrial Ca^{2+} of 5.6μM in the presence of ATP:***

Rather suprisingly, in view of previous studies (below), it was found that, pore opening and uncoupling induced by buffered Ca^{2+} required the presence of Mg^{2+} during the preincubation period. Fig 3.3a illustrates this clearly. Trace (A) shows that if Mg^{2+} is absent in the preincubation period, even if it is introduced just prior to addition of succinate as substrate, respiring mitochondria develop and maintain a high potential at a buffered Ca^{2+} concentration of 5.6μM. Trace (B) shows the familiar rapid collapse of $\Delta\Psi$ seen if 1.2mM Mg^{2+} is present during the preincubation. IMM pore opening (Fig. 3.3b) was assayed under the same conditions. When Mg^{2+} is absent from the preincubation (column 1), no sucrose entrapment occurs i.e. no pore opening occurs. Thus the mitochondria can establish and maintain a high $\Delta\Psi$. Raising the free Mg^{2+} to 1.2mM in the preincubation period allows entry and entrapment of [U-^{14}C]-sucrose. Thus Mg^{2+} exerts its effect only when present in the preincubation period.

Haworth and Hunter (1979b) found that if mitochondria were prepermeabilized to release endogenous ions and nucleotides and then resealed by chelation of Ca^{2+} with EGTA, addition of 1mM Mg^{2+} actually inhibited, in a competitive manner, subsequent Ca^{2+}-induced permeabilization caused by reexposure of these pretreated mitochondria to varying concentrations of buffered Ca^{2+}. However, in these studies pore state was examined indirectly by monitoring the shrinkage induced by the introduction of a non-permeant polyethylene glycol. The conclusions drawn from these studies are rather different from those drawn here. In addition, recent patch

Fig. 3.3: *The Mg^{2+} sensitivity of pore opening.* Mitochondria (1mg protein/ml) were preincubated for 6 min at 25°C in medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 3μM tetraphenylphosphonium ion (TPP^+) 40mM creatine phosphate plus 20 units of creatine phosphokinase. The free [Ca^{2+}] was maintained at 5.6μM throughout the preincubation and the free [Mg^{2+}] was varied by manipulating the conditions as indicated below. Respiration was started by the simultaneous addition of 0.5mM ATP, KH_2PO_4 and 3mM succinate. In Fig. 3.3(a) $\Delta\Psi$ was determined from the distribution of TPP^+ . Under the same conditions IMM pore opening was assayed (Fig. 3.3(b)) by measuring [U-^{14}C]-sucrose entry and entrapment in the matrix space (here indicated as sucrose accessible matrix space). Pore closure was induced 6min after addition of succinate by chelation of Ca^{2+} with EGTA when $\Delta\Psi$ was completely dissipated.

A. Ca^{2+} was maintained at 5.6μM during the preincubation by addition of a 1mM EGTA/ 1mM HEDTA Ca^{2+} buffer. Mg^{2+} was excluded from the preincubation but raised to 1.2mM by addition of a Mg^{2+}/HEDTA buffer just prior to addition of succinate. This buffer was chosen so as to maintain the free [Ca^{2+}] at 5.6μM throughout the run.

B. Free [Mg^{2+}] was maintained at 1.2mM and the free [Ca^{2+}] was maintained at 5.6μM throughout the preincubation and incubation by addition of a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer.

These manipulations are summarised below:

	Preincubation		Immediately before succinate addition	
	[Ca^{2+}] (μM)	[Mg^{2+}] (mM)	[Ca^{2+}] (μM)	[Mg^{2+}] (mM)
A:	5.6	-	5.6	1.2
B:	5.6	1.2	5.6	1.2

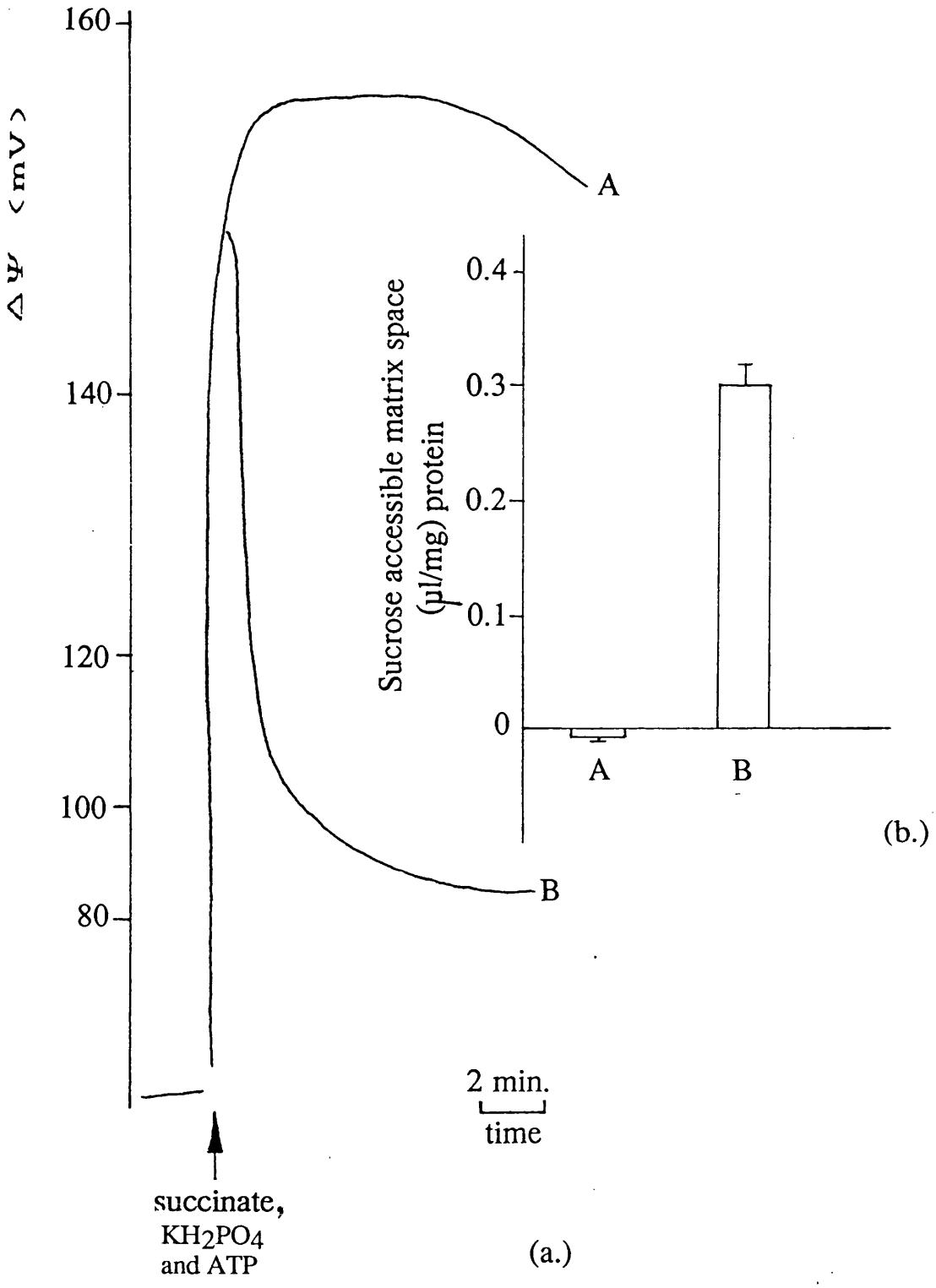

160
140
120
100
80
Δ Ψ (mV)
A
B
0.4
0.3
0.2
0.1
0
Sucrose accessible matrix space
(µl/mg) protein
A
B
(b.)
2 min.
time
succinate,
KH2PO4
and ATP
(a.)

Fig. 3.3

clamp studies on the "Multi Conductance Channel" which has been identified with the pore (Chapter 1; section 1.3.5) have shown that 1-2mM Mg^{2+} inhibits channel activity [Szabo and Zoratti, (1992)]. It is possible that chelation of ATP, which was clamped at 0.5mM, by this [Mg^{2+}] counteracts inhibition of Ca^{2+}-induced uncoupling by this concentration of free ATP. However, Mg^{2+}-ATP has been shown to oppose Ca^{2+}-induced permeabilization [Beatrice *et al.*, (1980); Leblanc and Clauser, (1974); Nicholls and Brand, (1980); Nicholls and Crompton, (1980); Crompton (1985)]. Clearly the role of Mg^{2+} as an effector of pore state needs further investigation.

[3.5] ***Pi and oxidative stress potentiate the Ca^{2+} induced uncoupling of mitochondria.***

Having established that 5.6μM Ca^{2+} (in the presence of 1.2mM Mg^{2+}) was sufficient to promote pore opening in the absence of detectable membrane phospholipid degradation, this concentration of Ca^{2+} was used routinely to trigger "permeabilization". The results of the sucrose entrapment experiments showing the Ca^{2+} and Mg^{2+} sensitivity of "permeabilization" correlate well with the assessment of "permeabilization" using $\Delta\Psi$ dependent parameters and in subsequent investigations the latter, more convenient assay, was employed. Firstly, Pi and oxidative stress, which are considered to be promoters of pore opening induced by high unbuffered Ca^{2+} were systematically examined to see if they showed significant stimulatory activity at a buffered [Ca^{2+}] of 5.6μM and in the presence of Mg^{2+}.

Fig. 3.4 shows the Ca^{2+} sensitivity of respiring rat liver mitochondria at two concentrations of Pi in the presence of a low [ATP]. In order to maintain the [Pi] constant this study was done in the absence of the ATP regenerating system in which, as already discussed, ATP is continuously hydrolysed to Pi. $\Delta\Psi$ is rapidly dissipated in the presence of 0.5mM Pi (curve B) at a free [Ca^{2+}] of 5.6μM. The mitochondria develop and maintain a high potential ($\Delta\Psi$; 150mV) if the [Ca^{2+}] is reduced to $<0.1\mu$M in the presence of 0.5mM Pi. Increasing the [Pi] to 10mM stimulates the rate of $\Delta\Psi$ dissipation seen at 5.6μM free Ca^{2+} (curve C).

The effects of *oxidative stress* were examined by gassing the incubation with 100% O_2, since mitochondrial superoxide production has been shown to increase linearly

Fig. 3.4: *The adverse effects of a high free [Ca^{2+}] in the presence of Pi and low ATP on respiring rat liver mitochondria.* Mitochondria (1mg protein/ml) were preincubated for 6 min at 25°C in medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 3μM tetraphenylphosphonium ion (TPP^+), 40mM creatine phosphate plus 20 units of creatine phosphokinase and a 1mM EGTA/ 1mM HEDTA buffer yielding finally 1.2mM free Mg^{2+} and the free [Ca^{2+}] concentration given below. TPP^+ uptake was started with 0.5mM ATP, 3mM succinate plus KH_2PO_4 (see below) .

A. Free Ca^{2+} < 0.1μM; 0.5mM Pi;
B. 5.6μM free Ca^{2+}; 0.5mM Pi;
C. 5.6μM free Ca^{2+}; 10.0mM Pi.

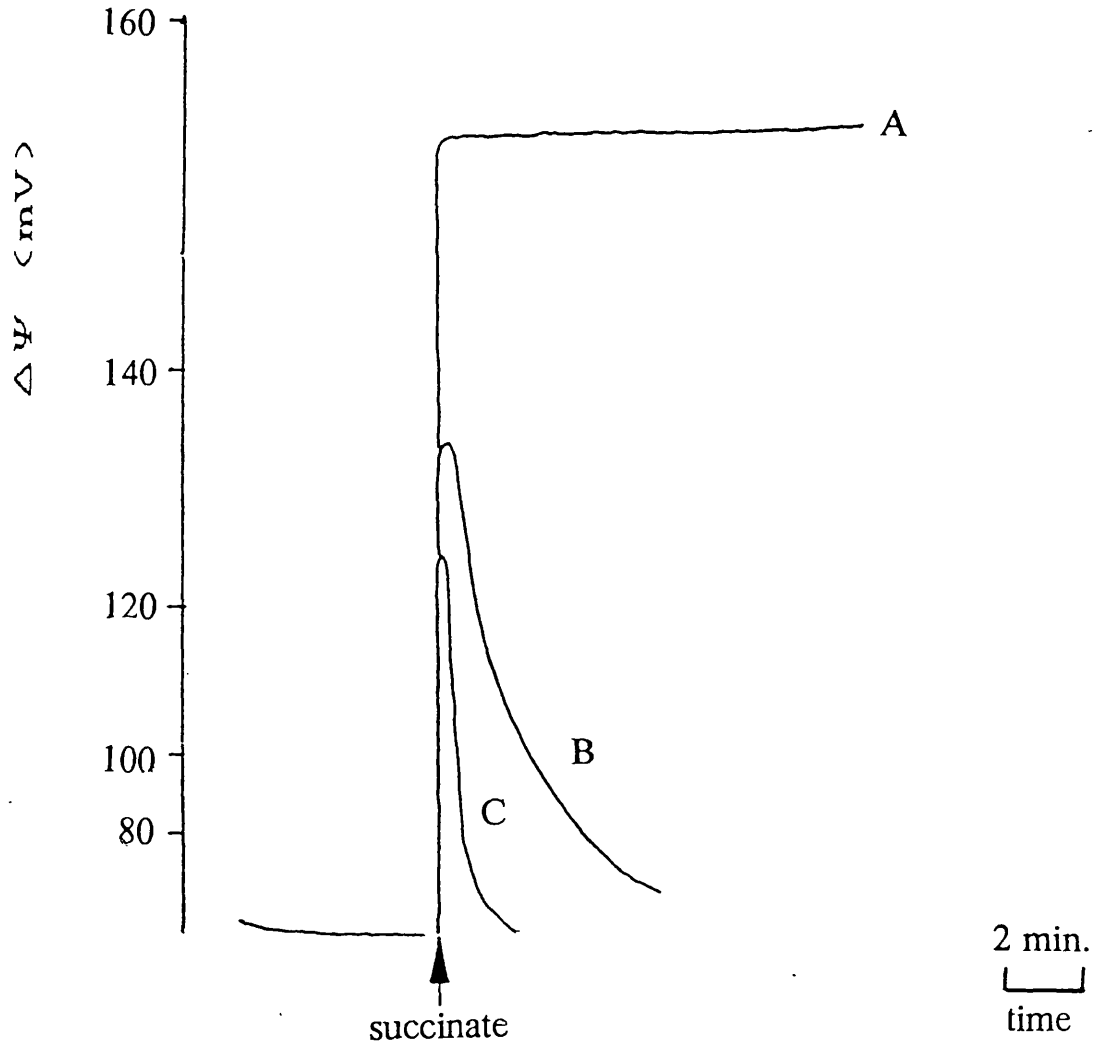


Fig. 3.4

with oxygen tension [Turrens *et al*, (1982)]. At least two components of the respiratory chain produce superoxide oxygen radicals ($O_2^{\cdot -}$), namely, the ubiquinone-cytochrome b segment [Nohl and Jordan, (1984)] and the NADH dehydrogenase complex [Krishnamoorthy and Hinkle, (1988); Turrens and Boveris, (1980)]. These highly reactive species can undergo rapid dismutation to H_2O_2, a reaction catalysed by superoxide dismutase [Chance *et al.*, (1979)]:

$$2H^+ + O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow[\textit{dismutase}]{\textit{superoxide}} H_2O_2 + O_2$$

Mitochondria lack catalase, and H_2O_2 is reduced to H_2O by glutathione peroxidase using GSH reductase. The GSSG produced is reduced by NADPH (glutathione reductase) yielding NADP which is reduced by NADH (energy linked transhydrogenase) [Oshino and Chance, (1977); Sies and Moss, (1978)]. This effect of H_2O_2 is often mimicked by addition of small amounts (a few nmol/mg of *tert*-butylhydroperoxide, (which is reduced to the alcohol by glutathione peroxidase), to respiring mitochondria. The deleterious effects of $O_2^{\cdot -}$ are thought to be due to the formation of the hydroxyl radical in the presence of transition metals such as iron [Halliwell (1987]:

$$Fe(III) + O_2^{\cdot -} \rightarrow Fe(II) + O_2$$

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

Fig. 3.5a shows the effect of increasing the oxygen tension on respiring mitochondria incubated in the presence of 1.5mM ATP and either 0.8μM or 5.6μM free Ca^{2+}. Whereas increasing the oxygen tension from 20% to 100% had no effect on $\Delta\Psi$ development at a low free Ca^{2+} concentration of 0.8μM (compare curves A and B), dissipation of $\Delta\Psi$ at a buffered Ca^{2+} concentration of 5.6μM was markedly potentiated (compare curves C and D). Fig. 3.5b shows that the effect of increasing the oxygen tension to 100% is reversed on reducing the oxygen tension to 20% again.In trace (B) succinate was reintroduced as indicated to ensure that the potential collapse was not due to a depletion of substrate.

Fig. 3.5(a): *A high oxygen tension potentiates the adverse effect of a high [Ca^{2+}] on respiring rat liver mitochondria.* Mitochondria (1mg protein/ml) were preincubated for 6 min at 25°C in medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 3μM tetraphenylphosphonium ion (TPP^+), 40mM creatine phosphate plus 20 units of creatine phosphokinase and a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer giving finally 1.2mM free Mg^{2+} and the free [Ca^{2+}] indicated below. Where indicated the oxygen tension was increased to 100% by blowing a stream of pure oxygen over the surface of the incubation. At the time indicated in the figure 3mM succinate was introduced simultaneously with 0.5mM ATP and 10mM KH_2PO_4 to initiate respiration.

A. Free Ca^{2+} < 0.8μM; oxygen tension 20%
B. " ; oxygen tension 100%
C. 5.6μM free Ca^{2+}; oxygen tension 20%
D. " ; oxygen tension 100%

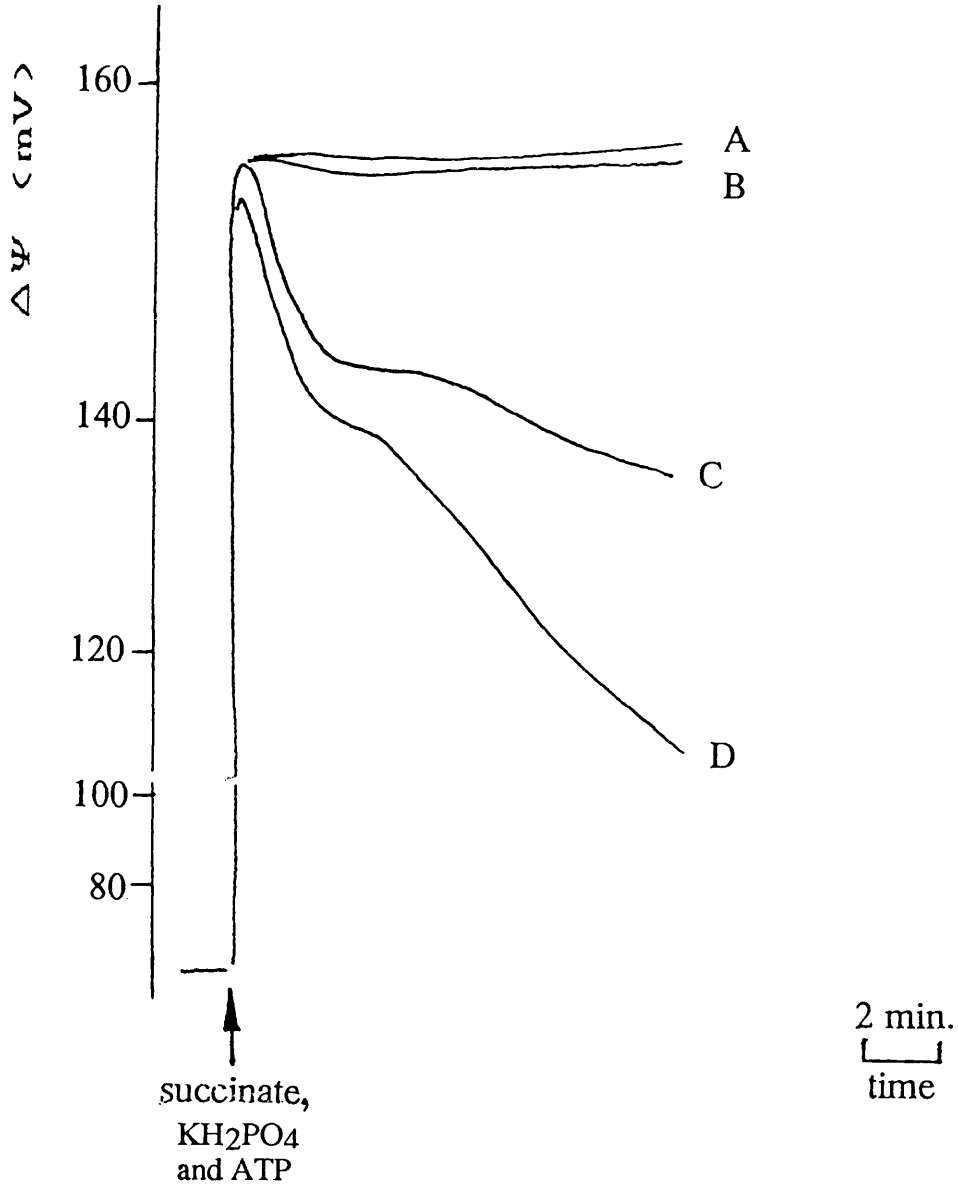

Δψ (mV)
160
140
120
100
80
A
B
C
D
2 min.
time
succinate,
KH2PO4
and ATP

Fig. 3.5 (a)

Fig. 3.5(b): *The effects of the oxygen are reversible.* Mitochondria were preincubated as described in the legend to Fig. 3.5(a). Where indicated, the oxygen tension was increased to 100% by blowing a stream of pure oxygen over the surface of the incubation. At the time indicated in the figure, 3mM succinate was introduced simulltaneously with 0.5mM ATP and KH_2PO_4.

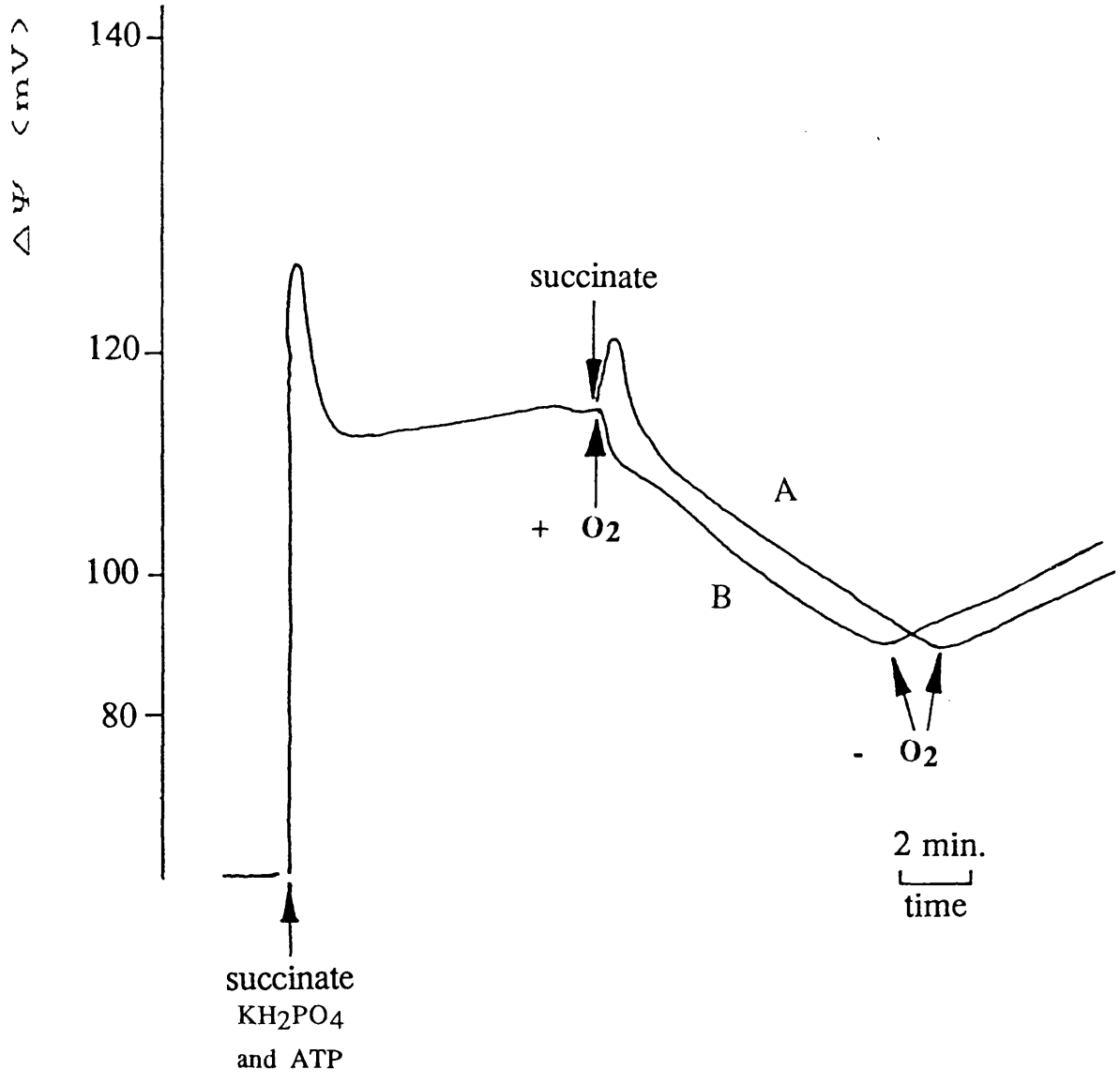

Δψ (mV)
140
120
100
80
succinate
+ O2
A
B
- O2
2 min.
time
succinate
KH2PO4
and ATP

Fig. 3.5 (b)

Thus, oxidative stress produced by increasing the oxygen tension to which respiring mitochondria are exposed acts in strict synergism with Ca^{2+} to stimulate pore opening. A similar synergism was observed by Crompton and Costi (1988) in respiring rat heart mitochondria, when pore opening was induced by the rather higher [Ca^{2+}] of 100nmol/mg of protein and addition of *tert*-butylhydroperoxide.

[3.6] ***Effects of adenine nucleotides on Ca^{2+} induced uncoupling of mitochondria.***

As described in the Chapter 1; section 1.3.5, non-specific permeability of the inner mitochondrial induced by relatively high amounts of unbuffered Ca^{2+} is favoured by a depletion of endogenous adenine nucleotides. The effect of supplementing the incubation with ATP, ADP or AMP was therefore examined to determine which adenine nucleotides, if any inhibit "permeabilization" induced by a buffered [Ca^{2+}] of 5.6μM in the presence of Mg^{2+}.

Fig. 3.6 shows that the adverse effect of Ca^{2+} and Pi was completely abolished by the addition of 5mM ATP. Lower concentrations of ATP, here 1.5 and 0.5mM ATP, were only partially effective. In the absence of ATP there is a rapid and complete dissipation of the potential.

In contrast supraphysiological concentrations of ADP (0.5mM) (Fig. 3.7) afforded no protection against the deleterious effects of Ca^{2+}. AMP (2mM) was also ineffective (results not shown).

Haworth and Hunter (1979b) concluded that endogenous ADP inhibits "permeabilization". More recently, Szabo and Zoratti (1992) have shown that the Multi Conductance Channel's activity (which has been identified with the pore) in excised mitoplast patches is inhibited by ADP. It may be that ADP only inhibits when added on the matrix side of the inner membrane or when it is allowed to equilibrate through the open pore and this is why it is effective in promoting pore closure in this instance.

Fig. 3.6: *Protection against the adverse effects of* Ca^{2+} *by ATP.* Mitochondria (1mg protein/ml) were preincubated for 6 min at 25°C in medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 3μM tetraphenylphosphonium ion (TPP^+), 40mM creatine phosphate plus 20 units of creatine phosphokinase and a 1mM EGTA/ 1mM HEDTA Ca^{2+}buffer giving finally 5.6μM free Ca^{2+} and 1.2mM free Mg^{2+}. This concentration of $Mg^{2+}$was maintained at all concentrations of ATP. After preincubation 10mM KH_2PO_4 was added followed immediately by ATP and 3mM succinate. The concentrations of ATP added are given in parentheses by each trace.

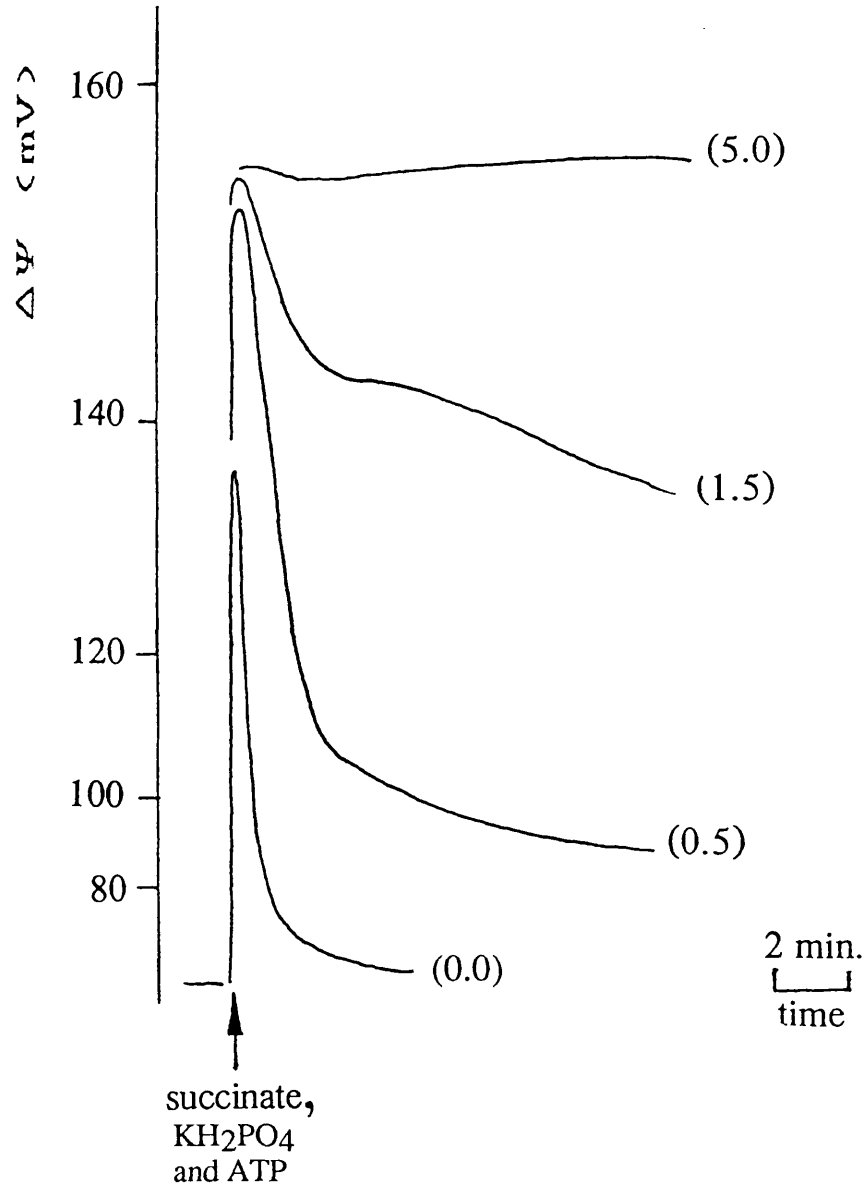

Δψ (mV)
160
140
120
100
80
(5.0)
(1.5)
(0.5)
(0.0)
2 min.
time
succinate,
KH2PO4
and ATP

Fig. 3.6

Fig. 3.7: *ADP fails to protect against the adverse effects of* Ca^{2+}. Mitochondria (1mg protein/ml) were preincubated for 6 min at 25°C in medium (pH 7.0) containing: 120mM KCl; 10mM Hepes (K^+ salt); 1μg rotenone/mg protein; 3μM tetraphenylphosphonium ion (TPP^+); and a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer giving finally 1.2mM free Mg^{2+} and the free [Ca^{2+}] indicated below. After preincubation 0.5mM ADP ; 50mM glucose; hexokinase (10 units) were added. Respiration was started with 3mM succinate:

A. Free $Ca^{2+} < 0.1\mu M$

B. 5.6μm free Ca^{2+}

C. 5.6μm free Ca^{2+} in the absence of the ADP regenerating system (control).

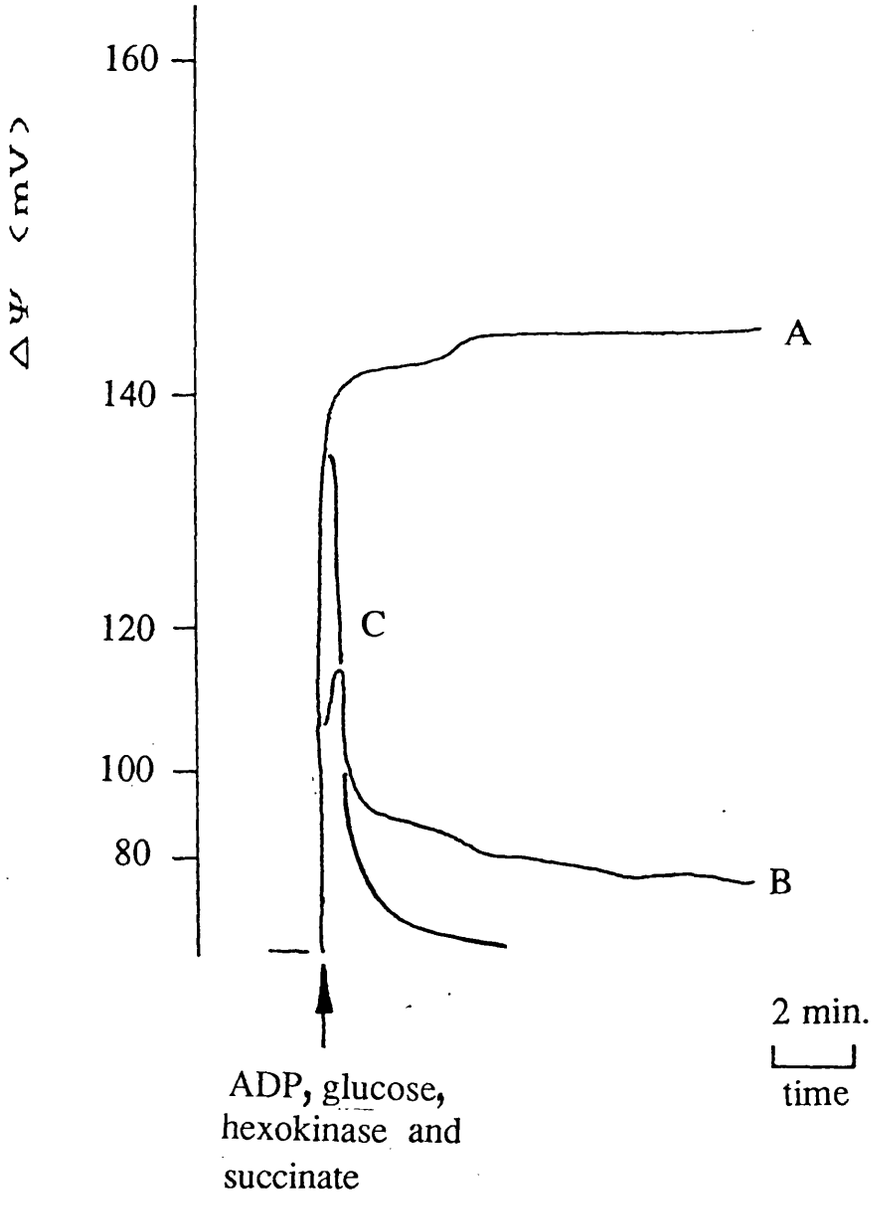

160
140
120
100
80
Δψ (mV)
A
B
C
2 min.
time
ADP, glucose,
hexokinase and
succinate

Fig. 3.7

[3.7] ***Cyclosporin A is a potent inhibitor of pore opening induced by a buffered free Ca^{2+} of 5.6μM.***

A principal aim of this investigation was to elucidate molecular components involved in pore opening. This objective would obviously be facilitated if tight binding inhibitors for the pore system were recognised. As a first step therefore it seemed reasonable to examine the effects on pore opening of compounds known to interact with Ca^{2+} binding proteins. These included the calmodulin antagonists, W7 (R24571) and trifluoperazine, and the plasma membrane voltage gated Ca^{2+} channel blockers nifedipin and verapamil. However, at a concentration of 10μM none of these antagonists had any effect on the Ca^{2+} and Pi-induced depolarization of $\Delta\Psi$. Broekmeier *et al* (1985) found that rather higher concentrations of trifluoperazine (50μM) and verapamil (200μM) inhibited the Ca^{2+}-induced swelling of mitochondria associated with "permeabilization" and it would have been better to titrate the incubation with increasing concentrations of these agents. However, the low potency of these agents makes them redundant as specific probes for the elucidation of the molecular mechanisms of "permeabilization", and they were not investigated further.

The immunosuppressant CsA had previously been shown to inhibit Ca^{2+} induced pore opening in heart mitochondria at the extremely low concentration of 30nM [Crompton *et al* (1988)]. Fig. 3.8 shows that, in rat liver mitochondria, with a high buffered Ca^{2+} concentration of 5.6μM, oxidative stress and in the absence of ATP, addition of 600nM CsA allowed the development of a potential almost equal to that developed at a low buffered [Ca^{2+}] of 0.3μM (*control*). Below this concentration, CsA's effectiveness diminished.

In subsequent experiments using ^{3}H-CsA it became apparent that a significant fraction of added CsA adheres to the incubation chamber and that much of the remainder is bound to the mitochondria. Fig. 3.8 includes an estimate of free [CsA] based on subsequent experiments on the partitioning of added CsA. With 1mg of mitochondrial protein/ml approximately 40% of added CsA (to 1μM) adhered to the sides of the incubation vessel, and this proportion was assumed to apply in Fig. 3.8. The amount of free CsA is estimated from the ^{3}H-CsA binding curves given in

Chapter 4. On these grounds about 100nM CsA was sufficient to prevent Ca^{2+} plus Pi induced depolarization.

0.6μM CsA also prevented entry of [^{14}C]-sucrose into the mitochondrial matrix via the inner membrane pore and its subsequent entrapment after chelation of Ca^{2+} with EGTA (results not shown). In fact, it was subsequently found that 30nM CsA was sufficient to maximally inhibit the pore opening induced by 5.6μM extramitochondrial free Ca^{2+} and Pi in the absence of added ATP. Fig. 3.9(A) shows the effect of 25nM free cyclosporin on the time course of mitochondrial pore opening. In this experiment the free [CsA] was determined separately by inclusion of [^{3}H]-cyclosporin in the incubate and measurement of supernatant ^{3}H after sedimentation. This data is thus a much more accurate representation of the sensitivity of "permeabilization" to CsA than that obtained using $\Delta\Psi$ dependent parameters.

The initial rates of [U-^{14}C]-sucrose permeation through open pores were used to determine the dependence of pore opening on free [CsA] and the results are shown in Fig. 3.9(B). CsA did not inhibit pore opening completely. Also the inhibition curve was strongly sigmoidal, suggesting either that (a) the cyclosporin binding component was not rate limiting for pore opening, or (b) the relevant component bound cyclosporin cooperatively.

It is evident, that rather more CsA is needed to inhibit Ca^{2+}-induced uncoupling than to inhibit "permeabilization". As already pointed out by Crompton *et al* (1988) this may relate to a previous observation that at mildly acid pH values (e.g. pH6.5), mitochondria become uncoupled in response to 100nM unbuffered Ca^{2+} and Pi before there is any significant sucrose entry [Al Nasser and Crompton (1986)]. It is possible that minimal pore opening causes uncoupling due to rapid H^{+} backflow through the inner mitochondrial membrane, particularly when the [H^{+}] is increased.

It has already been established by several groups that CsA does not inhibit uptake of Ca^{2+} via the uniporter [Fournier *et al.*, (1987); Crompton *et al.*, (1988); Vercesi *et al.*, (1988)]. Nor does it act, like butylated hydroxytoluene, by scavenging oxygen radicals [Chiara *et al.*, (1989)]. In view of its high potency as an inhibitor of "permeabilization", it is assumed it binds either directly or indirectly to the pore inhibiting pore opening.

Fig. 3.8: *Protection by Cyclosporin A (CsA).* Mitochondria (1mg protein/ml) were preincubated for 6 min at 25°C in medium (pH 7.0) containing 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 3μM tetraphenylphosphonium ion (TPP^+), varying concentrations of CsA as indicated and a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer giving finally 1.2mM free Mg^{2+} and 5.6μM free [Ca^{2+}]. In the control experiment, free Ca^{2+} was 0.3μM. After preincubation, 40mM creatine phosphate plus 20 units of creatine phosphokinase were added. At the time indicated in the figure 3mM succinate was introduced simultaneously with 0.5mM ATP and 10mM KH_2PO_4. The free concentration of CsA which was present in each incubation was estimated as indicated in the text and is given in parentheses (μM) alongside the actual concentration added.

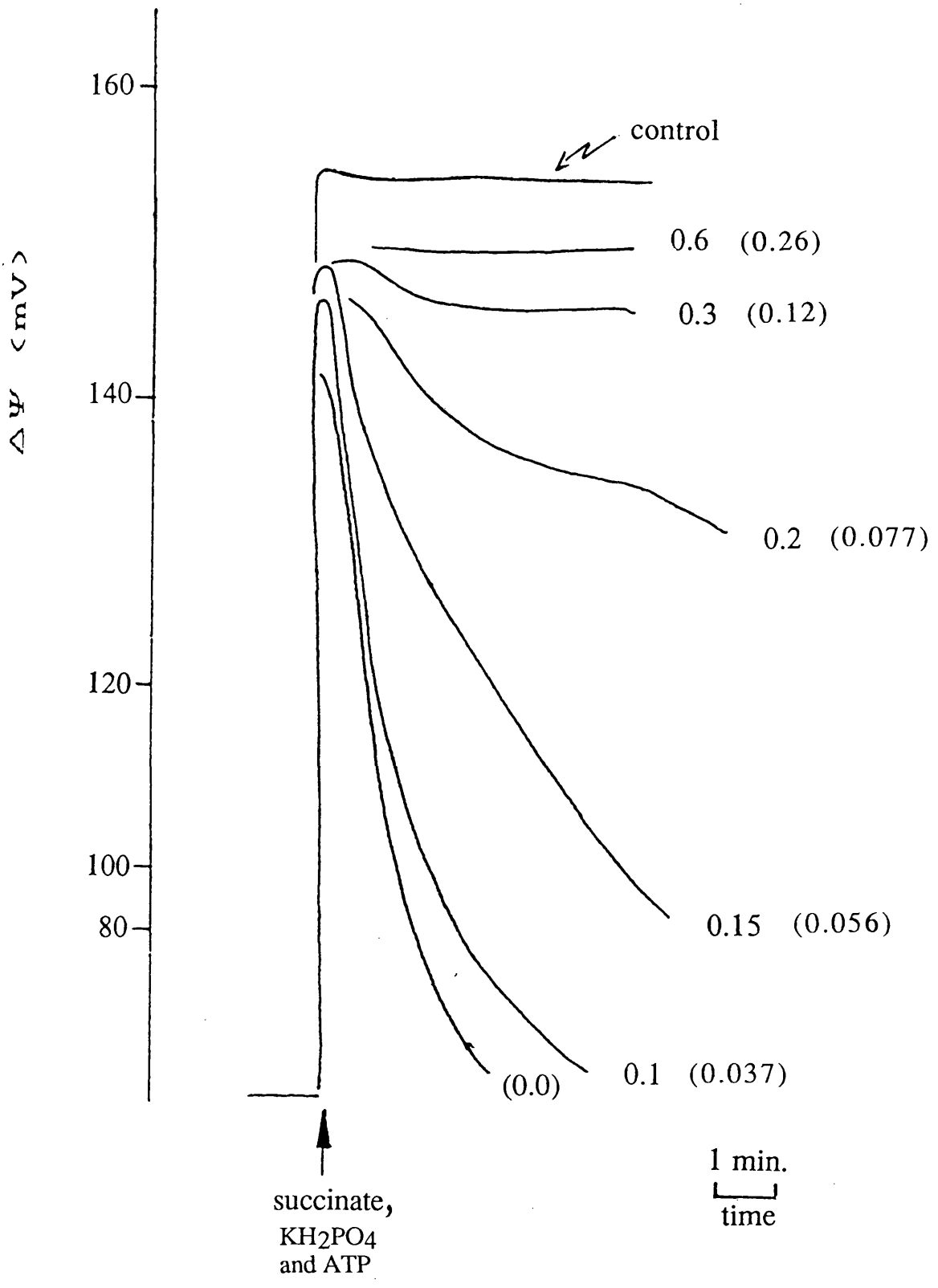

160
140
120
100
80
Δψ (mV)
control
0.6 (0.26)
0.3 (0.12)
0.2 (0.077)
0.15 (0.056)
0.1 (0.037)
(0.0)
succinate,
KH2PO4
and ATP
1 min.
time

Fig. 3.8

Fig. 3.9: *The inhibition of mitochondrial pore opening by cyclosporin A.* Pore opening was assessed by the degree of [U-^{14}C]-sucrose entrapped. (A) The time course of pore opening in the presence (○) and absence (●) of 25nM free cyclosporin. (B) The initial rates of pore opening at varied free [cyclosporin] were obtained from plots of the type shown in A. Means ± S.E.M. of three determinations with the same mitochondrial preparation are given.

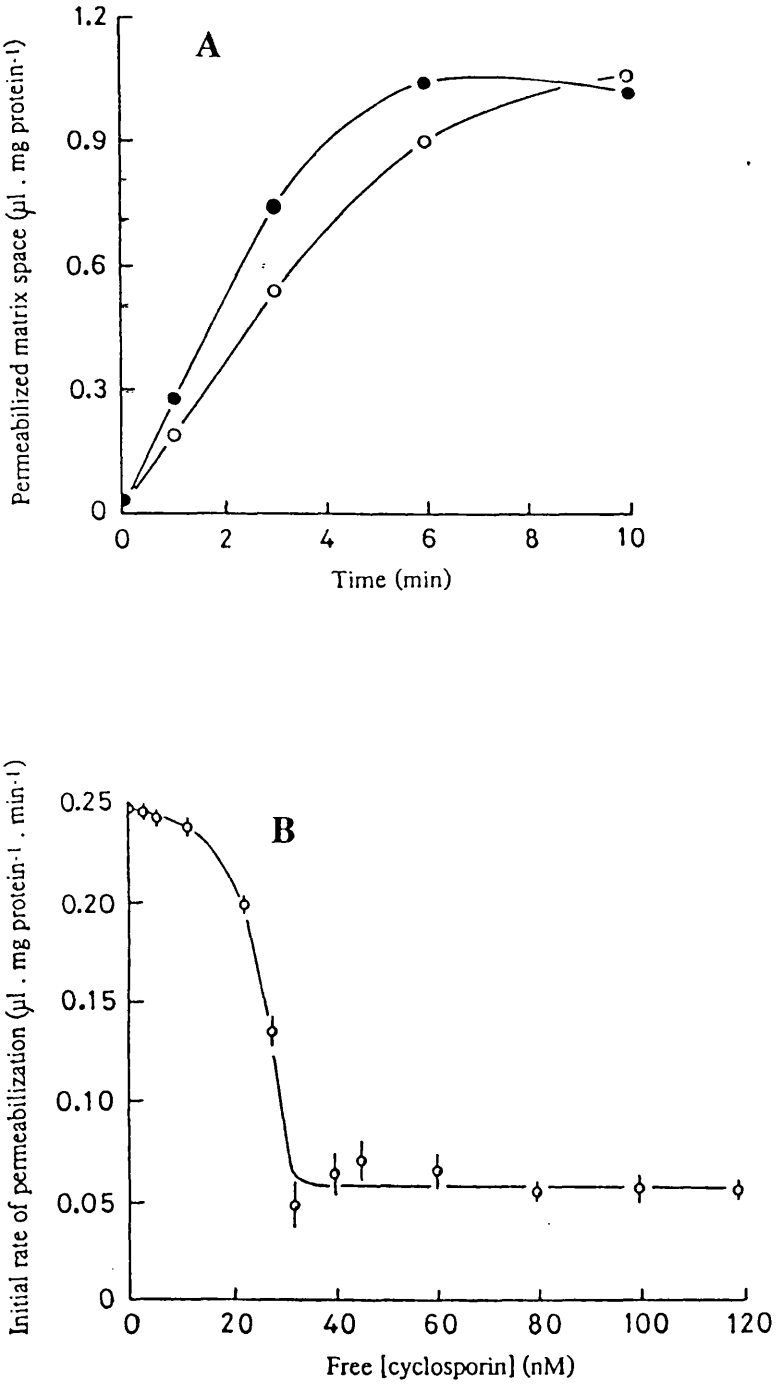

A
Permeabilized matrix space (µl . mg protein-1)
1.2
0.9
0.6
0.3
0
0
2
4
6
8
10
Time (min)
B
Initial rate of permeabilization (µl . mg protein-1 . min-1)
0.25
0.20
0.15
0.10
0.05
0
0
20
40
60
80
100
120
Free [cyclosporin] (nM)

Fig. 3.9

CHAPTER 4: DISSECTION AND INITIAL CHARACTERISATION OF THE CYCLOSPORIN A (CsA) BINDING COMPONENTS.

[4.1] *Background.*

The principal aim of this section was to further define the CsA-binding sites involved in Ca^{2+}-induced "permeabilization" of mitochondria. The initial approach to this investigation was two-fold. Firstly, cyclosporin binding to mitochondria was examined using tritiated ([^{3}H]-)CsA in order to determine the number of CsA binding components, and the relative affinities of the components for CsA. This would allow a comparison between CsA binding and the effect of CsA on Ca^{2+}-induced "permeabilization" presented at the end of the last chapter.

Secondly, as described in the introduction, it has been established that the ubiquitous protein cyclophilin, binds to CsA and mediates its immunosuppressive activity [Handschumacher *et al.*, (1984); Harding *et al.*, (1988)]. Cyclophilin is in fact identical to peptidyl-prolyl *cis-trans* isomerase (PPIase) [Takahaishi, *et al.* (1989), Fischer *et al.*, (1989)] an enzyme which is believed to be involved in the correct folding of proteins. CsA abolishes the activity of PPIase by binding to the active site [Fischer and Schmid, (1990)]. The above information, together with the fact that cyclophilin was shown in *Neurospora Crassa* to have a dual localisation in the cytosol and in the mitochondrial matrix [Tropschug *et al.*, (1988)] made it important to determine whether rat liver mitochondria also contain cyclophilin/PPIase and whether or not this was the CsA binding component implicated in Ca^{2+}-induced "permeabilization" of mitochondria.

[4.2] *Cyclosporin A [CsA] binding to mitochondria.*

Studies of [^{3}H]-CsA binding were carried out with rat liver mitochondria prepared in the standard manner. To ensure a valid comparison between CsA binding and the effects of CsA on Ca^{2+}-induced "permeabilization", all measurements were made after a standardized preincubation with CsA (8 min at 25°C) identical to that in the studies of the effect of CsA on "permeabilization". Indeed preliminary experiments indicated that CsA binding was essentially complete after incubation for 8 min as shown in Fig. 4.1. In the studies in which [CsA] was varied "cold" CsA was changed while a fixed amount of ^{3}H-CsA was present in order to quantify binding

Fig. 4.1: *Cyclosporin binding to mitochondrial proteins during the 8 min preincubation period.* The amounts of [^{3}-H]-cyclosporine bound to mitochondria at various times after addition of 1μM cyclosporine (means $\pm$ SEM; three measurements).

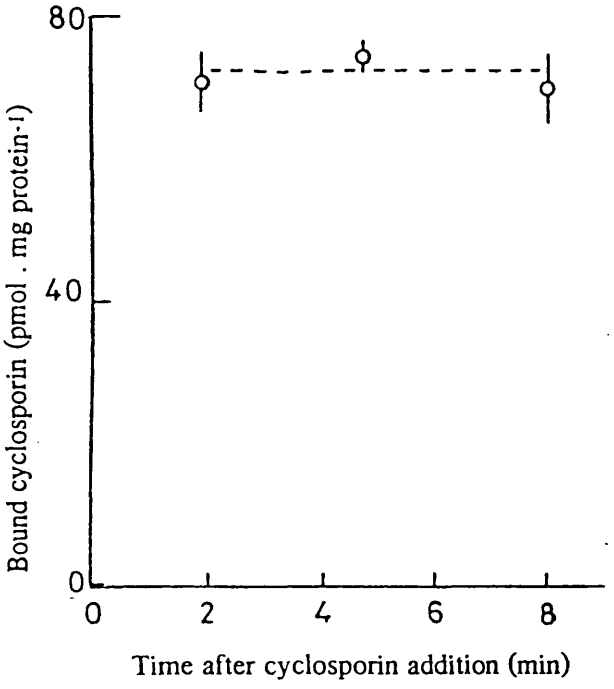

80
40
0
0
2
4
6
8
Bound cyclosporin (pmol . mg protein-1)
Time after cyclosporin addition (min)

Fig. 4.1

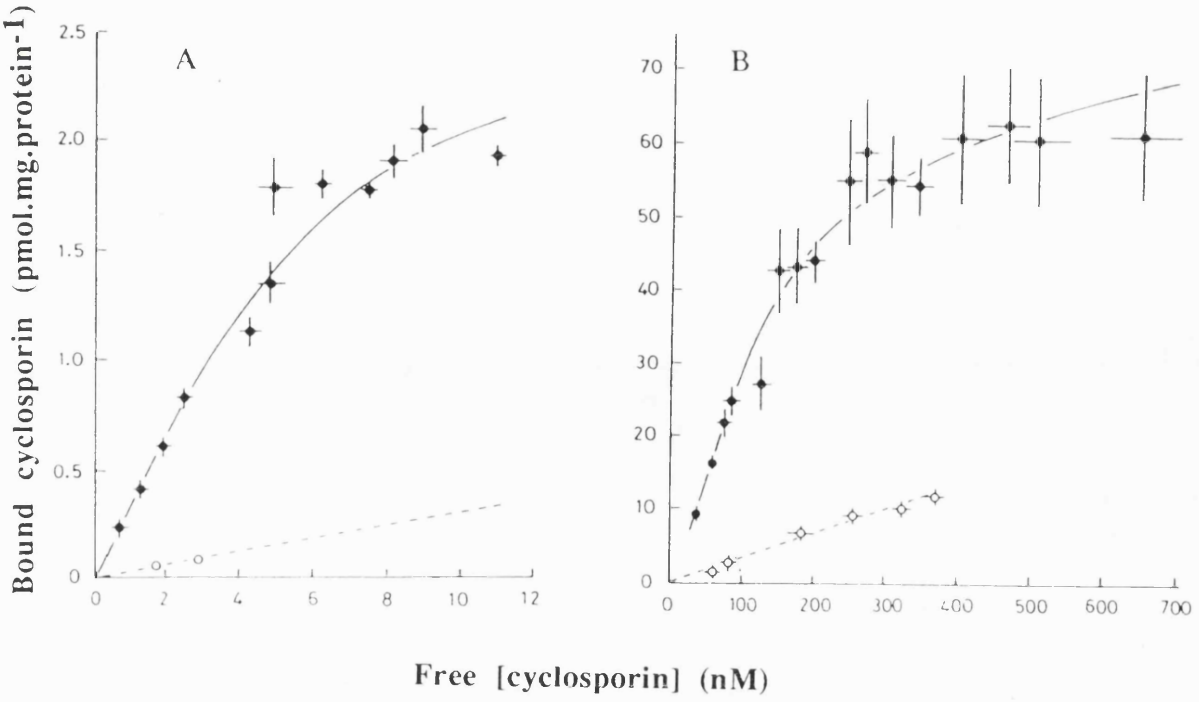
(Chapter 2; section 2.9). The task proved more difficult than expected, as CsA in aqueous solution bound variably to glass and plastic ($\simeq$ 20-40% adhered). Calculations of the amount bound to mitochondria were therefore based on the free ^{3}H-CsA in samples withdrawn from the incubate before and after sedimentation of mitochondria. The amounts that adhered to the sides of the incubation vessel were obtained from the difference between the ^{3}H-CsA added and the total CsA recovered in the incubate from the sample before mitochondrial sedimentation.

Fig. 4.2(A) illustrates the binding of CsA to rat liver mitochondria over the range 0.7-12nM CsA and reveals a binding component of high affinity. Binding over the extended range 35-700nM CsA, where binding again approached saturation, is shown in Fig. 4.2(B).

As CsA is a highly lipophilic molecule, and may simply dissolve in the lipid bilayer, the degree of CsA partitioning into mitochondrial phospholipids was also estimated. This was achieved using extracted rat liver mitochondrial phospholipids in the form of sonicated vesicles. To facilitate the comparison of ^{3}H-CsA partitioning into phospholipids with ^{3}H-CsA binding to mitochondria the phospholipid content of mitochondria was determined (Chapter 2; section 2.11). The phospholipid content of liver mitochondria amounted to $140\pm31\mu$g/mg of mitochondrial protein (mean $\pm$ S.E.M; four mitochondrial preparations). This value compares well with that measured in liver mitochondria from other species (145-159μg/mg mitochondrial protein [Munn, 1974]). CsA binding to phospholipid was therefore expressed in pmoles/140μg of phospholipid corresponding to the amount of CsA/mg of protein that would be expected to partition into the bilayer in mitochondria. It can be seen from Fig. 4.2(A) and (B) that CsA binding to phospholipid (dotted line) was non-saturable and constituted only a small fraction of the total CsA bound. The "solubility" of CsA in the phospholipid phase was 0.3pmol/mg mitochondrial protein.nM^{-1} (Table 4.1).

CsA binding components were resolved by Scatchard analysis, as shown in Fig. 4.2(C). Thus two CsA binding components, a high affinity component (I) and a lower affinity component (II) exist as well as non-specific partitioning of CsA into phospholipid (III). In the latter instance the Scatchard plot is parallel to the χ axis confirming that the partitioning was non-saturable over this range of CsA. This mixture of components makes the Scatchard plot difficult to analyse quantitatively.

Fig. 4.2. *[^{3}H]-Cyclosporin binding to liver mitochondria. (A;B)* The relationship between free and bound CsA in mitochondria (●) and in mitochondrial phospholipid (○, pmol cyclosporin/ 140μg phospholipid, see text). Values are means ± S.E.M. with four mitochondrial preparations and four determinations with mitochondrial phospholipids. (C) Data presented as a Scatchard plot. All incubations were carried out in medium containing 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 2mM KH_2PO_4, 5mM succinate and a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer giving finally 1.2mM free Mg^{2+} and 0.1μM free Ca^{2+}.


A
B
Bound cyclosporin (pmol.mg.protein-1)
Free [cyclosporin] (nM)

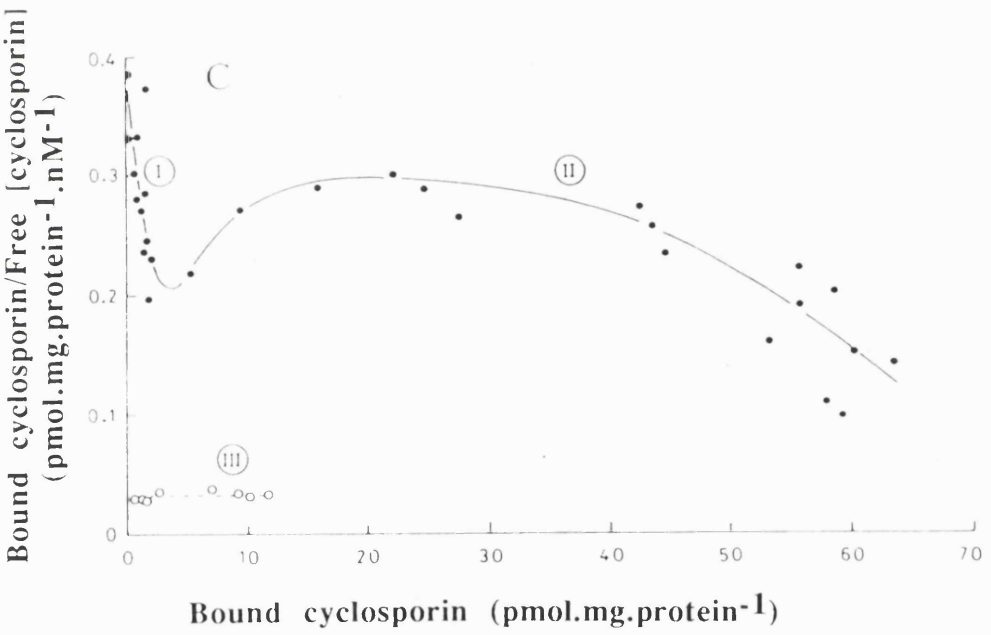

C
I
II
III
Bound cyclosporin/Free [cyclosporin] (pmol.mg.protein-1.nM-1)
Bound cyclosporin (pmol.mg.protein-1)

Fig. 4.2

However, some conclusions can be drawn. Component I bound <5pmol/mg of mitochondrial protein with high affinity (kd<12nM), in a hyperbolic manner. Component II bound around 60pmol/mg of mitochondrial protein with a lower affinity. It is half saturated at ≈100nM CsA. In this instance the Scatchard plot exhibits a maximum at 20-30pmol of CsA bound/mg of mitochondrial protein which is indicative of cooperative binding.

Table 4.1: *Summary of cyclosporine A interactions*
Affinities are given as K_d values or the [CsA] yielding half maximal occupancy or inhibition ($K_{0.5}$).

Component	CsA affinity (nM)	No. of CsA binding sites (pmol/mg mitochondrial protein)
Component I	$<12(K_d)$	< 5
Component II	$\approx 100(K_{0.5})$	≈70
Phospholipids		0.03 ($.nM^{-1}$)
Pore opening	$25(K_{0.5})$	<6
PPIase[a]	$7.8\pm1.0(K_d)$	4.5 ± 0.4[b]

a Measurement of PPIase(peptidyl-prolyl-*cis-trans*-isomerase) activity is described below.
b Calculated as 2xtotal bound CsA giving half maximal inhibition (mean±SEM; 4 preparations).

The apparent CsA "affinity" of mitochondrial "permeabilization" under buffered Ca^{2+} conditions (Fig. 3.9(B)) did not correlate with the CsA binding capacity of either component I or component II. Involvement of component II, the membrane component, is certainly excluded. Thus, maximal inhibition of pore opening was obtained with 30nM CsA (Fig. 3.9(B); Table 4.1). From Fig. 4.2(B) it is clear that at 30nM free CsA about 6 pmoles of CsA are bound to component I plus component II. Thus, CsA inhibition of pore opening (as assessed by ^{14}C-sucrose entrapment)

was maximal when $<10\%$ of component II (maximal binding capacity $\approx$ 70 pmole/mg) was occupied by CsA.

[4.3] ***Ca^{2+} depresses binding of cyclosporin A to component I:***

However, participation in "permeabilization" of component I is not excluded. Firstly, looking at a summary of the cyclosporin interactions in Table II, there was a good correlation between the total amount of bound CsA yielding maximal pore inhibition (6 pmol/mg) and the maximal capacity of component I. Secondly, in causing resealing in the presence of Ca^{2+} (section 4.3), CsA in effect renders the mitochondria Ca^{2+}-insensitive. It is conceivable that one way in which this might occur is by an overall competition between Ca^{2+} and CsA for the relevant component. CsA binding to mitochondria was therefore re-examined over the range 0.5-30nM CsA with 0.1μM and 5.6μM extramitochondrial Ca^{2+}. These Ca^{2+} concentrations were chosen since they are those that lead to mitochondrial Ca^{2+} accumulation that is sufficient (5.6μM external Ca^{2+}) and insufficient (0.1μM external Ca^{2+}) to induce pore opening in the absence of CsA (Chapter III). A Scatchard analysis of the results is shown in Fig. 4.3. In agreement with the above hypothesis it can be seen that increased Ca^{2+} depressed the binding of CsA to component I by 30-50%. This Ca^{2+}-dependent decrease in CsA binding to component I further implicates this component in the "permeablization" process.

[4.4] ***Cyclosporin A inhibition of mitochondrial peptidyl-prolyl-cis-trans-isomerase (PPIase) activity.***

PPIase activity was measured using an assay based on the ability of chymotrypsin to cleave a test peptide N-succinyl-ala-ala-pro-phe-nitroanilide only when the ala-pro is *trans* (Chapter 2; section 2.1.3). Initial experiments indicated that rat liver mitochondria do contain isomerase activity. This activity was largely latent being increased approximately 6-fold on disruption of mitochondria by sonication after which 90% of the activity resided in the soluble fraction .

Fig. 4.4(A) shows the typical absorbance changes which occur in the period beginning 1s after starting the PPIase assay with test peptide. Pre-existing *trans* (85%) is cleaved in the mixing-time, but cleavage of the *cis* peptide is limited by the rate of isomerization of the ala-pro bond and this hydrolysis is represented by the

Fig. 4.3: *The effect of Ca^{2+} on [^{3}H]-cyclosporin binding to mitochondria.* A Scatchard analysis of the relationship between free and bound cyclosporine A in mitochondria incubated with insufficient (0.1μM, ○) and sufficient (5.6μM, ●) extramitochondrial free Ca^{2+} to promote "permeabilization". All incubations were carried out in medium containing 120mM KCl, 10mM Hepes (K^+ salt), 1μg rotenone/mg protein, 2mM KH_2PO_4, 5mM succinate and a 1mM EGTA/ 1mM HEDTA Ca^{2+}/Mg^{2+} buffer giving the free [Mg^{2+}] and [Ca^{2+}] indicated.

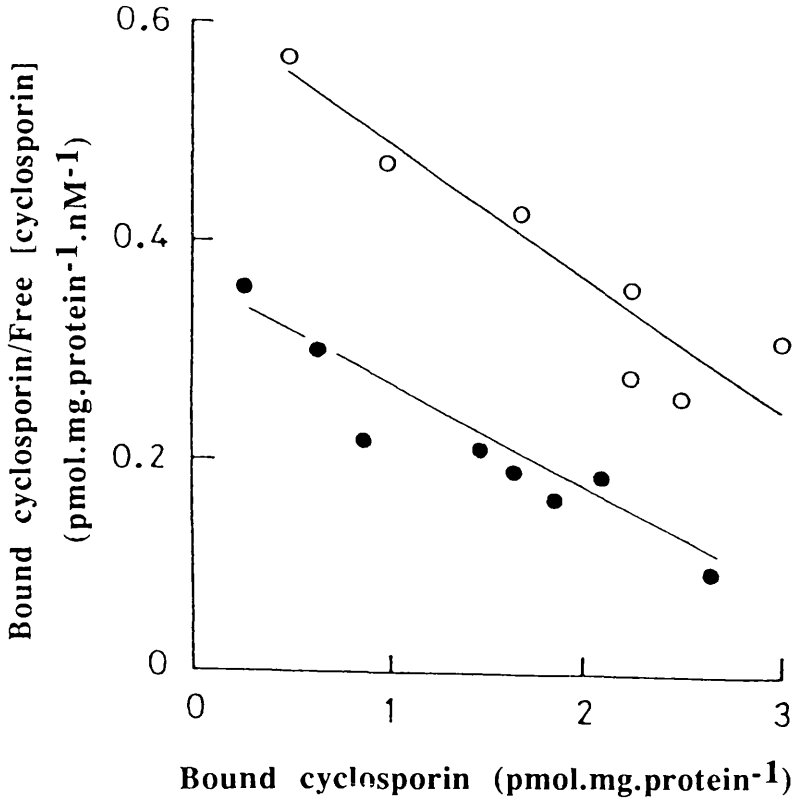

Bound cyclosporin/Free [cyclosporin]
(pmol.mg.protein-1.nM-1)
0.6
0.4
0.2
0
0
1
2
3
Bound cyclosporin (pmol.mg.protein-1)

Fig. 4.3

Fig. 4.4. *Cyclosporin inhibition of peptidyl-prolyl*-cis-trans *isomerase activity in sonicated mitochondria.* (A) Absorbence versus time after addition of test peptide with (i) no mitochondria, (ii) sonicated mitochondria, and (iii) sonicated mitochondria plus 10nM free CsA. The absorbance change due to hydrolysis of preexisting *trans*-peptide occurred within the mixing time (1s) and is not shown. (B) Data from A are plotted according to the relation $\ln(A_t/A_0)=-kt$ where A_t is the absorbance change that occurred subsequent to any time t , and A_0 is the maximal absorbance change recorded in curve (i). (C) The rate constants (k_{isom}) for *cis-trans*-isomerization were obtained from plots of the type shown in B; they were corrected for the rate of isomerization in the absence of mitochondria and refer to isomerase activity.

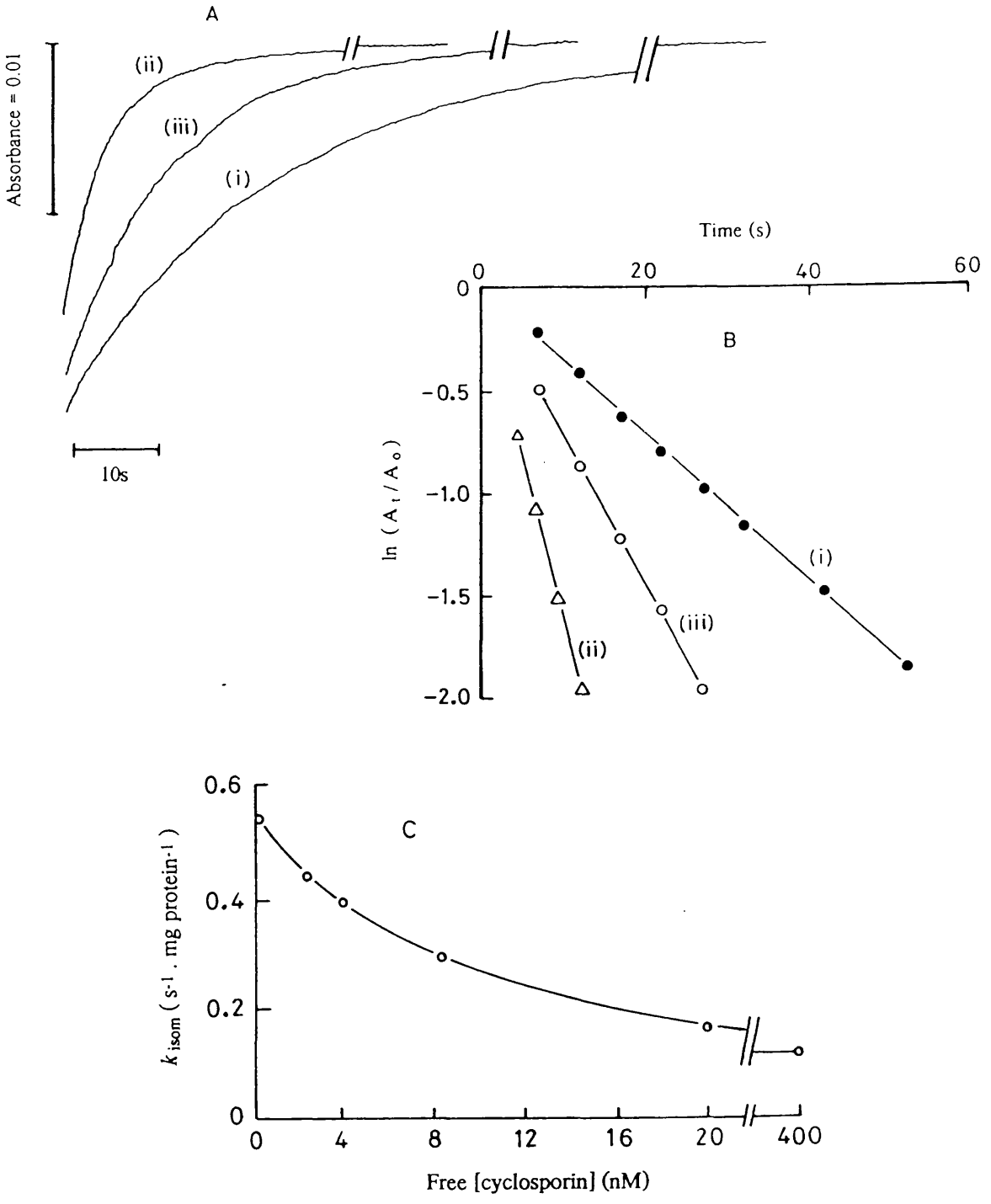

A
Absorbance = 0.01
(ii)
(iii)
(i)
10s
Time (s)
0
20
40
60
B
0
-0.5
-1.0
-1.5
-2.0
ln (A t / A o)
(i)
(iii)
(ii)
C
0.6
0.4
0.2
0
k isom (s-1 . mg protein-1)
0
4
8
12
16
20
400
Free [cyclosporin] (nM)

Fig. 4.4

absorbance changes shown here. Some isomerization occurred spontaneously in buffer alone (curve (i)). In the presence of sonicated rat liver mitochondria the rate of isomerization was stimulated (curve (ii)). 10nM free cyclosporine inhibited the isomerase (curve (iii)), but had no effect on isomerization in the absence of mitochondrial protein (not shown).

As shown in Fig. 4.4(B) these curves were resolved by plotting semi-logarithmic plots according to the relation (Chapter 2; section 2.1.3)):

$$\ln(A/A_{\infty}) = -\kappa t$$

where A is the absorbance change that occurred subsequent to any time t, and A_{∞} is the maximal absorbance change recorded. The shift A_0 was always determined from the slower rate of isomerization seen in the presence of buffer alone (curve (i)). The fact that the first second of the reaction was unrecorded introduced a small innaccuracy in determining the total absorbance shift associated with isomerization (A_{∞}). However this was insufficient to introduce significant error as judged by the fact that semi-logarithmic plots were obtained. This analysis allowed first-order rate constants to be determined.

Fig. 4.4(C) shows the sensitivity of mitochondrial PPIase to the free [CsA]. The free CsA was estimated from the CsA binding curves (Fig. 4.1 A;B), knowing the total [CsA] added and the quantity of mitochondrial protein in the assay. In other words, it was assumed that intact mitochondria and sonicated mitochondria bound CsA to the same extent at any [CsA]. Note that the isomerase activity is incompletely inhibited by CsA. Approximately 6.5nM free [CsA] yielded half maximal inhibition, and with four preparations the mean value was 7.8 ± 1.0 nM. The fact that the PPIase is soluble and that it is inhibited by CsA indicates that it is probably cyclophilin. Later results (Chapter 6) showed that the PPIase has a MW of $\approx 18{,}000$ confirming that it is indeed cyclophilin. The affinity of CsA for the PPIase reported here is similar to that reported for whole kidney cyclophilin (3-20nM [Fischer *et al.*, (1989) and Takahashi *et al.*, (1989)].

The affinity of the PPIase for CsA is consistent with it being component I which has a K_d of <12nM (Table 4.1). The number of CsA binding sites for PPIase, calculated as 2 x total bound CsA giving half-maximal inhibition was 4.5 ± 0.4

pmol/mg of mitochondrial protein is again consistent with it being component I which has a maximal binding capacity of <5 pmol CsA/mg of mitochondrial protein. (Table 4.1). Unlike component I however, the sensitivity of PPIase activity to cyclosporine was unaffected by $[Ca^{2+}]$ (to 5.6 μM; data not shown). Thus if PPIase and component I are indeed identical, there would need to be a mechanism which renders CsA binding to this protein Ca^{2+}-sensitive in whole mitochondria. One possibility is that in intact mitochondria PPIase binds to a protein (possibly a pore component) when that protein is complexed with Ca^{2+} and that when bound in this way, no longer interacts with CsA. In other words, there would be effective competition between Ca^{2+} and CsA for the PPIase in intact mitochondria and, for some reason, the competition is lost on sonication.

[4.5] ***The effect of cyclosporin A on pore-state interconversion.***

Although the *in vivo* function of PPIase is unknown, its isomerase activity accelerates the refolding of a number of proteins *in vitro* [Fischer and Schmid, (1990)]. As already mentioned in Chapter 3; section 3.2 there is already evidence for a continual interconversion of pores between the closed and open states in "permeabilized" mitochondria [Al Nasser and Crompton (1986a, 1986b); Crompton and Costi (1990), Antonenko *et al.*, (1991)], and since any such interconversion would entail reversible conformational change in the pore protein, it was relevant to examine isomerase involvement.

Fig. 4.5 shows the results of an experiment , in which mitochondria were subjected to a 15min permeabilization (sufficient to induce maximal pore opening, Fig 3.9(A)) before addition of CsA. In the absence of CsA, sucrose entered the permeabilized mitochondria rapidly, but CsA depressed sucrose entry provided that a few minutes exposure to the inhibitor was allowed. After an 8 min exposure, the rate of sucrose entry was no greater than when cyclosporine was present throughout the incubation period. The fact that CsA reversed permeabilization excludes direct catalysis of pore closure by the isomerase since, if that were the case, CsA would be predicted to maintain the permeabilized state rather than reverse it.

Fig. 4.5: *The effect of cyclosporin on [^{14}C]-sucrose entry into permeabilized mitochondria..* (●) Mitochondria were "permeabilized" maximally (15-min incubation) before addition of [^{14}C]-sucrose at zero time in the figure; CsA was not added. (○, ▲) The "permeabilized" mitochondria were further preincubated with 1μM cyclosporine for either 2 min (○) or 8 min (▲) before addition of [^{14}C]-sucrose at time zero. (▵) 1μM CsA was added before permeabilization was started.

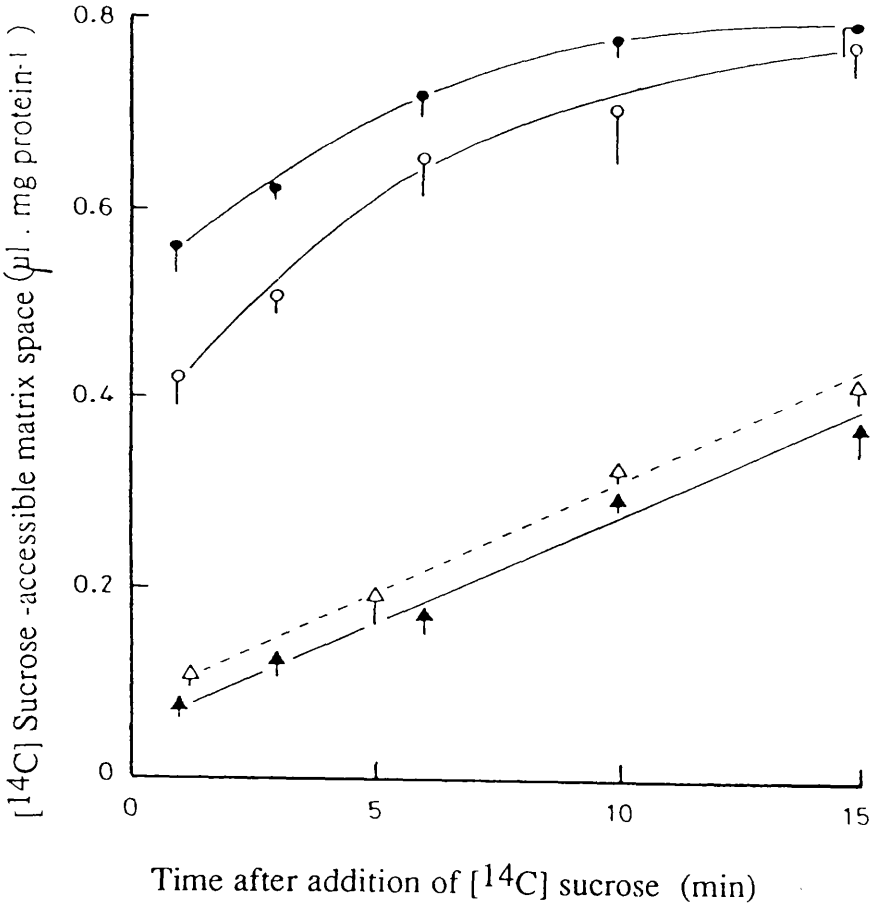

0.8
0.6
0.4
0.2
0
0
5
10
15
[14C] Sucrose -accessible matrix space (µl . mg protein-1)
Time after addition of [14C] sucrose (min)

Fig. 4.5

[4.6] ***Cyclosporin A binding to extensively washed sub-mitochondrial particles.***

As already stated, PPIase activity is largely latent in intact mitochondria and is increased three-fold on sonication. Fig. 4.6 shows the sensitivity of the soluble matrix fraction, and the membrane fraction to the total [CsA]. Here 7.5nM CsA yielded half-maximal inhibition of the soluble activity; on the other hand, the PPIase activity in the membrane fraction was essentially CsA-insensitive, being decreased by $<20\%$ by 25-450μM CsA. Thus $>97\%$ of the CsA sensitive isomerase activity was soluble.. Further experiments indicated that the remaining 3% of CsA sensitive activity could be removed from the membrane fraction by extensive washing.

The question of whether or not CsA binding component I is PPIase was pursued by determining whether or not component I is also soluble. This involved examining whether or not component I is lost from the membrane on sonication. Fig. 4.7 (A,B) reports the binding of ^{3}H-CsA to sonicated sub-mitochondrial particles and compares this to the binding of ^{3}H-CsA to whole mitochondria (data taken from Fig. 4.1). To facilitate comparison, the binding data are given in the same units (per mg mitochondrial protein) and are corrected for binding to phospholipids. It is evident that sub-mitochondrial particles bind ^{3}H-CsA in a sigmoidal manner with increasing [^{3}H-CsA] (Fig. 4.6A), so that the quantity bound/free (Scatchard plot, Fig. 4.7B) increases with increasing free CsA. Although a complete binding curve was not obtained, the sigmoidicity is reminiscent of binding to component II (Fig. 4.1), suggesting that this component may be membrane bound. On the other hand, there is no evidence (Fig. 4.7B) for the presence of component I in sub-mitochondrial particles. It seems therefore that component I, like PPIase is released into the soluble fraction on sonication, which is consistent with their being the same protein.

[4.7] ***Cyclosporin A binding to mitochondrial fractions assessed by equilibrium dialysis:***

From the data presented in previous sections, it appears that CsA binding component I may be PPIase since both components are soluble (Figs 4.6 and 4.7B). and the amount of component I estimated from Scatchard plots of binding to whole mitochondria (Table 4.1) is comparable with the amount of PPIase (table 4.1; again the estimation is based on CsA binding to whole mitochondria). In view of the problems inherent in trying to quantify binding to individual components from

Fig. 4.6. *Cyclosporin inhibition of peptidyl-prolyl*-cis-trans *isomerase activity* . Inhibition in :(●) the soluble matrix fraction and ;(○) in sub-mitochondrial particles. The rate constants for isomerization were obtained as described in the legend to Fig. 2.2..

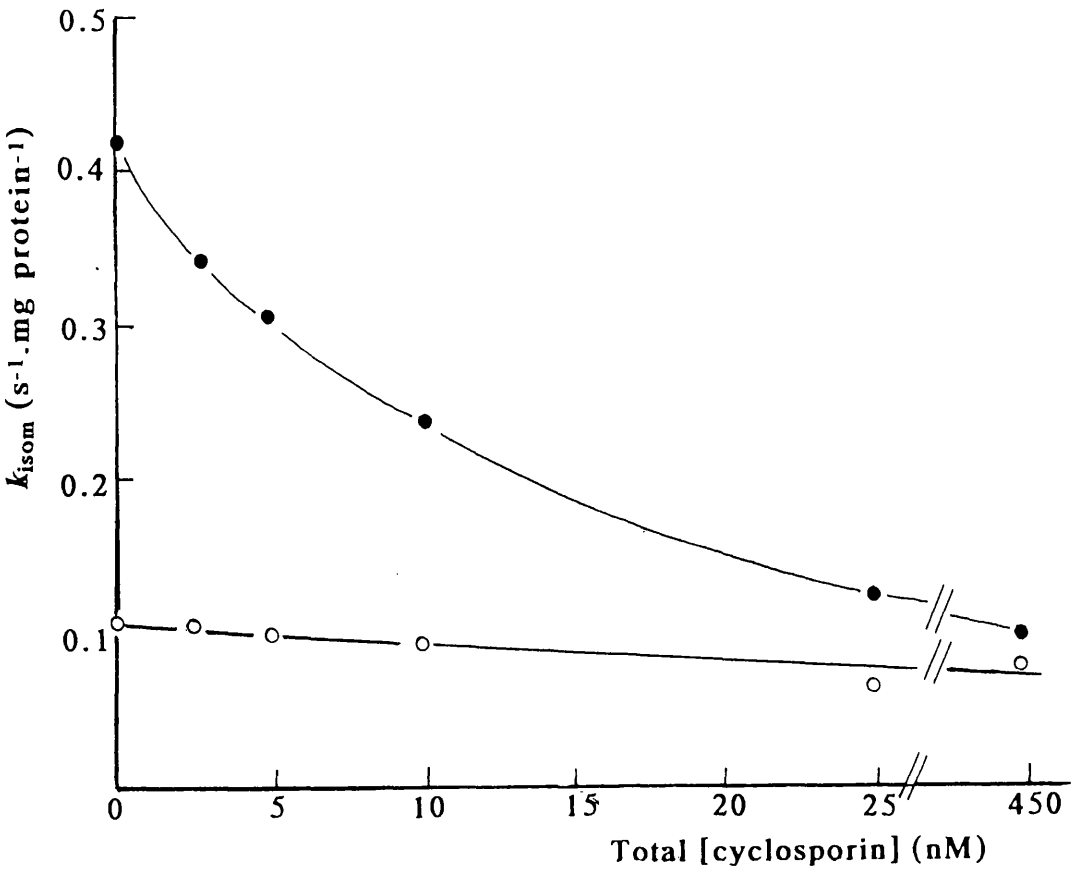

0.5
0.4
0.3
0.2
0.1
k_{isom} (s^{-1}.mg protein^{-1})
0
5
10
15
20
25
450
Total [cyclosporin] (nM)

Fig. 4.6

Fig. 4.7. *[^{3}H]-Cyclosporin binding to washed (8x) sub- mitochondrial particles.* In (A) the binding of ^{3}H-CsA to the membranes of submitochondrial particles (●) is given in terms of total mitochondrial protein (membrane protein/total mitochondrial protein = 0.33). Data are given as means ± S.E.M. (bars) of four determinations with separate particle preparations. In (B) the binding of ^{3}H-CsA to the membranes of submitochondrial particles (○) is again given in terms of total mitochondrial protein. These units are used to enable ready comparison with CsA binding to whole mitochondria (●). Here data are presented as a Scatchard plot. All data are corrected for the binding of ^{3}H-CsA to mitochondrial phospholipids (0.3 pmol CsA/mg mitochondrial protein, nM; Fig. 4.2).

Bound cyclosporin (pmol.mg.protein-1)
0
1
2
3
4
0
5
10
15
Free [cyclosporin] (nM)
A.
Bound cyclosporin/Free [cyclosporin]
(pmol.mg.protein-1.nM-1)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
Bound cyclosporin
(pmol.mg.protein-1)
B.

Fig. 4.7

(complex) Scatchard plots, it seemed important to measure CsA binding activity directly to the soluble component(s). This was approached by equilibrium dialysis. Preliminary experiments used whole mitochondria to determine the incubation time required for equilibration of ^{3}H-CsA using this technique.

Fig. 4.8 shows the amounts of free and bound CsA at various intervals during dialysis ± the protease inhibitor phenyl-methyl-sulphonyl fluoride (PMSF). It should be noted that in these experiments the amount of bound plus free CsA was only a small fraction (< 12%) of the total cyclosporin added. Presumably the bulk of the CsA stuck to the Perspex of the dialysis apparatus itself and to the dialysis membrane itself. Interestingly PMSF, a covalently binding inhibitor of serine proteases which is reactive at nucleophilic serines destroys the CsA binding potential of the components. At face value, one might postulate that a nucleophilic serine residue is involved in the binding of CsA to its mitochondrial ligands. However, the half-life of PMSF as a serine protease inhibitor in aqueous buffers is only a couple of hours [James (1978)], so the loss of binding seen here after 40hr cannot be explained in this way. That CsA binding was being measured despite the fact that only a small fraction of the CsA added was involved in binding is confirmed by the complementary changes in free CsA seen.

The results show that > 30 hr incubation was required for equilibration of binding in the equilibrium dialysis set-up. However the amounts of CsA bound were greater than those obtained after mitochondrial sedimentation (Fig. 4.1A). Thus equilibrium dialysis yielded 4-6pmole CsA bound at 4-5nM free CsA which is about 4x that observed previously.

In order to examine the contribution of the soluble matrix and membrane components to binding, the binding of [^{3}H]-CsA (with 12nM and 300nM "cold" CsA) to washed sub-mitochondrial particles and to the soluble matrix fraction was again measured by equilibrium dialysis (40hrs). The results of this study are summarised in Table 4.2 below:

Table 4.2: *Cyclosporin A binding to the soluble mitochondrial matrix fraction and to the mitochondrial membrane fraction measured by equilibrium dialysis.*

Fraction	[CsA][a] (nM)	Bound (pmol/mg) (nM)	Free	Bound/Free (pmol/mg.nM^{-1})
Matrix	*12*	*0.46± 0.05*	*0.92± 0.07*	*0.5*
	300	*12.59± 1.2*	*23.2± 1.4*	*0.52*
Membrane	12	0.68± 0.08	0.75± 0.07	0.91
	300	10.9± 0.89	14.1± 2.2	0.77

[a]This was the total [CsA] added to the incubation.

[b]Values are given as the mean +S.E.M. (3 determinations).

As can be seen, the amount bound in pmol/mg by each fraction is roughly the same. Again the amounts bound were several fold higher than those measured in previous studies. This suggests either that : (a.) 8 min preincubation of mitochondria in the binding (Fig. 4.2) and inhibition (Fig. 3.9) studies was insufficient to equilibrate the binding proteins (notwithstanding (Fig. 4.1), or (b.), that significant protein denaturation occurred during the 40 hr equilibrium dialysis incubations and that a significant proportion of the binding measured occurred to denatured proteins. CsA is highly lipophilic and, it might be anticipated, would equilibrate rapidly across the lipid bilayer. Nevertheless it was decided to explore whether non-equilibration was the cause of the lower binding measurements obtained earlier by introducing an aqueous pore forming agent, calmidazolium, into the inner mitochondrial membrane. These studies are described in the next section.

Fig. 4.8: *[^{3}H]-Cyclosporin binding to mitochondria measured by equilibrium dialysis.* These assays were performed ± the serine protease inhibitor PMSF as indicated.

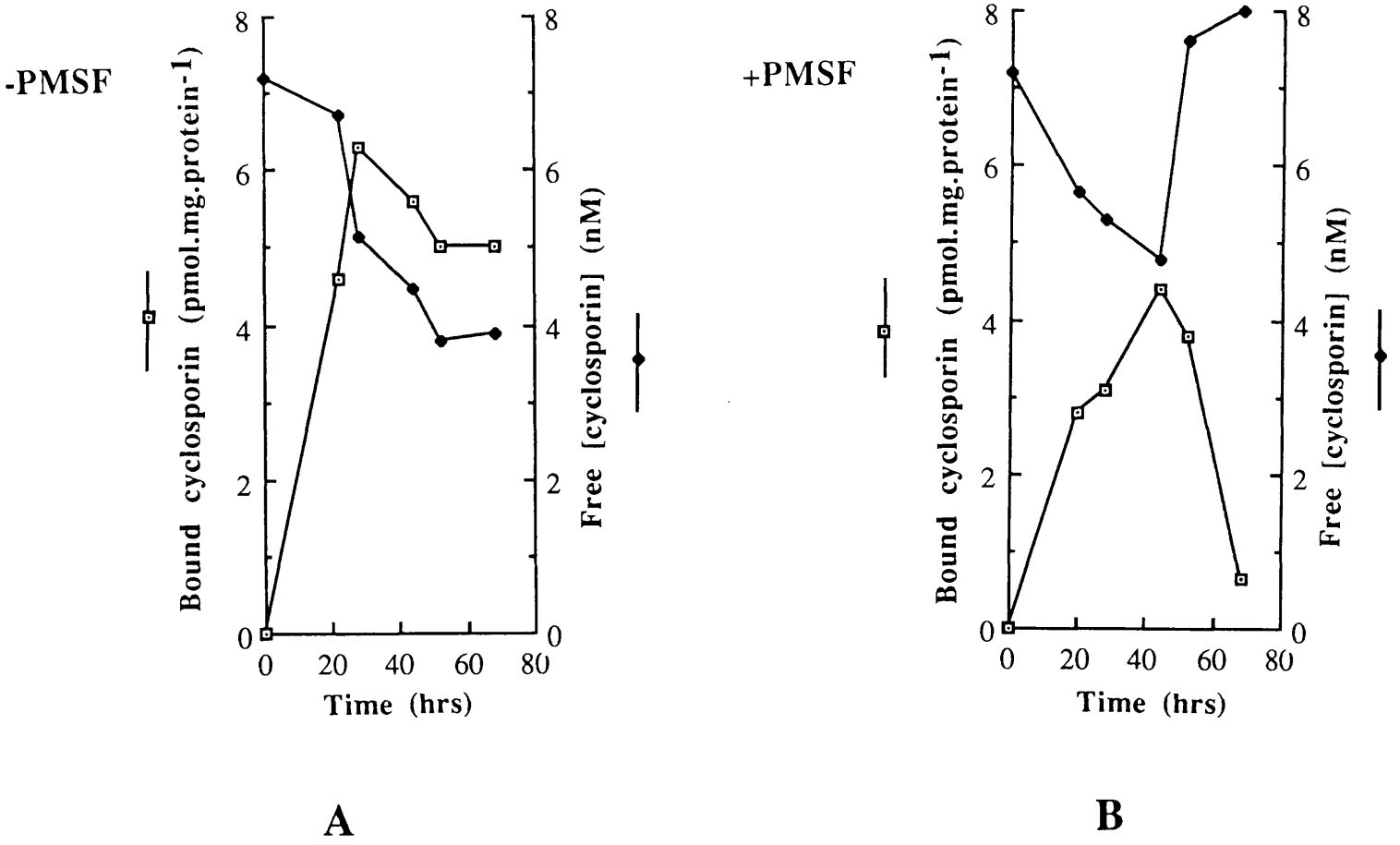

-PMSF
+PMSF
Bound cyclosporin (pmol.mg.protein-1)
Free [cyclosporin] (nM)
Time (hrs)
0
2
4
6
8
0
20
40
60
80
A
B

Fig. 4.8

[4.8] ***The effect of the calmidazolium on cyclosporin binding to mitochondria.***

There is evidence that calmidazolium permeabilizes cells. Lükhoff *et al* (1989) have shown that calmidazolium releases the Ca^{2+} indicator indo-1 from endothelial cells, and eventually kills the cells, leading to losses of protein e.g. lactate dehydrogenase. Studies in the M. Crompton laboratory (unpublished) have shown that calmidazolium ($>1\mu M$) induces rapid release of the polar fluorescent indicator calcein (MW) entrapped in phospholipid vesicles. Indeed, in the next chapter (Chapter 5) it is shown that calmidazolium releases ^{14}C-sucrose entrapped in submitochondrial particles. Calmidazolium was therefore used as the pore forming agent that might allow CsA to equilibrate more rapidly with its binding sites. CsA binding to rat liver mitochondria was therefore analysed over the range 0-160nM CsA in the presence of $10\mu M$ calmidazolium. The results are illustrated in Fig. 4.9 and it can be seen that calmidazolium increases CsA binding to mitochondria across the range by $\simeq$ three-fold.

These findings mean that the earlier measurements without calmidazolium may have underestimated the binding capacities of both component I and/or component II. However, it is not possible to deduce from the Scatchard plot, which component, or whether a further binding component binds increased CsA in the presence of calmidazolium. Also, it must be stressed that the calmidazolium induced increase in CsA binding does not negate the conclusions drawn regarding which component is affected during CsA-inhibition of pore activation, since both the binding studies and the inhibition studies were conducted after an incubation period with CsA of 8 min. Fig. 4.1 would appear to indicate that CsA has equilibrated with the component involved in inhibition of "permeabilization".

Finally, the effects of a range of calmodulin antagonists and Ca^{2+} channel blockers on CsA binding to rat liver mitochondria was examined at the total CsA concentrations of 4nM and 75nM to ensure that the increases in binding seen with calmidazolium were not due to the more familiar property of this drug as a calmodulin antagonist. The histogram in Fig 4.10 illustrates that calmidazolium only had a pronounced effect on CsA binding to mitochondria at the two concentrations shown.

Fig. 4.9: *[^{3}H]-Cyclosporin binding to mitochondria in the presence of the calmodulin antagonist calmidazolium resolved by Scatchard analysis.* The relationship between bound and bound/free CsA in the presence (○) and absence (♦) of calmidazolium.

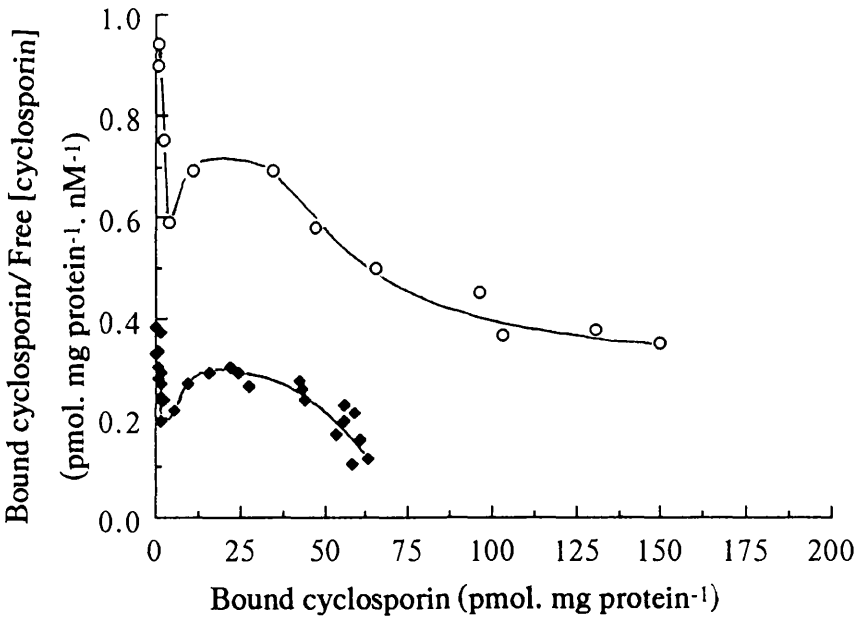

Bound cyclosporin/ Free [cyclosporin]
(pmol. mg protein-1. nM-1)
1.0
0.8
0.6
0.4
0.2
0.0
0
25
50
75
100
125
150
175
200
Bound cyclosporin (pmol. mg protein-1)

Fig. 4.9

Fig. 4.10: *The effect of a range of* Ca^{2+}*/calmodulin antagonists on CsA binding to rat liver mitochondria..*

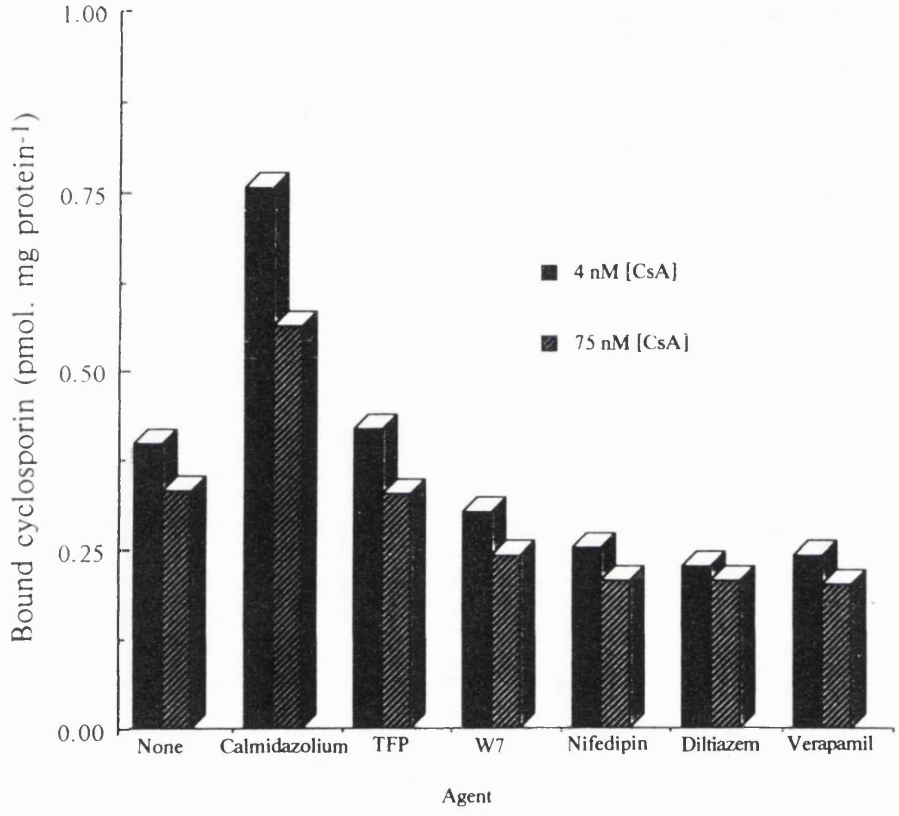

1.00
0.75
0.50
0.25
0.00
Bound cyclosporin (pmol. mg protein-1)
4 nM [CsA]
75 nM [CsA]
None
Calmidazolium
TFP
W7
Nifedipin
Diltiazem
Verapamil
Agent

Fig. 4.10

As shown in the Table 4.3 below, neither calmidazolium nor the two calmodulin antagonists W7 and trifluoperazine had any effect on CsA binding to phospholipid vesicles after incubation for 8 min.

Incubation	[CsA] Bound (pmol/mg)	[CsA] Free (nM)	Bound/Free (pmol/mg.nM^{-1})
Control	2.34±0.10	62.6±9.8	0.037
Calmidazolium	2.69±0.04	58.1±1.6	0.045
Trifluoperazine	2.52±0.21	63.6±1.7	0.039
W7 (R24571)	2.02±0.31	74±5.5	0.028

Table 4.3. *The effect of various calmodulin antagonists on ^{3}H-CsA binding to phospholipid vesicles.*

Thus the increase in binding caused by calmidazolium is not due to an increased partitioning of CsA into phospholipid vesicles. Part of component I, II or other CsA binding components normally masked become readily accessible to CsA on permeabilization of mitochondria by calmidazolium.

CHAPTER 5: INVESTIGATION OF PORE ACTIVITY IN SUB-MITOCHONDRIAL PARTICLES.

[5.1] *Background*.

The discovery that CsA probably interacts with soluble mitochondrial cyclophilin to induce pore blockage, prompted an investigation of pore activity in sub-mitochondrial particles. Patch clamp studies of the 1.3 nanosiemens pore/channel in rat liver mitoplasts have shown that CsA acts on the matrix side of the inner membrane [Szabo and Zoratti (1990)]. Inhibition takes place erratically when CsA is added to the matrix side of excised patches. This could be due to the random loss of cyclophilin, or some other matrix constituent that confers CsA sensitivity. A demonstration of pore activity in sub-mitochondrial particles would provide a simple assay system to assess whether or not it was the CsA-cyclophilin complex which caused pore blockage or, alternatively, demonstrate cyclophilin's direct involvement in pore opening.

Sub-mitochondrial particles were prepared by ultrasonic disintegration of mitochondria as described in the Chapter 2; section 2.3. The bulk of the sub-mitochondrial particles produced in this way are "inside-out" i.e. the inner face of the inner mitochondrial membrane normally inaccessible is exposed to the incubation medium. Proteins and other low molecular weight constituents including nucleotides normally present in the matrix space are released during sonication. These constituents could thus be selectively reintroduced at what would normally be the inner face of the inner mitochondrial membrane allowing an investigation of their possible role in pore activity. Another consequence of the inverted orientation of sub-mitochondrial particles is that the potential they develop across their membranes on addition of succinate as substrate is in the reverse orientation to that developed in mitochondria. Ca^{2+} is thus electrophoretically expelled from sub-mitochondrial particles (rather than being accumulated) via the *uniporter*. As will be explained in the Chapter 7; section 7.1, rises in buffered extramitochondrial [Ca^{2+}] cause a disproportionate increase in the matrix [Ca^{2+}] due to a phenomenon known as *relay amplification*. For this reason, one needs to add 100-200μM unbuffered Ca^{2+} to an incubation of sub-mitochondrial particles in order to mimic what happens to mitochondria exposed to 5.6μM buffered free Ca^{2+}.

[5.2] ***Sub-mitochondrial particles are insensitive to the unbuffered concentrations of Ca^{2+} and Pi which normally induce pore opening in mitochondria.***

As already stated in Chapter III, H^+ ions permeate the open pore rapidly causing uncoupling in energized mitochondria, thus allowing pore state to be monitored using $\Delta\Psi$ dependent parameters. Assuming that the pore is operative in respiring sub-mitochondrial particles one should be able to apply the same assay for an investigation of its activity. A TPB^--selective electrode was therefore constructed (Chapter 2; section 2.5) for potential measurements in sub-mitochondrial particles. Sub-mitochondrial particles were prepared as for the equilibrium dialysis experiments described in the last chapter i.e. rat liver mitochondria prepared as before were sonicated in 5mM Pi and the final particle pellet was resuspended in the same buffer. This technique proved very problematic. The first hitch encountered was that the TPB^- salt formed an insoluble complex with KCl (presumably $K^+.TPB^-$) resulting in the formation of a white precipitate in the incubation. Thus the 120mM KCl, 10mM Hepes (K^+ salt) buffer was replaced with a buffer containing 20mM Hepes (Tris salt), 10mM NaCl at pH7.0, and separated from the reference electrode with a salt bridge of the same composition. Although it was possible to make measurements with this arrangement it was unsatisfactory since the measured potential drifted. This needs some explanation.

When using a reference electrode, it is essential that the liquid junction (the area of contact between the solution with which the electrode is filled and the test-solution) be free flowing i.e. that there is some flow of electrolytes into and out of the electrode. Free flow in turn generates a potential at the liquid junction which depends on the profiles of the ions in the regions where the two solutions mix i.e. on the relative mobilities of the anion and the cation [McInnes, (1961)]. The *transference number* of each ion of a solute in an electrolytic solution is the fraction of the total current carried by that ion and is given by the ratio of the mobility of the ion to the sum of the mobilities of the ions constituting the electrolytic solution. The *transference number* for K^+ in KCl is 0.4907 indicating that very nearly half the current is carried by each of the individual ions (K^+, Cl^-) constituting the electrolyte. In other words the mobilities of K^+ and Cl^- in aqueous solution are very nearly the same so that very little diffusion potential is developed across the interface with a KCl gradient. In contrast, the *transference number* for Na^+ in NaCl is 0.3918, indicating that the current is carried disproportionately by Cl^- in this electrolytic

Fig. 5.1. *Potential measurements in sub-mitochondrial particles using a TPB^--selective electrode.* Sub-mitochondrial particles (0.75 mg/ml) were preincubated for 4 min in medium (pH 7.4) containing 20mM Tris (Hepes salt), 10mM NaCl, 2mM $MgCl_2$ and oligomycin (4μg/mg of protein). Respiration was initiated by addition of 5mM succinate.

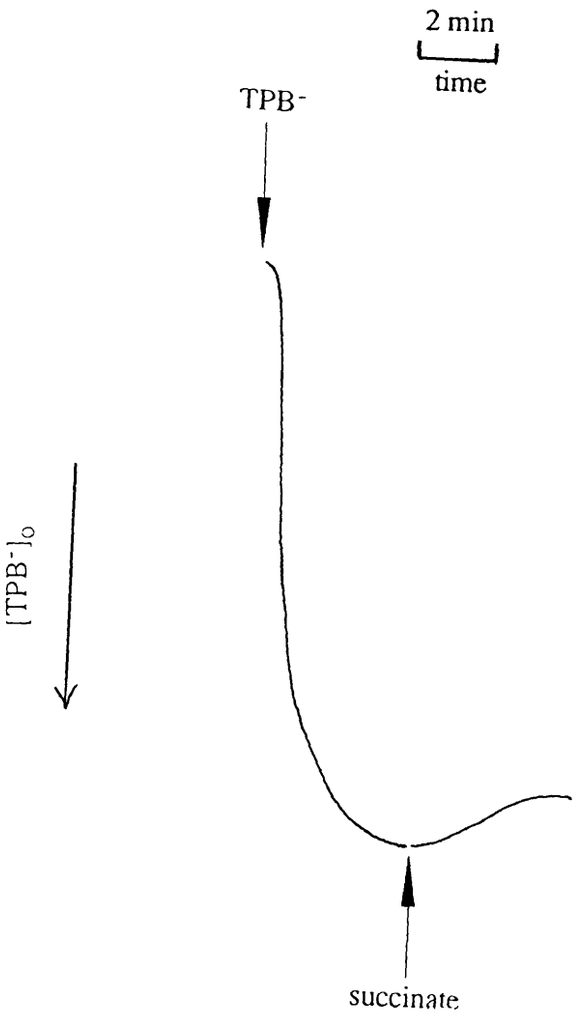


Fig. 5.1

Fig. 5.2. *Potential measurements in sub-mitochondrial particles using a TPB^--selective electrode.* Sub-mitochondrial particles (0.75 mg/ml) prepared by the method of Schatz and Racker (1966) were preincubated for 4min in medium (pH 7.0) containing 20mM Tris (Hepes salt), 10mM NaCl, 2mM $MgCl_2$, 10μM TPB^- and oligomycin (4μg/mg of protein).Succinate was added as indicated stimulating uptake of TPB^-. The time in min indicates the time the sub-mitochondrial particles were on ice before each experiment was performed. 0.1μM FCCP was added to the respiring sub-mitochondrial particles as indicated.

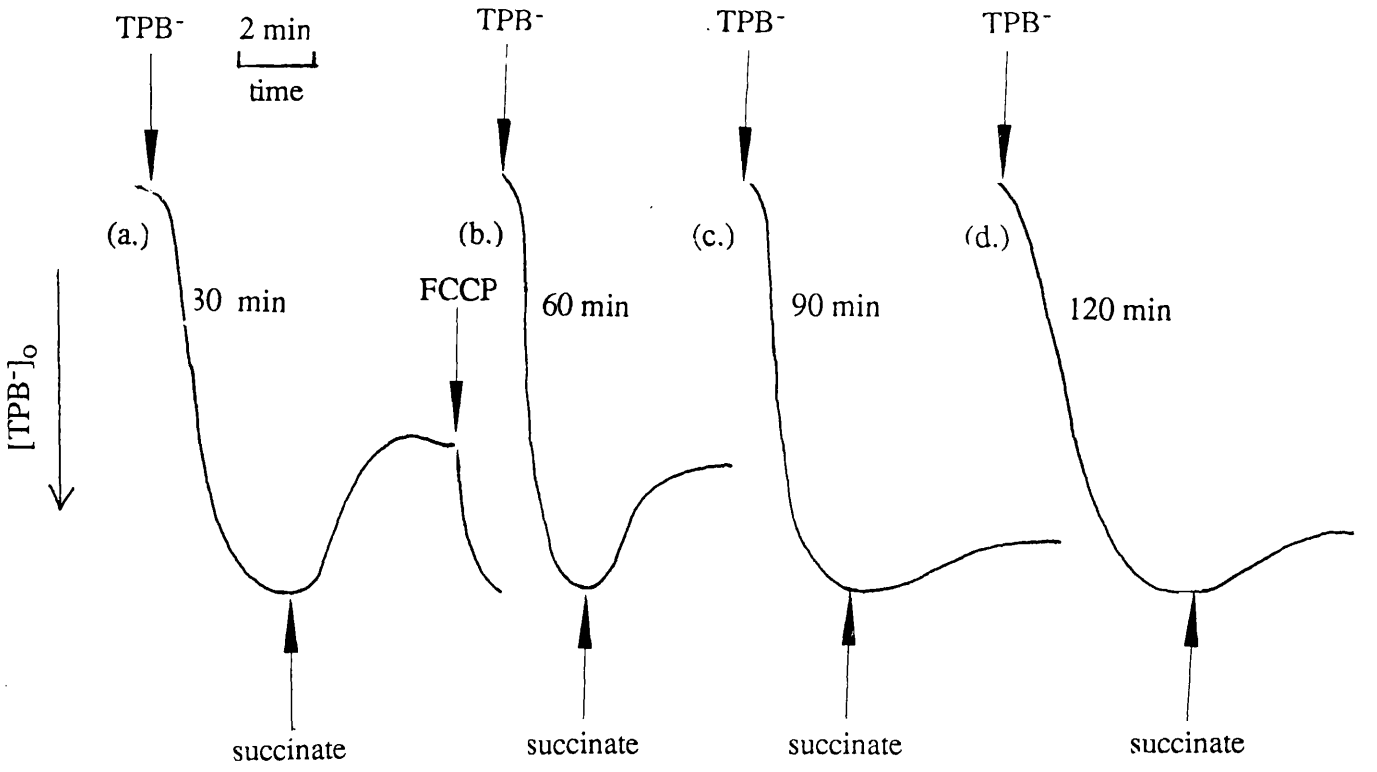

TPB⁻
2 min
time
TPB⁻
TPB⁻
TPB⁻
(a.)
(b.)
(c.)
(d.)
30 min
FCCP
60 min
90 min
120 min
[TPB⁻]o
succinate
succinate
succinate
succinate

Fig. 5.2

solution. It is this difference in mobilities of the ions constituting the solute that causes the potential drift in NaCl.

Apart from the potential drift, the % changes in potential being measured were very small and perhaps not sensitive enough to detect "permeabilization" on addition of the normal modulators of pore opening. Fig 5.1 shows the potential change measured on addition of 5mM succinate to sub-mitochondrial particles (0.75mg/ml) in medium containing 20mM Hepes (Tris salt), 10mM NaCl, 2mM $MgCl_2$ and oligomycin (4μg/mg of protein). A slight improvement was obtained by using an adaptation of the method of Schatz and Racker (1966) to prepare the sub-mitochondrial particles. Rat liver mitochondria (RLM), at a concentration of 10mg/ml were sonicated in 10mM H_2SO_4 (Tris salt), pH 7.4 and the final particle pellet was resuspended in 2mM Hepes (Tris salt),pH 7.8. The potential changes being measured are shown in Fig. 5.2. As can be seen, the ability of the particles to establish and maintain a membrane potential deteriorated rapidly making comparisons between consecutive incubations difficult. No improvement was gained by supplementing the sonication buffer with 4mM $MgCl_2$ and 1mM ATP and/or 4mM $MnCl_2$, 2mM GSH and 100μM EGTA which is supposed to improve the coupling of sub-mitochondrial particles [Hansen and Smith (1964)]. It was possible that some proteolytic degradation was occurring, however, inclusion of the serine protease inhibitor PMSF did not ameliorate the rapid deterioration of the particles. In every instance, the potential developed on addition of succinate as substrate was unaffected by 100μM Ca^{2+} and 10mM Pi, agents that cause pore opening in mitochondria.

The use of the TPB^- electrode to monitor potential changes in sub-mitochondrial particles was finally abandoned as apart from the potential drift occurring, as already pointed out, the % changes in potential being measured were very small and these measurements were perhaps not sensitive enough to detect "permeabilization" on addition of the mormal modulators of pore opening.

[5.3] ***Potential measurements in sub-mitochondrial particles using potential sensitive dyes:***

The problem of measuring potential in sub-mitochondrial particles was next approached by using fluorescence spectroscopy in conjunction with "potential

sensitive dyes". These are charged molecules whose fluorescence changes on moving from an aqueous into the lipid environment of the membrane in response to the development of surface charge.

Initially, fluorescence changes were monitored using the fluorescent cationic dye, 3,3'-dipropylthiodicarbocyanine dye (diS-C_3-(5)) described by Laris *et al* [Hoffman and Laris, P.C.(1975)]. Excitation was at 622nm and emission was at 670nm. However, no change in potential was observed on addition of succinate to sub-mitochondrial particles (0.75mg/ml of incubate) in medium (pH 7.4) containing 10mM H_2SO_4 (Tris salt), 2mM $MgSO_4$, oligomycin (4μl/mg of protein) and diS-C_3-(5) (1μg/mg of protein). Studies with erythrocytes have shown that changes in fluorescent intensity are highly dependent upon the relative concentrations of the dye and the cells. However in this study, varying the concentration of the dye and the amount of protein did not make any difference.

The method of Azzi *et al.,* (1971), using the fluorescent anionic dye amino-napthalene sulphonate (ANS), proved successful. Experiments with optical probes have indicated, that where the anion (ANS^-) or the cation (di-S-$C_3(5)^+$) redistributes with an organelle under investigation in response to the development of a potential, α larger change in fluorescence is observed when the dye is accumulated rather than ejected into the surrounding medium. Thus Azzi *et al* (1971) found that the fluorescence decrease on succinate addition to mitochondria incubated in ANS was only 50% of the fluorescence increase observed on succinate addition to sub-mitochondrial particles incubated in ANS. In contrast Hoffman and Laris, (1975) found that the % change in fluorescence on addition of succinate to sub-mitochondrial particles incubated in di-S-$C_3(5)$ was 20% as opposed to the 70% change in fluorescence seen on addition of succinate to mitochondria incubated in di-S-$C_3(5)$. Thus, this may explain the relative success of the use of ANS as an optical probe in these experiments as opposed to di-S-$C_3(5)$.

The fluorescence changes of ANS were monitored by exciting at 366nm and measuring the emitted light at 470nm. Sub-mitochondrial particles were prepared as described by Schatz and Racker (1966) as described in section 5.2 and as described by Azzi *et al., (1971).* In the latter case, RLM (10mg/ml) were sonicated in 2mM EDTA (Tris salt), pH8.4 and the final sub-mitochondrial particle pellet was resuspended in buffer containing 10 mM HCl (Tris salt) and 250mM sucrose at pH

7.4. Inclusion of sucrose in the resuspension medium was subsequently found to improve the lifetime of the coupled particles. Fig. 5.3 shows the increase in fluorescence which occurred on addition of succinate to the two different preparations of particles incubated as described in the legend. This response, however, was much attenuated after storage of particles on ice for several hours especially in the case of the particles prepared by the method of Schatz and Racker (1966). The method of Azzi *et al.*, (1971) was therefore adopted routinely for preparation of sub-mitochondrial particles. As expected there is a rapid decrease in fluorescence on addition of the uncoupler FCCP (0.6μg/ml). A second addition of uncoupler had no effect confirming that this effect is indeed due to membrane depolarization and not some artefactual change. In the absence of oligomycin the potential developed was much smaller than that developed in its presence on addition of succinate as substrate (results not shown). This is probably because the sonication process knocked off some of the F_1 particles loosely attached to the sub-mitochondrial particles, leaving a leaky F_o channel which uncouples the particles. Oligomycin blocks the F_o channels restoring the integrity of the membrane needed for efficient energy transduction.

Supplementing the incubation with 100μM Ca^{2+} and 10mM Pi caused no detectable change in $\Delta\Psi$ as measured by ANS fluorescence (results not shown). Thus conditions which readily depolarize mitochondria were quite ineffective with SMP. One might envisage that there is a soluble matrix component needed, in addition to Ca^{2+} and Pi, to induce pore opening. The incubate was therefore supplemented with soluble matrix fraction i.e. the supernatant after sub-mitochondrial particle preparation (undialysed or dialysed for 18 hrs overnight in 10mM HCl (Tris salt), 250mM sucrose; 2-3mg protein/ml incubate). This concentration of matrix proteins was chosen in an effort to compensate for the dilution which occurs when these proteins are released from their normally concentrated environment in the matrix space. Under these conditions, the potential developed with SMP were the same with and without Ca^{2+} and Pi (results not shown). Further experiments in which the particles were supplemented with purified cyclophilin (Chapter 6) again failed to display any effect of cyclophilin with or without Ca^{2+} and Pi.

Fig. 5.3. *Potential measurements in sub-mitochondrial particles using ANS fluorescence.* In (A) sub-mitochondrial particles were prepared by the method of Schatz and Racker (1966), while in (B) the method of Azzi *et al.*, (1971)] was used. In both cases particles (0.75 mg/ml) were preincubated for 4 min in medium containing 10mM HCl (Tris salt)) at pH 7.4, 250mM sucrose, 2mM $MgSO_4$, 20μM ANS and oligomycin (4μg/mg of protein). Fluorescence changes were monitored on addition of 5mM succinate as substrate. 0.1μM FCCP was added as indicated.

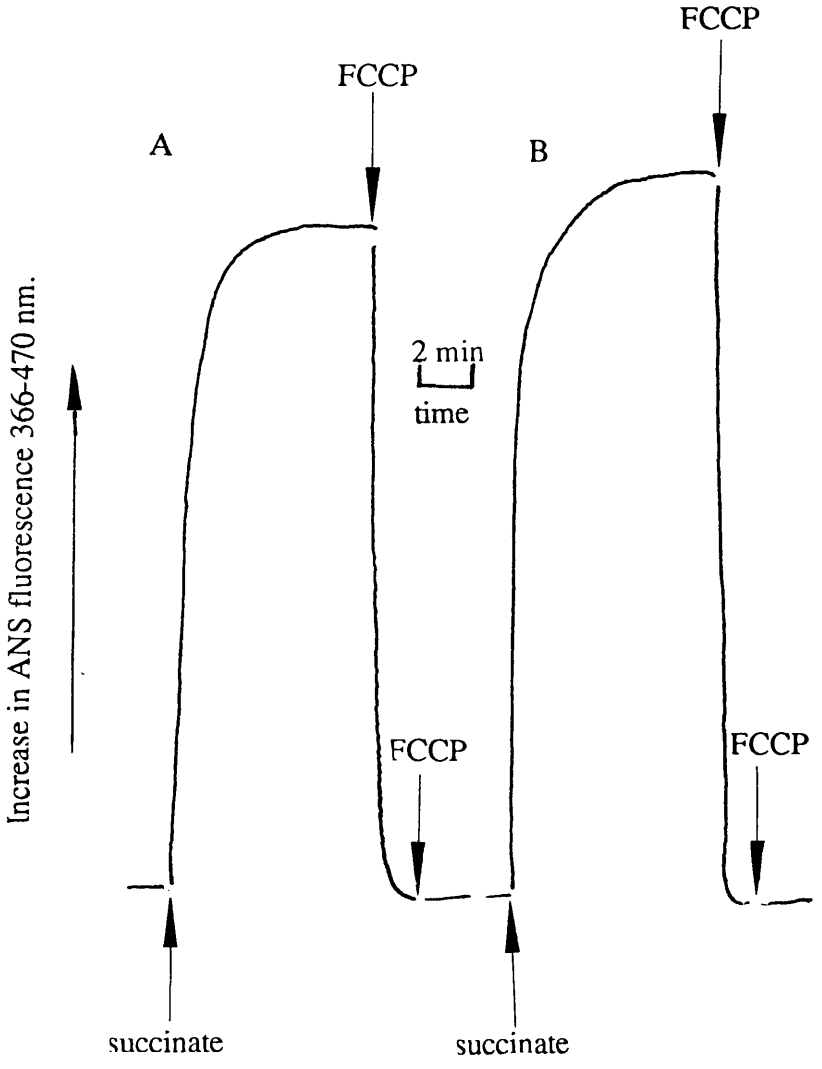

FCCP
FCCP
A
B
2 min
time
Increase in ANS fluorescence 366-470 nm.
FCCP
FCCP
succinate
succinate

Fig. 5.3

Fig. 5.4. *Fractionation of [U-^{14}C]-sucrose loaded sub-mitochondrial particles by gel filtration on a Bio-Gel P-6DG desalting column:* The graph shows the elution profile for protein assayed by measuring the absorbance at 280nm and for [U-^{14}C]-sucrose assayed by scintillation counting.

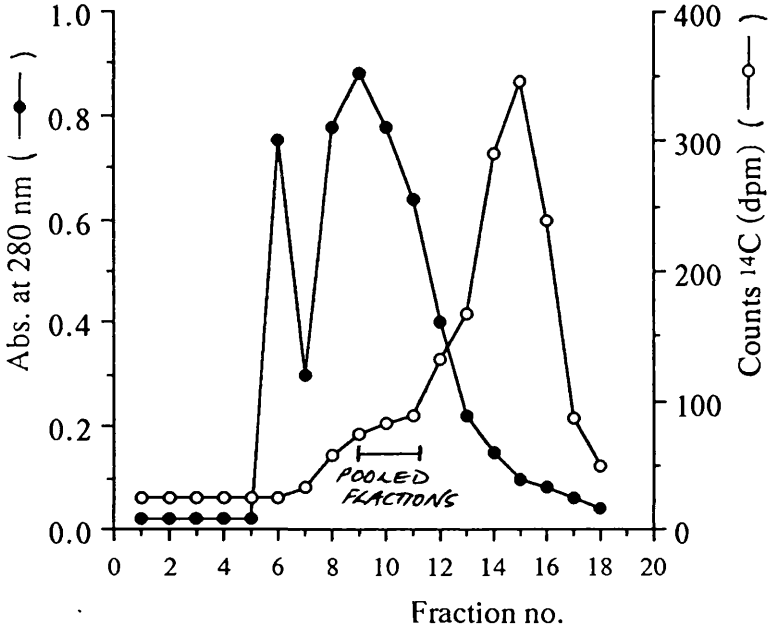

1.0
0.8
0.6
0.4
0.2
0.0
Abs. at 280 nm (—●—)
400
300
200
100
0
Counts ^{14}C (dpm) (—○—)
POOLED FRACTIONS
0 2 4 6 8 10 12 14 16 18 20
Fraction no.

Fig. 5.4

[5.4] ***Assessment of pore activity in sub-mitochondrial particles using ^{14}C-sucrose entrapment.***

Pore activity was also assessed in sub-mitochondrial particles from the permeability to [^{14}C]-sucrose. As sub-mitochondrial particles are "inside-out", this involved initially entrapping [^{14}C]-sucrose inside the particles, any release of [^{14}C]-sucrose being indicative of pore opening. To do this, sub-mitochondrial particles were prepared by the method of Azzi *et al.*, (1971) (Chapter 2; section 2.3) except that 5μCi of [U-^{14}C]-sucrose was added to suspensions before sonication. The final particle pellet was resuspended in 0.5ml of buffer containing 10mM HCl (Tris salt), 250mM sucrose, pH 7.4, and loaded onto a Bio-Gel P-6DG desalting column (10cm x 1cm) pre-equilibrated in two column volumes of the same buffer, to remove extraparticulate sucrose. One millilitre fractions were collected and assayed for [U-^{14}C]-sucrose by scintillation counting and for protein by measuring the absorbance at 280nm. The elution profile for the protein and [U-^{14}C]-sucrose is shown in Fig. 5.4. Fractions containing both protein and [U-^{14}C]-sucrose were pooled as indicated and used for entrapment studies. The results of these studies are summarised in Table 5.1 below.

	t=0		t=25	
Incubation Conditions	^{14}C activity in SN (cpm)	% 14C in pellet	14C activity in SN (cpm)	%14C in pellet
(a.)Control	74.8 ± 5.5	55	121.5 ±3.3	27.5
(b.)Ca^{2+},Pi	81.6 ± 2.8	51	118.0 ±9.2	29.5
(c.)Ca^{2+},Pi,Mg^{2+}	89.4 ± 3.7	47	127.7 ±6.1	23.8
(d.)Ca^{2+},Pi,CsA	84.2 ± 0.8	50	125.8 ±2.8	24.9
(e.)Ca^{2+},Pi,Mg^{2+} and CsA	89.5 ± 2.8	47	125.9 ±6.0	24.9
(f.)Calmidazolium	100.5 ± 13.6	40	152.0 ±4.8	9.0

Table 5.1 *The permeability of sub-mitochondrial particles to [^{14}C]-sucrose under various conditions.* Duplicate samples of submitochondrial particles loaded with [^{14}C]-sucrose (the total activity in cpm in each sample was 167.6 ± 2.06 S.E.M. (mean of 3 determinations) were incubated at pH 7.4 in basic incubation medium comprising, 10mM

HCl (Tris salt), 250mM sucrose, oligomycin (4μg/mg of protein), and 5mM succinate alone (control) or supplemented with either, 5mM Pi, 200μM free Ca^{2+}, 2mM Mg^{2+}, 2μM CsA or 10 μM calmidazolium as indicated. Half the samples were immediately (t=0) spun in a Beckman Airfuge at 100,000g for 15 min to sediment the sub-mitochondrial particles. Aliquots of the supernatant were then removed into Ecoscint for liquid scintillation counting. After a 25 min incubation period this procedure was repeated with the remaining duplicate samples. The activity of [^{14}C]-sucrose in the SN is given as the mean $\pm$ S.E.M. (three determinations).

It can be seen that in all instances approximately 50% of the entrapped sucrose was lost during centrifugation. After 25 min a further 25% of the entrapped [U-^{14}C]-sucrose had leaked out. Only calmidazolium effected a significant release of entrapped radiolabelled sucrose, 90% of the entrapped [U-^{14}C]-sucrose being lost in total during the incubation and centrifugation periods.

It is evident from these results, that there is a slow release of radiolabelled sucrose occurring continuously from the loaded particles. The calmodulin antagonist calmidazolium permeabilizes sub-mitochondrial particles increasing the slow rate of sucrose release seen in its absence. This result corroborates the conclusion drawn in the last chapter, namely, that permeabilization of mitochondria with calmidazolium allows cyclosporine A to equilibrate more rapidly with its mitochondrial binding components. However, no additional release of sucrose was detected under conditions which normally activate the pore in mitochondria. The slow release of sucrose seen in the absence of these effectors does not reflect permanent activation of the pore as it was unaffected by 2μM cyclosporine A with or without purified cyclophilin (Chapter VI; section 6.5). It would appear therefore, that there is no expression of pore acticvty in sub-mitochondrial particles.

[5.5]*An investigation of the involvement of ADP-ribosylation in "permeabilization" using sub-mitochondrial particles.*

As described in Chapter 1; section 1.3.2, pore activity is also influenced by oxidative stress, when the redox couples NADH/NAD, NADPH/NADP and GSH/GSSG become more oxidised. These low molecular weight components are removed

Fig. 5.5. *NAD induces a rapid dissipation of* $\Delta\Psi$ *in sub-mitochondrial particles.* Sub-mitochondrial particles (0.75 mg/ml) were preincubated for 4min in medium (pH 7.4) containing 10mM HCl (Tris salt), 250mM sucrose, 2mM $MgSO_4$, 20μM ANS and oligomycin (4μg/mg of protein). 5mM succinate was added to energize the particles and the fluorescence changes monitored. On energization the concentrations of NAD and ADP-ribose below were added as indicated.

(A) Control, no additions;
(B) NAD (60μM); and
(C) ADP-ribose (60μM).

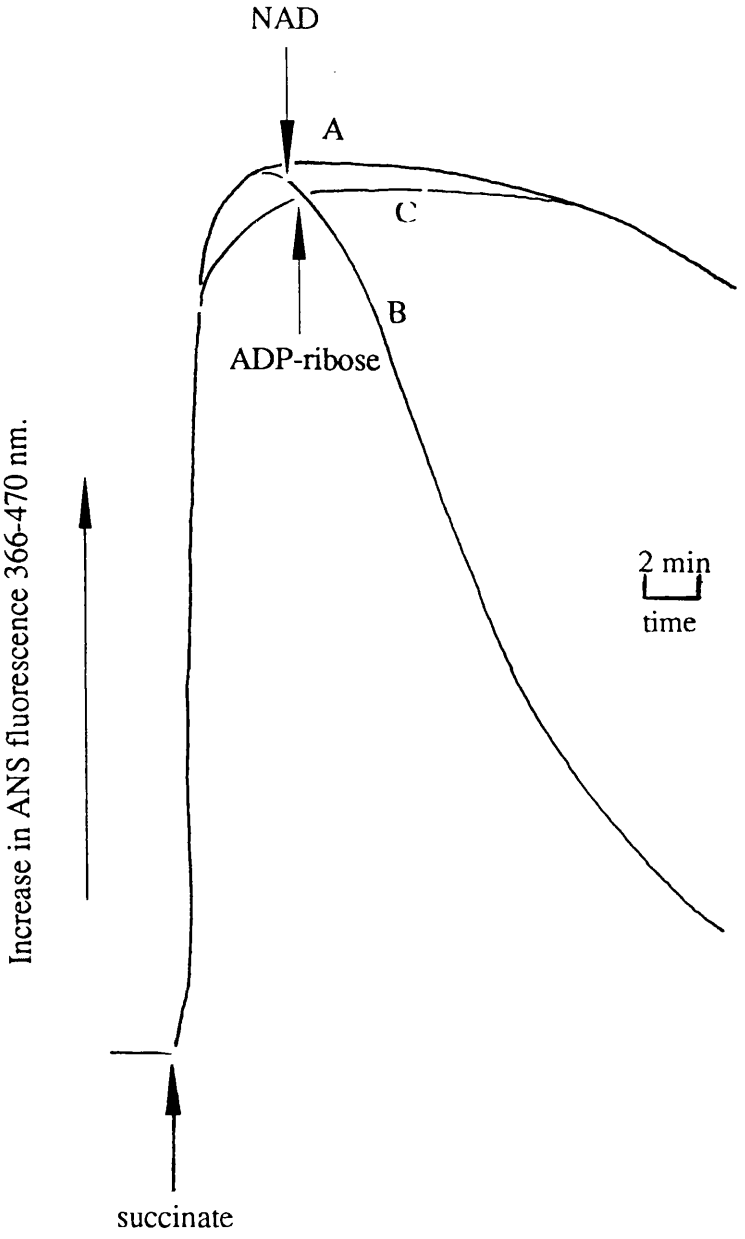

NAD
A
C
B
ADP-ribose
2 min
time
Increase in ANS fluorescence 366-470 nm.
succinate

Fig. 5.5

during dialysis of the crude soluble extract, so the effect of each of these compounds on respiring sub-mitochondrial particles ± 100μM Ca^{2+} and 10mM Pi and ± soluble matrix protein i.e. the supernatant after sub-mitochondrial particle preparation (undialysed or dialysed for 18 hrs overnight in 10mM HCl (Tris salt), 250mM sucrose; 2-3mg protein/ml incubate), was examined using the potential sensitive dye ANS. Only NAD had an effect. It induced a rapid dissipation of $\Delta\Psi$ at a concentration of 60μM as shown in Fig. 5.5. The potential collapse also occurred in the presence of rotenone indicating that it was not due to reverse electron transport. Fluorescence measurements at 340(ex)/450(em) confirmed that it was not due to reduction of NAD.

NAD is a cosubstrate in numerous hydride transfer reactions central to intermediary metabolism. It is also a substrate for a second distinct class of enzymes that catalyse the cleavage of a glycosidic linkage between nicotinamide and ribose and transfer of the ADP-ribosyl moiety to an acceptor protein. Moser *et al.*, (1983) have purified a NAD glycohydrolase from rat liver sub-mitochondrial particles which hydrolyzes NAD^+ to ADP-ribose and nicotinamide. As described in Chapter 1 (section 1.5), Richter *et al.*, [Lotcher *et al.*, (1979); Lotcher *et al.*, (1980); Winterhalter and Richter, (1985)], have shown that oxidative stress leads to Ca^{2+} release from mitochondria and is also associated with release of nicotinamide, suggesting that with these conditions the glycohydrolase is active. CsA inhibits the release of both Ca^{2+} and nicotinamide [Richter *et al.*, (1991)]. Richter *et al.*, propose that this is due to inhibition of NAD hydrolysis by CsA (although they have not shown that the glycohydrolase is inhibited directly). In addition, incubation of sub-mitochondrial particles with NAD effected ADP-ribosylation of an inner membrane protein. Richter interprets these findings in terms of regulation of mitochondrial Ca^{2+} release via ADP-ribosylation of the Ca^{2+} release transport protein. However, there is no evidence that Ca^{2+} release under conditions of oxidative stress does not proceed via the non-selective pore. It was quite relevant therefore to examine whether ADP-ribosylation of the pore protein might lead to its activation.

However CsA at a concentration of 1μM did not inhibit the depolarization caused by NAD in sub-mitochondrial particles . This result suggested that the depolarization caused by NAD (Fig. 5.5) did not reflect "permeabilization". Furthermore, Richter found that ADP-ribose alone could substitute for NAD in causing ADP-ribosylation of the inner membrane protein implicated in Ca^{2+} release. Here, ADP-ribose

unlike NAD had no effect on $\Delta\Psi$ in sub-mitochondrial particles (trace (C)). This again suggests that the effect of NAD in these studies is not due to the ADP-ribosylation of the inner membrane protein Richter proposes to be implicated in Ca^{2+} release.

As shown in Fig. 5.6, both ATP (2mM) and ADP (2mM) counteracted the effect of NAD on the mitochondrial membrane potential. Further investigation revealed that NAD was in fact inhibiting respiration and as shown by the oxygen electrode trace in Fig 5.7. Singer *et al.*, (1973) have shown in sub-mitochondrial particles that succinate dehydrogenase can produce oxaloacetate in the presence of NAD. The accumulating oxaloacetate could cause a feedback inhibition of succinate dehydrogenase. In support of this hypothesis, as shown in Fig. 5.7 traces (C) and (D), addition of ADP (2mM) or ATP (2mM), activators of succinate dehydrogenase counteracted the effect of NAD on respiration. As shown in Fig. 5.8 addition of glutamate together with glutamate oxaloacetate transaminase, which would remove the oxaloacetate by transamination had a similar effect.

Bearing in mind the results of Richter *et al.*, it was still important to establish whether or not NAD induced pore opening, although clearly this could not be done in a respiration-dependent assay. Instead, passive permeability was measured in deenergised sub-mitochondrial particles using potassium and valinomycin to induce transient diffusion potentials. The results of the diffusion potential experiments are shown in Fig. 5.9. NAD (60μM) did not change the profile of the diffusion potential obtained in control experiments i.e. a more rapid decay indicative of increased permeability was not observed. Increasing the preincubation time with NAD to 30min or increasing the concentration of NAD to 200μM did not change this result, nor did preincubation with Ca^{2+}(100μM) and Pi (2.5mM) agents that normally induce pore opening in intact mitochondria.

In entrapment studies on deenergized submitochondrial particles loaded with [U-^{14}C]-sucrose, prepared as described in the previous section, 60μM NAD ($\pm$ 100μM Ca^{2+} and 2.5mM Pi; $\pm$ dialysed matrix extract) induced no significant loss of particle [U-^{14}C]-sucrose with four separate particle preparations. Control experiments were done to ensure that entry and entrapment of sucrose i.e. permeation via the pore occurred in mitochondria under these conditions. Thus in no instance was NAD found to induce pore activity in sub-mitochondrial particles.

Fig. 5.6. *Protection by ADP and ATP.* Sub-mitochondrial particles (0.75 mg/ml) were preincubated for 4 min in medium (pH 7.4) containing 10mM HCl (Tris salt), 250mM sucrose, 2mM $MgSO_4$, 20μM ANS and oligomycin (4μg/mg of protein) and the concentrations of ATP and ADP indicated below. 5mM succinate was added to energize the particles and the fluorescence changes monitored. 60μM NAD was added on energization.

(A) Control, no additions:
(B) ATP (2mM); and
(C) ADP (2mM).

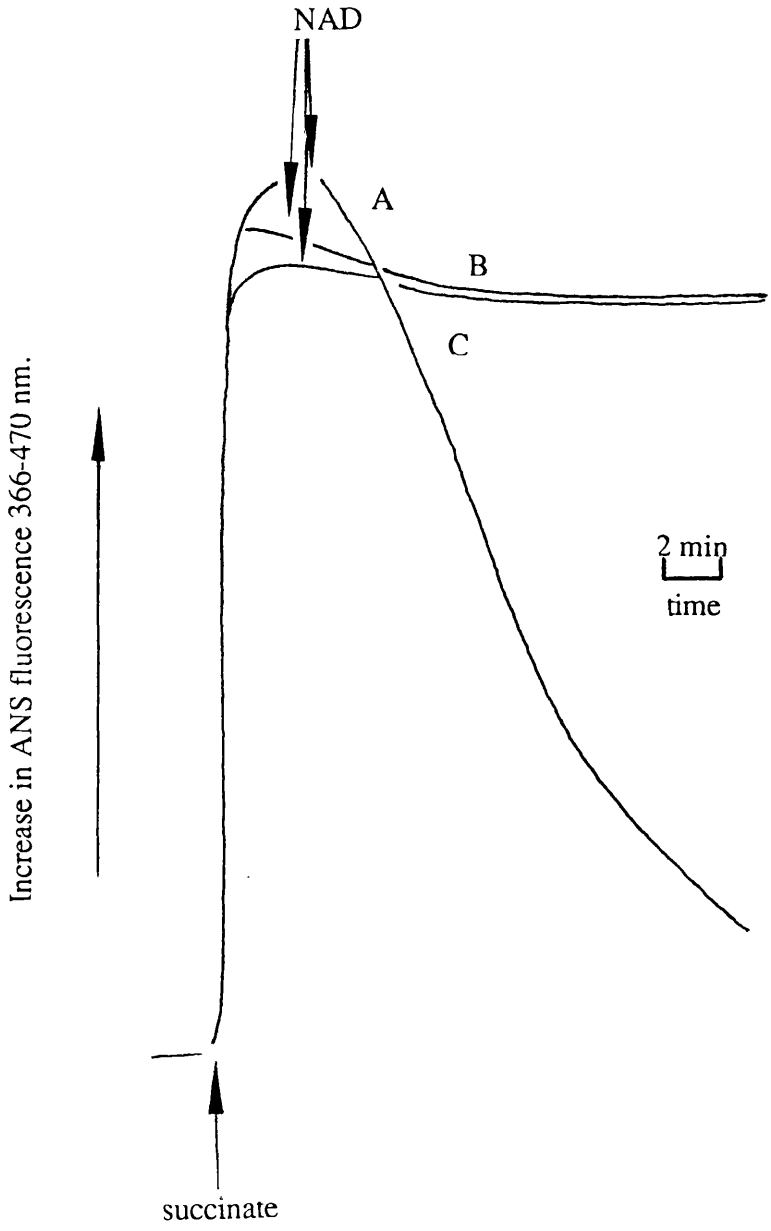

NAD
A
B
C
2 min
time
Increase in ANS fluorescence 366-470 nm.
succinate

Fig. 5.6

Fig. 5.7. *NAD inhibits respiration in sub-mitochondrial particles.* Sub-mitochondrial particles (0.75 mg/ml) were preincubated for 4 min in medium (pH 7.4) containing 10mM HCl (Tris salt), 250mM sucrose, 2mM $MgSO_4$, 20μM ANS, oligomycin (4μg/mg of protein), and 2mM ADP or 2mM ATP as indicated below. In addition, where indicated, 60μM NAD was added on energization. Oxygen consumption was monitored using a Clark type oxygen electrode.

(A) Control, no additions;
(B) NAD;
(C) ADP; NAD;
(D) ATP; NAD.

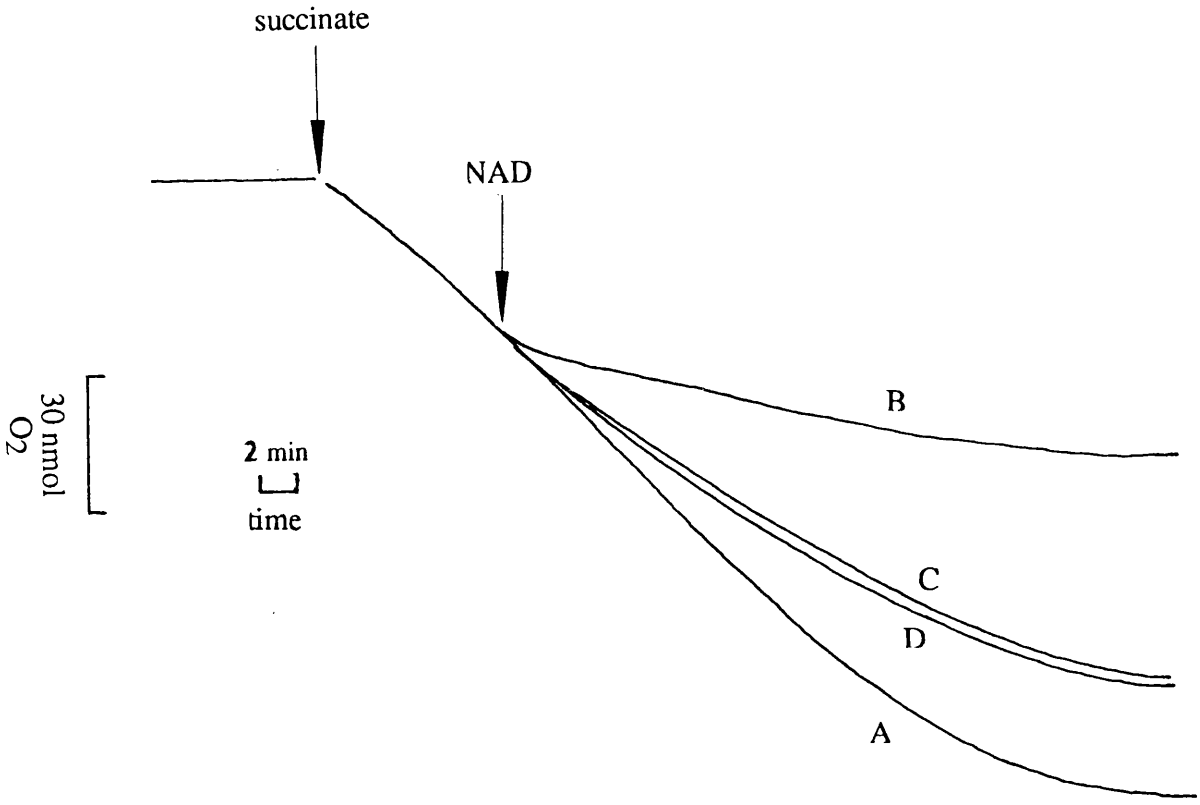


Fig. 5.7

Fig. 5.8 *Protection by glutamate together with glutamate oxaloacetate transaminase.* sub-mitochondrial particles (0.75mg/ml) were preincubated for 4 min in medium pH (7.4) containing 10mM HCl (Tris salt), 250mM sucrose, 2mM $MgSO_4$, 20 μM ANS and oligomycin (4μg/mg of protein), 2mM sodium glutamate and glutamate oxaloacetate transaminase. 5mM succinate was added to energize the particles and the fluorescence changes monitored.

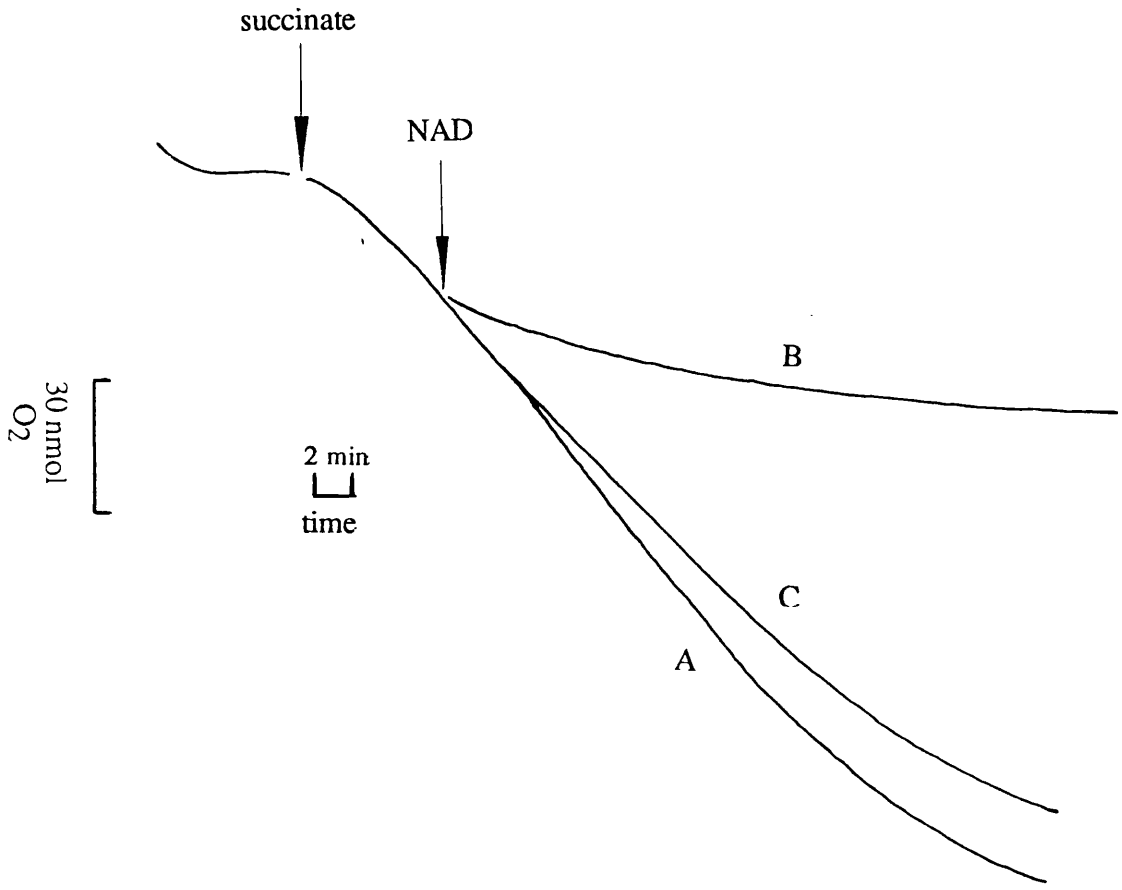


Fig. 5.8

Fig. 5.9. *Generation of transient diffusion potentials using potassium and valinomycin.* Sub-mitochondrial particles (0.75 mg/ml) were preincubated for 4 min in medium (pH 7.4) containing 10mM HCl (Tris salt), 250mM sucrose, 2mM $MgSO_4$, 20μM ANS, oligomycin (4μg/mg of protein) and valinomycin (0.3μg/mg) and 100μM NAD as indicated below. 50mM KCl was added as indicated to induce the transient diffusion potential and any changes in fluorescence monitored using the wavelength pair 366-470nm.

(A) Control, no additions,
(B) Valinomycin;
(C) Valinomycin; NAD.

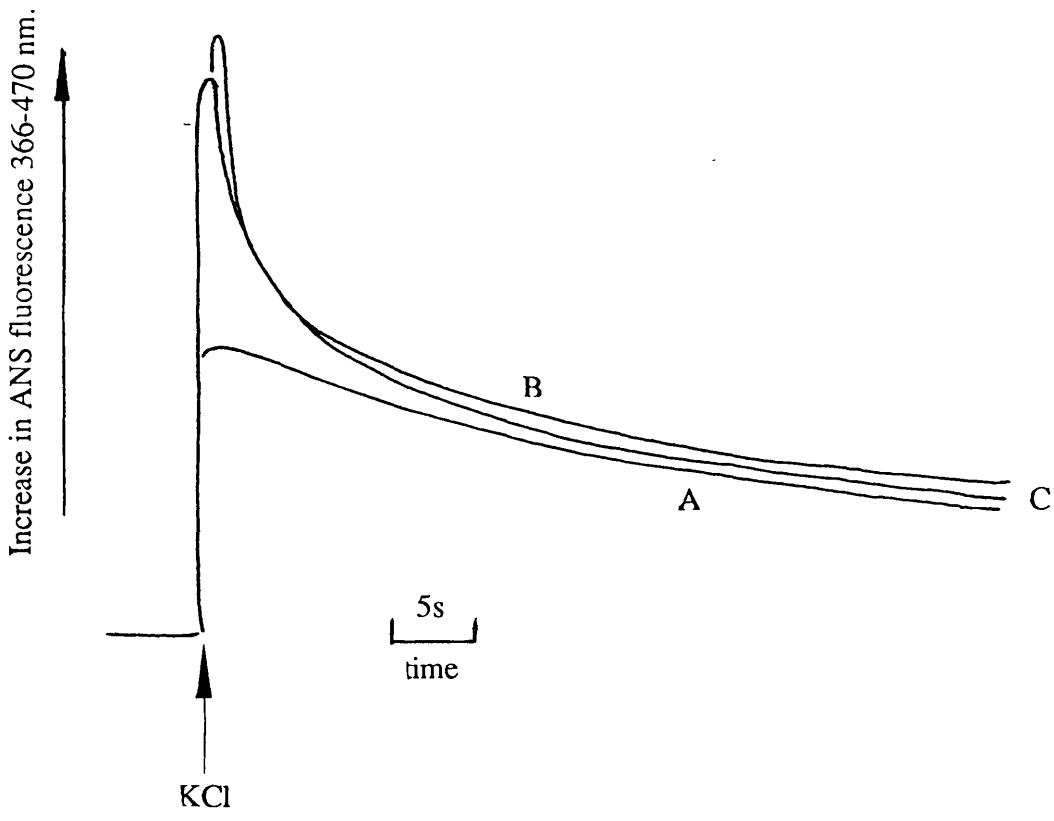


Fig. 5.9

[5.6] ***Protein ADP-ribosylation in sub-mitochondrial particles.***

In view of Richter's data it was therefore considered important to assess whether ADP-ribosylation of mitochondrial proteins actually occurred under the conditions of the above experiments. Protein ADP-ribosylation in intact mitochondria has not been studied in detail because the inner mitochondrial membrane is impermeable to NAD^+. One can only obtain indirect evidence for a functional link between ADP-ribosylation and pore opening in these experiments by showing that the agents which modulate pore opening affect the degree of protein ADP-ribosylation.

In previous studies, ADP-ribosylation was directed almost exclusively to a protein with a MW of 30,000 Da in washed sub-mitochondrial particles prepared from rat liver [Richter and Frei (1988)] and bovine heart [Hilz *et al.*, (1984)]. Incubation of sub-mitochondrial particles under standard conditions with [*adenylate* ^{32}P]-NAD (Chapter 2; section 2.14) resulted in the radiolabeling of one membrane polypeptide migrating with an apparent MW of approximately 34KDa. The intensely labelled band is seen after low pH lithium-dodecylsulphate gel electrophoresis and autoradiography is reported in Fig. 5.10. When proteins were fractionated using traditional sodium-dodecyl sulphate gel electrophoresis at alkali pH [Laemmli (1970)], no labelled bands were observed on autoradiography of the gel.

In this and subsequent fractionations labeled bands were cut out of the gel and incorporation of radiolabeled ^{32}P was quantified by scintillation counting. Fig. 5.11 shows the time dependence of the incorporation of radiolabel into sub-mitochondrial particles. Incorporation exhibits a lag phase and reaches $\simeq$ 127pmol/mg at 30min when labelling is still incomplete. This is very close to the degree of incorporation of 100pmol/mg observed by Richter and Frei (1988) after this period of incubation. In view of the discrepancy in apparent MW however, it is not clear that the band being radiolabeled under these conditions is the 30KDa protein described by Richter and Frei. These investigators have also purified a membrane glycohydrolase of 32KDa not observed in their previous radiolabelling experiments, which undergoes a time dependent auto-ADP-ribosylation [Richter *et al.*, (1991)] and this may be the protein which is being labelled. Also, although this result may indicate ADP-ribosylation of a membrane protein, protein modification by 5'-AMP, 5'-ADP or by phosphorylation is not excluded. This ambiguity could have been clarified by the addition of unlabeled ADP-ribose to the incubation of sub-mitochondrial particles to

Fig. 5.10. *^{32}P labelled proteins in the membrane plus soluble matrix fraction of rat liver sub-mitochondrial particles.*

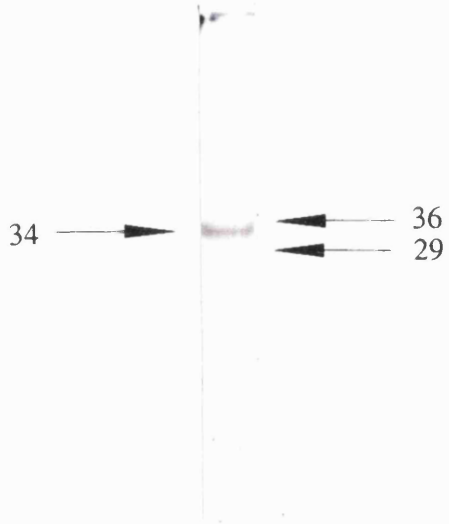


Fig. 5.10

Fig. 5.11. Time course of incorporation of ^{32}P into rat liver sub-mitochondrial particles.

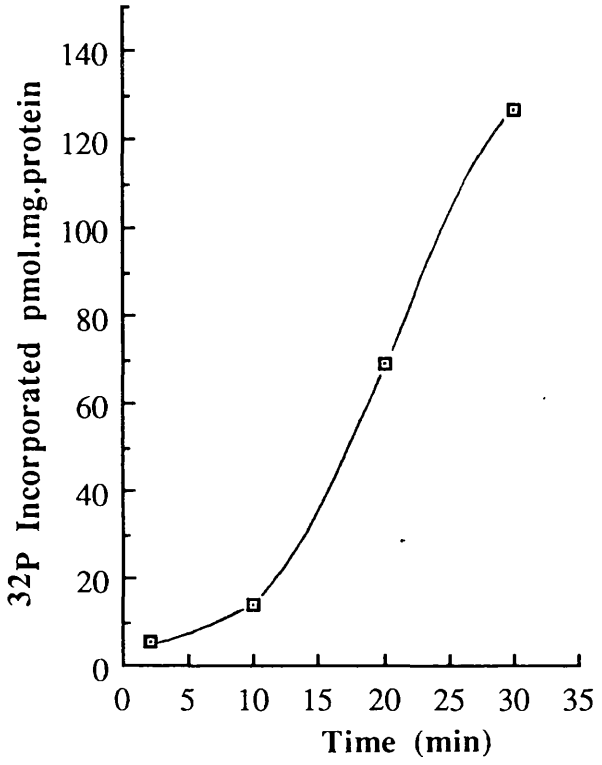

140
120
100
80
60
40
20
0
32P Incorporated pmol.mg.protein
0
5
10
15
20
25
30
35
Time (min)

Fig. 5.11

compete with labeled ADP-ribose derived from radiolabeled NAD. If ADP-ribosylation were implicated, this would have reduced the extent of labelling. ADP ribosylation could also have been unambiguously demonstrated by using [*ribose*(NMN)-^{14}C]NAD as substrate.

Whatever the nature of the covalent linkage, it is presumably labile under alkali conditions as no radiolabeled bands were observed when proteins were electrophoresed at pH 8.8. In this respect, it is interesting that the 30K ADP-ribose protein conjugate studied by Richter and Frei (1988) was stable for at least 12hr at pH 4.0 and 4°C whereas at basic pH radioactivity was released rapidly from the protein ($t_{1/2}$=50min).

Further studies investigated whether or not ADP-ribosylation might be involved in pore regulation. Agents that promoted (Ca^{2+}, Pi) and inhibited (adenine nucleotides, CsA) pore activation were included in the incubation to determine their effects on the level of ADP-ribosylation. In each case the labeled protein migrating at 34K was excised from the gel and ^{32}P incorporation quantified. The results are reported in Table 5.2.

Incubation	^{32}P Incorporated (pmol/mg protein)
(1.)Control	130
Ca^{2+}/Pi 100μM/1.25mM	71.5
(2.)Control	127.7± 10.18 S.E.M. (n=4)
2mM ATP	49.5
2mM ADP	57.0
(3.)Control	150.0
0.3μM CsA	190.0
1.4μM CsA	178.0

Table 5.1: *The effect of (1.) Ca^{2+} and Pi; (2.) adenine nucleotides; and (3.) CsA on the amount of 32P incorporated into the 34,000 Da protein.*

It is evident that there was no correlation between the effects of these agents on ADP-ribosylation in sub-mitochondrial particles and their effects on the pore in whole mitochondria. Whereas adenine nucleotides inhibit both ADP-ribosylation (Table 5.1) and pore activation, Ca^{2+} had opposite effects on the two processes and the pore inhibitor CsA tended, if anything to increase ^{32}P labelling.

The reduction in incorporated radiolabel seen in the presence of ATP may be due to the inhibition of the membrane bound glycohydrolase. Richter *et al* (1985) have shown that this enzyme is inhibited by 50% in the presence of 10mM ATP. Most important of all, if labelling is a reflection of protein modification by ADP-ribosylation, CsA did not inhibit hydrolysis of NAD to nicotinamide and ADP-ribose as no decrease in the extent of radiolabelled protein was observed in the presence of the immunosuppressant. This contradicts Richter's claim that CsA inhibits the hydrolysis of NAD (Richter *et al* (1991). However, this conclusion was based on indirect evidence as will be discussed (Chapter 7; section 7.6).

CHAPTER 6: ISOLATION OF MITOCHONDRIAL CYCLOPHILIN AND RECONSTITUTION STUDIES WITH SUB-MITOCHONDRIAL PARTICLES.

[6.1] *Background*.

Previous studies (Chapter 5; section 5.4) failed to detect any pore activation in sub-mitochondrial particles by the crude soluble matrix fraction. It is possible that there are other proteins present in this crude extract which interfere with pore activity on reconstitution with sub-mitochondrial particles. Thus the possibility that soluble cyclophilin was necessary in addition to Ca^{2+} and Pi to induce pore opening in sub-mitochondrial particles had not been excluded. Mitochondrial cyclophilin was therefore isolated in order to investigate its possible role in pore activity in a more refined system.

CsA may work as an immunosuppressant, not by blocking cyclophilin, but by forming an "active" cyclophilin-CsA complex that in turn blocks other enzyme activities. In particular, the cyclophilin-CsA complex inhibits calcineurin, a Ca^{2+}/calmodulin dependent protein phosphatase whereas both free cyclophilin and free CsA are ineffective Liu *et al.*, (1991). It is possible that the same principle is involved in CsA's inhibition of the inner membrane pore. Unfortunately, without an appropriate assay system demonstrating $Ca^{2+}$and Pi induced pore opening in sub-mitochondrial particles, this hypothesis could not be tested. However, if the complex binds to the inner membrane pore, it was considered that it should be possible to demonstrate that cyclophilin promotes CsA binding to sub-mitochondrial particles. Finally, isolation of cyclophilin would also allow the amount of cyclophilin, presumably the high affinity CsA binding component to be quantified empirically as the binding studies described in Chapter IV had given ambiguous results.

[6.2] *Purification of mitochondrial cyclophilin.*

The purification protocol used was that described by Fischer *et al.*, (1984) for the isolation of cyclophilin from porcine kidney was followed. Cyclophilin was detected from its PPIase activity as described in Chapter IV. Sub-mitochondrial particles were prepared by the method of Azzi *et al.*, (1971) and the soluble matrix fraction was retained after particle sedimentation. An ammonium sulphate fractionation of the crude extract was carried out. The pH was carefully maintained

at 7.0 during the fractionation. Cyclophilin was precipitated at 40-80% ammonium sulphate. However this procedure resulted in a 50% loss of activity. As the mitochondrial matrix fraction already contains fewer proteins than the crude cell extract used by Fischer and and his colleagues this step was abandoned.

The next procedure tried was ion exchange FPLC (Fast Protein Liquid Chromatography) using a Pharmacia Mono-S cation exchange column. Ion exchange chromatography discriminates between proteins on the basis of accessible surface charges and their corresponding electrostatic interaction with the sorbent. As the crude matrix extract was in 2mM EDTA (Tris salt; the sonication buffer used in the fractionation of RLM), it was initially dialysed for 18hrs (with two buffer changes) in 10mM Tricine (Cl^- salt), pH 8.4. The dialysed extract (4ml containing 20mg of protein) was loaded onto the Mono-S cation exchange column pre-equilibrated in two column volumes (8ml) of the above buffer. The isomerase activity appeared in the void fraction along with the bulk of the protein. It was concluded that the protein must be uncharged or negatively charged under these conditions.

Next time therefore, the crude extract was first dialysed for 18hrs, with two buffer changes in Tris (Cl^- salt), pH 8.4 and was loaded onto a Pharmacia Mono-Q anion exchange column pre-equilibrated in two column volumes (8ml) of the same buffer. Again the isomerase activity appeared in the void fraction.

As cation exchange had been successfully used by other investigators in the isolation of cyclophilin a second attempt was made to use the Mono-S cation exchange column. This time the pH of the buffer was varied in an attempt to find a pH at which the protein was positively charged. NAP gel filtration columns (G25; Sigma) were used to quickly exchange the buffer of 5mg samples of the crude extract with 10mM MOPS (morpholino propane sulphonate) (Cl^- salt) at pH 8.0, pH 7.5 and pH 7.0. These samples were then sequentially loaded onto the Mono-S cation exchange column pre-equilibrated in two column volumes (8ml) of buffer at the same pH. Cyclophilin bound to the column at pH 7.0. Cyclophilin is thus positively charged at neutral pH but carries little net charge between pH 7.5 and pH 8.4. The protein was eluted with a 0-500mM gradient of NaCl. The elution profile is shown in Fig. 6.1. Isomerase activity eluted at 80mM NaCl. The fractions containing the isomerase activity were pooled and concentrated under pressure in an Amicon

Fig. 6.1: *Fractionation of the dialysed soluble matrix fraction on Pharmacia's "Mono-S" cation exchange column.* The graph shows the elution profile for protein assayed by measuring the absorbance at 280nm and the peptidyl-prolyl-*cis-trans*-isomerase activity of protein containing fractions. The basis of the assay for this enzymic activity is described in Chapter IV. The volume of each fraction was 2 ml.

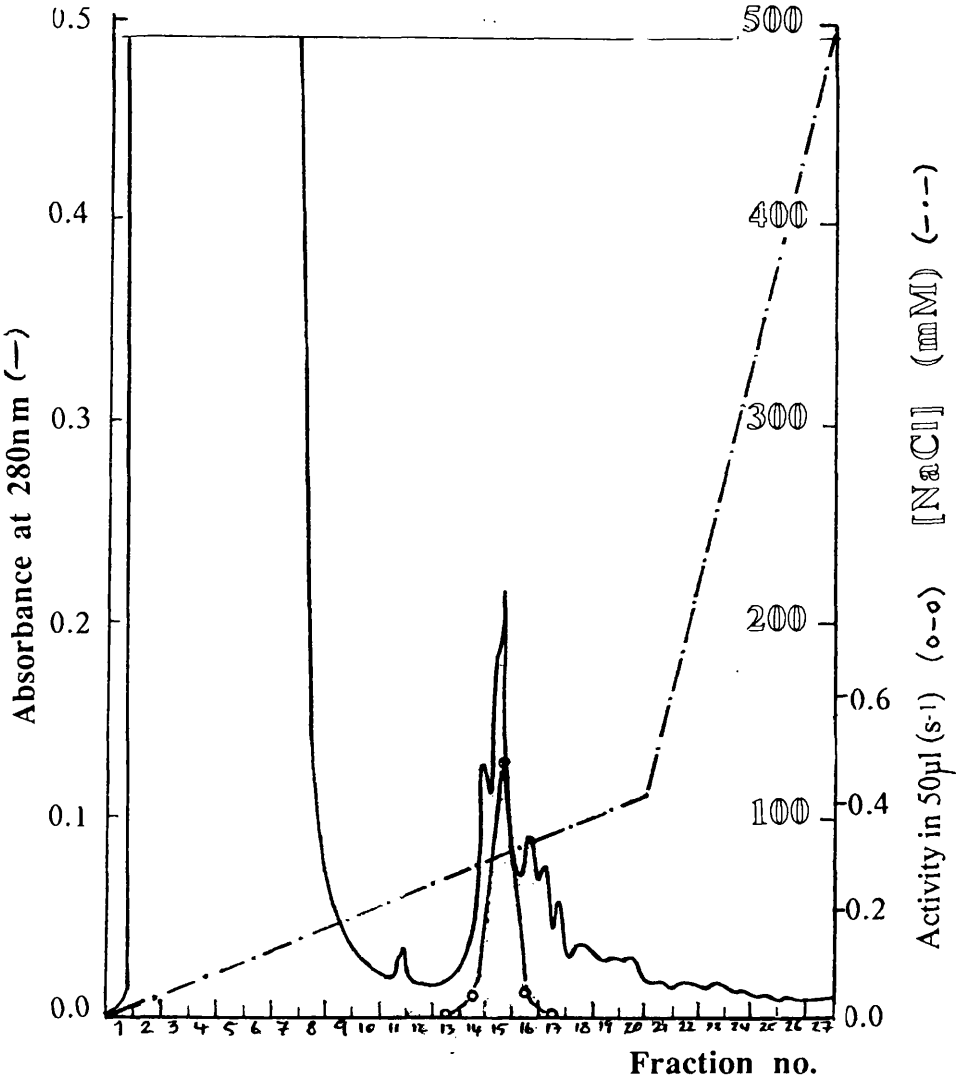

Absorbance at 280nm (—)
0.5
0.4
0.3
0.2
0.1
0.0
500
400
300
200
100
0.6
0.4
0.2
0.0
[NaCl] (mM) (-·-)
Activity in 50µl (s-1) (o-o)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Fraction no.

Fig. 6.1

stirred ultrafiltration cell (membrane; 10,000 MW cutoff) at 4°C in the cold room to 200μl.

The isomerase containing fraction was then further fractionated by gel filtration FPLC. The concentrated fraction from the ion-exchange step was loaded onto a Pharmacia Superdex-75 (fractionation range 5,000-75,000 Da) gel filtration column. Initially the column was pre-equilibrated in two column volumes (48ml) of 10mM MOPS (Cl^- salt) at pH 7.0. Isomerase activity was not detected in the eluate and cyclophilin must therefore have adhered to the column. It was found that inclusion of 100mM NaCl in the buffer prevented cyclophilin from sticking to the column under these conditions. Isomerase activity eluted at 12.5-13.5ml. The elution profile is shown in Fig 6.2.

The fractions containing isomerase activity were pooled and concentrated to 40μl using Centricon-3 microconcentrators (3000 MW cut-off; Amicon). These are small inserts for preparative rotors which use centrifugation to drive the solvent through the dialysis membrane. The concentrated fraction was frozen at -60°C. Isomerase activity was stable at this temperature and also resistant to freeze-thawing.

The gel filtration column was calibrated using a set of Pharmacia markers comprising: BSA (MW;67,000); ovalbumin (MW;43,000); chymotrypsinogen A (MW; 25,000) and ribonuclease A (MW; 13,700). The elution profile and a plot of k_{AV} vs MW is shown in Fig. 6.3 where:

$$k_{AV} = V_e - V_o / V_t - V_o$$

and V_e=elution volume of standard or unknown, V_o=the void volume of the column (here 6ml) and V_t=the total column volume. k_{AV} is a column-independent measure of the protein behaviour and is the fraction of stationary gel volume which is accessible to the protein. From the calibration curve the peak with the isomerase activity has a MW of 15,000 to 19,000 Da.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) of this fraction as shown in Fig. 6.4 confirms its purity with only one band running at $\simeq$18,000 Da which corresponds to the MW of the various subcellular forms of cyclophilin previously isolated from other tissues [Harding *et al.*, (1986)].

Fig. 6.2: *Fractionation of the pooled fractions from the cation exchange step on Pharmacia's "Superdex-75" gel filtration column.* The graph shows the elution profile for protein assayed by measuring the absorbance at 280nm and the peptidyl-prolyl-*cis-trans*- isomerase activity of the protein containing fractions. The basis for this enzymic assay is described in Chapter IV. The volume of each fraction was 1ml.

Absorbance at 280nm (—)
0.05
0.1
0.15
0.2
0.25
Fraction no.
0.1
0.2
0.3
Activity in 50µl (s^{-1}) (o—o)

Fig. 6.2

Fig. 6.3: *Calibration of Pharmacia's "Superdex-75" gel filtration column:* (A) shows the elution profile for the four molecular weight markers assayed by measuring the absorbance at 280nm. These comprised :(1.) BSA (MW; 67,000); (2.) ovalbumin (MW; 43,000);chymotrypsinogen A (MW; 25,000) and ribonuclease A (MW; 13,700). The volume of each fraction was 1 ml. (B) is a plot of K_{AV} versus MW (see text) and allows MWs to be estimated from a knowledge of the elution volumes of the unknowns.

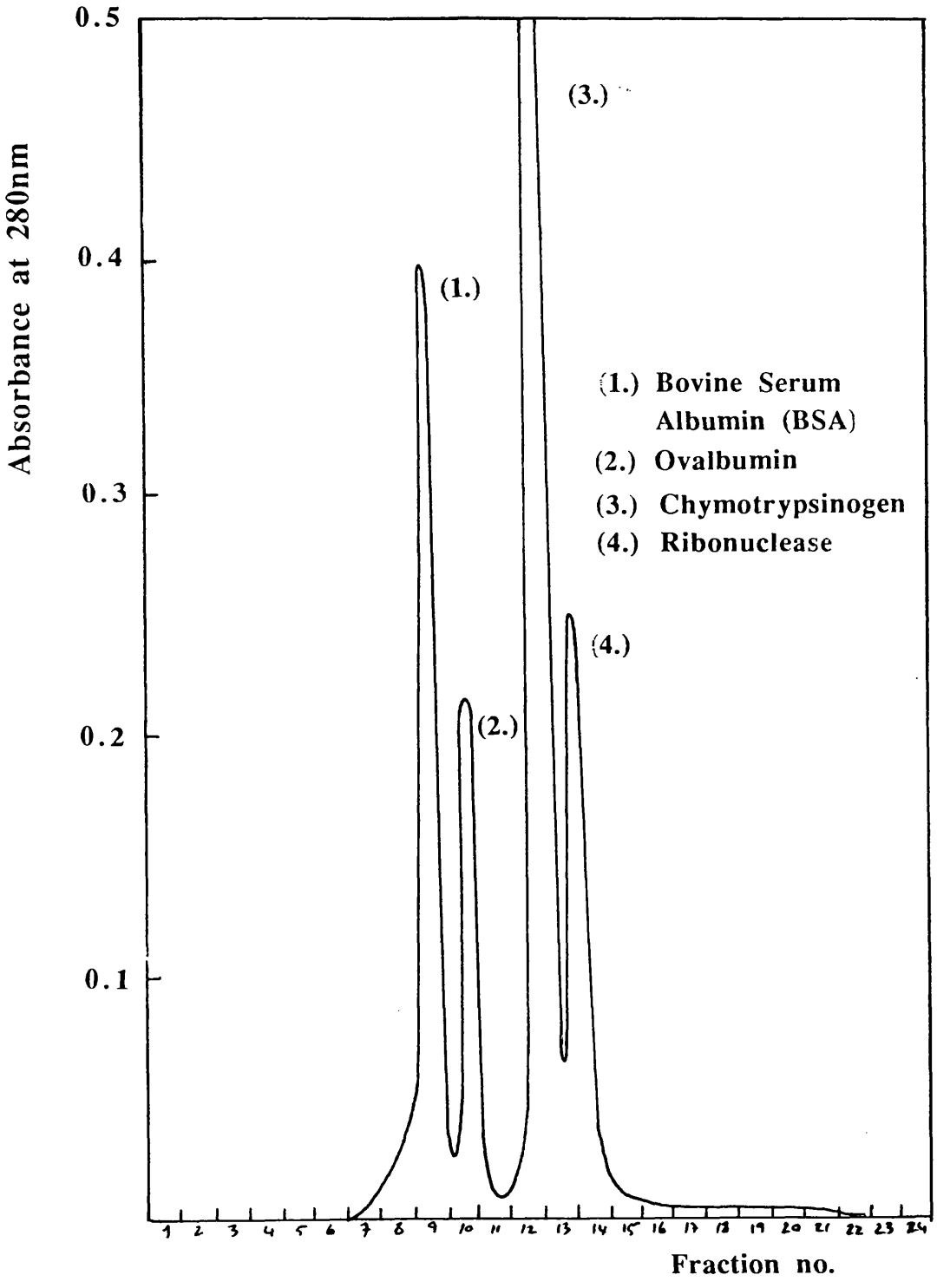

Absorbance at 280nm
0.5
0.4
0.3
0.2
0.1
(1.)
(2.)
(3.)
(4.)
(1.) Bovine Serum Albumin (BSA)
(2.) Ovalbumin
(3.) Chymotrypsinogen
(4.) Ribonuclease
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Fraction no.

Fig. 6.3 (a)

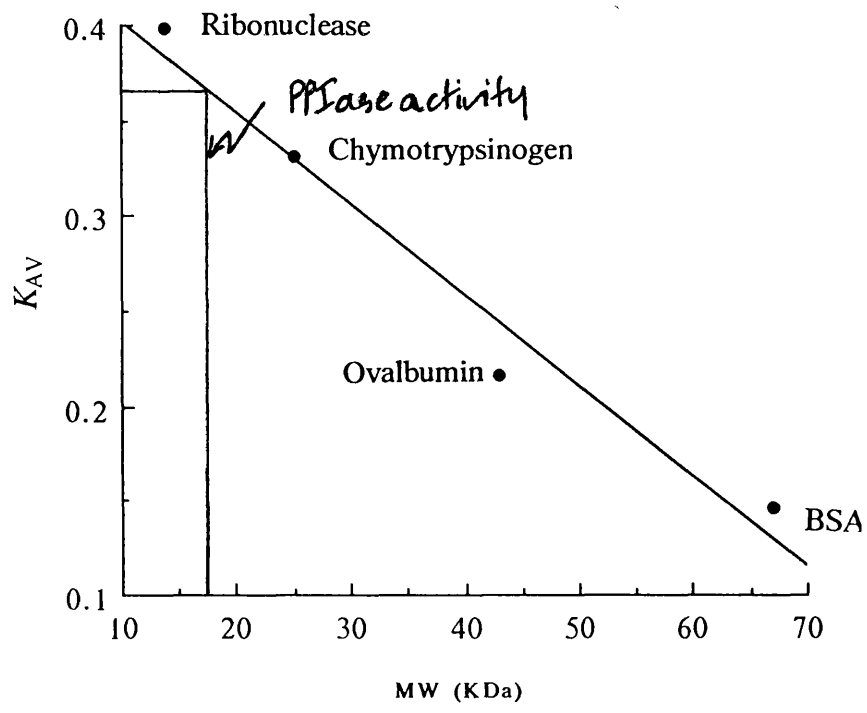

0.4
0.3
0.2
0.1
K_{AV}
Ribonuclease
PPIase activity
Chymotrypsinogen
Ovalbumin
BSA
10
20
30
40
50
60
70
MW (KDa)

Fig. 6.3 (b)

Fig. 6.4. ***SDS PAGE of purified mitochondrial cyclophilin.*** Proteins were analysed by SDS PAGE as described in the Methods section. Lane (1.), crude soluble matrix extract. Lane (2.), fraction obtained after subjecting this extract to cation exchange (Mono-S; Pharmacia) and gel filtration (Superdex-75; Pharmacia) FPLC. The molecular weight markers flanking the 18K cyclophilin are indicated.

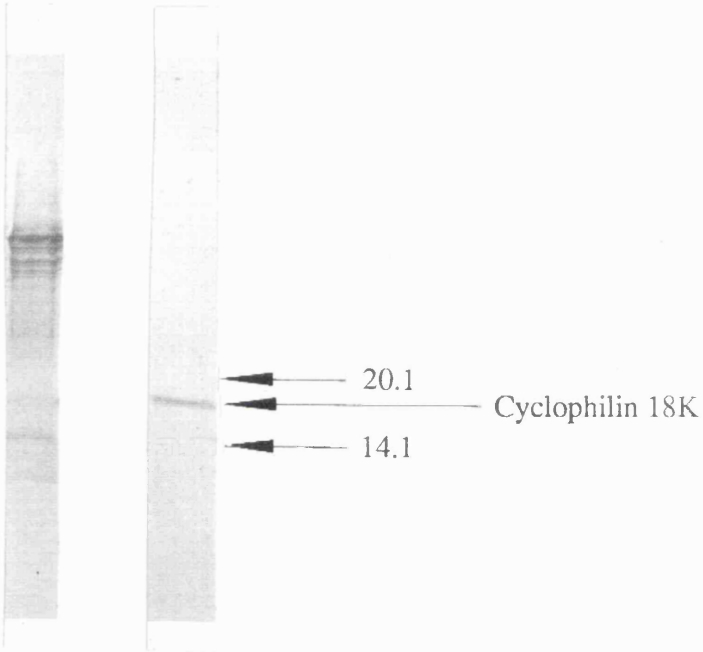


Fig. 6.4

Table 6.1 below is representative of 7 preparations of cyclophilin and shows the activity and amount of protein recovered at each step :

	Activity (s^{-1})	[Protein] (mg)	Specific activity ($s^{-1}.mg^{-1}$)
Undialysed extract	103.00	150.00	0.68
Dialysed extract	70.36	150.00	0.46
Cation exchange	36.89	1.57	25.93
Gel filtration	30.54	0.034	898.00

Table 6.1 *Recovery of mitochondrial cyclophilin at each purification step from the soluble matrix fraction (undialysed extract) of RLM.*

This is a 1320 fold purification. Assuming the MW of cyclophilin is 18,000 Da then a recovery of 34μg corresponds to 1.923 nmoles. Now, the soluble matrix protein was estimated to be 67% of the total mitochondrial protein (results not shown). Therefore, the amount of cyclophilin recovered was 0.67/150 x 1923, i.e. 8.59pmoles/mg of mitochondrial protein. Since only 30% of the activity was recovered the total amount of cyclophilin could amount to 28.98pmoles/mg of protein. This tends to corroborate the equilibrium dialysis data (see Chapter IV), and the ^{3}H-CsA binding data obtained in the presence of calmidazolium, suggesting that the initial estimation of the number of CsA binding sites attributable to cyclophilin, as 5pmol/mg underestimated the true value.

[6.4] ***Cyclophilin does not promote binding of cyclosporin to the inner mitochondrial membrane.***

As discussed at the beginning of this chapter, if a cyclophilin-CsA complex binds to the pore to mediate pore blockage, one might envisage that cyclophilin would promote CsA binding to the inner mitochondrial membrane. ^{3}H-CsA binding (at a [CsA] of 5nM) to sub-mitochondrial particles was therefore assessed in the presence and absence of purified cyclophilin (120 pmols/mg of sub-mitochondrial protein). Binding studies were carried out as described in Chapter 2; section 2.9 with a few alterations. The total volume of the incubation in these experiments was 50μl so that only small aliquots of purified cyclophilin needed to be used. Particles were sedimented in a Beckman Airfuge by spinning at 100,000g for 30 min. 5nM cyclosporine was used in these experiments as this is the concentration at which component I, presumably cyclophilin is half-saturated after 8 min.in the previous binding studies. It was found that the same amount of cyclosporin bound whether cyclophilin was present or not (results not shown) so the notion that the cyclosporine-cyclophilin complex may bind to some membrane component of the pore to mediate pore blockage was not borne out.

[6.5]***Cyclophilin alone does not sensitise sub-mitochondrial particles to the unbuffered concentrations of Ca^{2+} and Pi which activate pore activity in mitochondria:***

The effect of purified cyclophilin on respiring sub-mitochondrial particles was monitored using the potential sensitive dye, aminonapthalene sulphonate and fluorescence techniques as described in Chapter 5. Sub-mitochondrial particles were incubated at pH7.4 in 10mM HCl (Tris salt) with either cyclophilin (30pmols/mg of mitochondrial protein) alone or in conjunction with 100μM Ca^{2+} and 2.5mM Pi. In both instances the particles were able to develop and maintain a potential on addition of succinate as substrate (results not shown). Thus cyclophilin had no detectable effect on $\Delta\Psi$ in sub-mitochondrial particles.

Possible effects of cyclophilin alone, or in conjunction with CsA, on pore activity were also assessed in sub-mitochondrial particles from the permeability to ^{14}C-sucrose. These studies were carried out as described in Chapter 5; section 5.4 and the results are summarised in Table 6.2.

	t=0		t=25	
Incubation Conditions	^{14}C in SN(cpm)	%^{14}C in pellet	^{14}C in SN(cpm)	%14C in pellet
(a)Control	74.8 ± 5.5	55	121.5 ±3.3	27.5
(b)Ca^{2+},Pi, CP	81.2 ± 3.1	52	121.5 ±2.8	27.5
(c)Ca^{2+}, Pi, CP, CsA	85.1 ± 2.7	49	123.7 ±4.1	25.9

Table 6.2 *The permeability of sub-mitochondrial particles to [^{14}C]-sucrose under various conditions.* Duplicate samples of submitochondrial particles loaded with [^{14}C]-sucrose (the total activity in cpm in each sample was 167.6 ± 2.06 S.E.M. (mean of 3 determinations) were incubated at pH 7.4 in basic incubation medium comprising, 10mM HCl (Tris salt), 250mM sucrose, oligomycin (4μg/mg of protein), and 5mM succinate alone (control) or supplemented with either, 5mM Pi, 200μM free Ca^{2+}, 2mMMg^{2+}, 2μM CsA or cyclophilin (CP; 10μg/mg mitochondrial protein) as indicated. Half the samples were immediately (t=0) spun in a Beckman Airfuge at 100,000g for 15 min to sediment the sub-mitochondrial particles. Aliquots of the supernatant were then removed into Ecoscint for liquid scintillation counting. Atfer a 25 min incubation period this procedure was repeated with the remaining duplicate samples. The activity of [^{14}C]-sucrose in the SN is given as the mean ± S.E.M. (three determinations).

As seen previously (Chapter 5; section 5.4), approximately 50% of the entrapped sucrose was lost during centrifugation. A further 25% of the entrapped [^{14}C]-sucrose had leaked out after 25 min. However this slow release of [^{14}C]-sucrose was not accelerated by either cyclophilin alone or the combination of cyclophilin and CsA.

[6.6] ***Mitochondrial cyclophilin stimulates uncoupled respiration in sub-mitochondrial particles.***

As described in the Introduction there is evidence that the pore can exist in several sub-states which vary in their conductivity and permeability properties. It was considered possible that a smaller pore, permeable to H^+ but not to [^{14}C]-sucrose, could open in response to Ca^{2+} and Pi, or to these agents plus cyclophilin. In addition, if, as indicated in previous studies the pore constantly flickers open and closed, causing only a slight uncoupling in sub-mitochondrial particles, potential measurements with ANS may not be sensitive enough to detect the pore's activity. Partial uncoupling of sub-mitochondrial particles should result in an increase in the respiratory rate. The effect of purified cyclophilin on respiring sub-mitochondrial particles was therefore monitored by measuring its effect on the oxygen consumption of the particles.

Since the effects on respiration (below) were small, it was necessary to amplify the effect. This was achieved by setting up two identical incubations in parallel, adding test substances (cyclophilin, CsA) to one incubation, and amplifying the respiration rate difference between the two incubations. Representative traces are shown in Fig. 6.5. Initially, before addition of cyclophilin or Ca^{2+}, the two incubations were identical and so there was no difference recorded with time. In the complete absence of Ca^{2+} (chelated by addition of EGTA; trace A) cyclophilin at a concentration of 100μM stimulated respiration. The stimulation was not immediate but exhibited a lag phase. Addition of Ca^{2+} (Fig. 6.5; trace B) caused a slight stimulation of respiration. The further addition of cyclophilin (100 pmol/mg) caused an immediate increase in the rate of respiration. Cyclosporine (1μM), which would completely inhibit pore activity under these conditions in mitochondria, did not inhibit this stimulation of respiration.

Respiration was also monitored in sub-mitochondrial particles using an assay which coupled fumarate production via fumarase and malic enzyme to the reduction of NADP, which was monitored spectrophotometrically. The increase in absorbance due to the reduction of NADP was monitored at 340nm. The reactions involved in the assay are illustrated schematically below:

Fig. 6.5. ***Cyclophilin stimulates respiration.*** Sub-mitochondrial particles (0.75-1mg protein/ml) were suspended in medium (pH 7.4) containing 10mM HCl (Tris salt), 120mM sucrose, 1.2mM $MgSO_4$ and 3mM succinate. (A) contained in addition, 30μM EGTA. The incubate was split between two Clark type oxygen electrodes set up in parallel. Additions were then made to one of the incubates as shown and at the concentrations indicated below and the difference in oxygen consumption between the two incubates was monitored.

(A.) Cyclophilin (CP)(100 pmol/ml);
(B.) Ca^{2+} (100μM); cyclophilin (CP) (100 pmol/ml); cyclosporine A (CsA) (1μM).

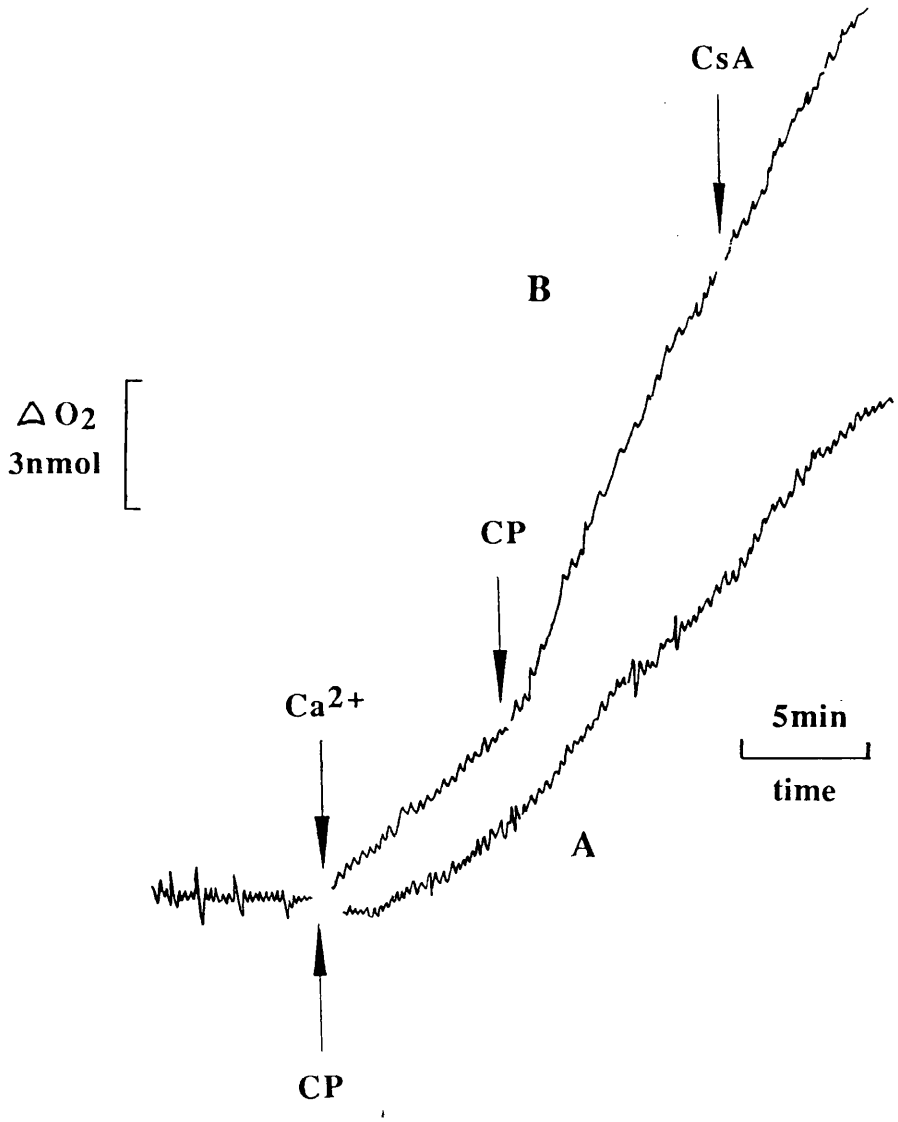
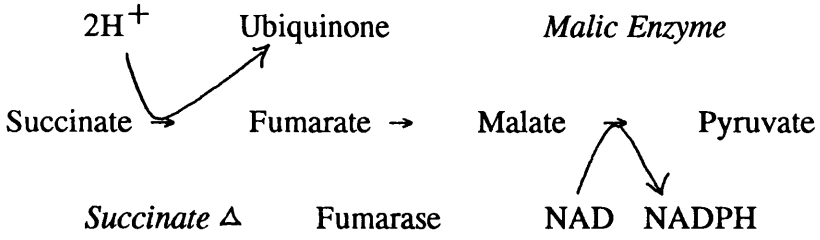


Fig. 6.5



This enabled differences in respiration to be monitored more reliably since differences between control and test incubations could be measured in a split beam spectrophotometer.The previously observed stimulation of respiration could either be due: to an increase in proton permeability due to pore opening as respiration is normally rate-limited by the rate of proton reentry across the inner mitochondrial membrane; or due to a direct effect on one of the enzymes of the redox chain. This question was addressed by examining the effect of cyclophilin on the rate of respiration in the presence of the uncoupler CCCP. Fig. 6.6 illustrates that cyclophilin actually stimulates uncoupled respiration and thus must be interacting with one of the enzymes of the respiratory chain to induce this effect. Thus, again, these results show that cyclophilin alone ($\pm Ca^{2+}$ and Pi) does not change the permeability of sub-mitochondrial particles and no sign of pore activity can be detected in these particles.

Although the stimulation of respiration observed is quite small ($\simeq$ 10%), the possibility that cyclophilin is interacting directly with some component of the respiratory chain is intriguing. Furthermore ADP an important modulator of pore activity inhibits the stimulation of respiration conferred by cyclophilin. Thus,as shown in Fig. 6.7 ADP at concentrations of 1mM and 100μM inhibit completely while 30μM ADP is ineffective. There may thus be a connection between cyclophilin's effect on redox components and its role in pore activity although this possibility needs further investigation.

Fig. 6.6. ***Cyclophilin stimulates uncoupled respiration.*** Sub-mitochondrial particles (0.7-1mg protein/ml) were suspended in medium (pH 7.0) containing 10mM HCl (Tris salt), 1.2mM $MgSO_4$, 100μM $CaCl_2$, fumarase (4 units), malic enzyme (1 unit), 0.25mM NADP , 100μM CCCP and 3mM succinate. The incubate was split between two 3ml glass cuvettes. Cyclophilin (CP; 100 pmol/ml) was added as indicated and the difference in absorbance between the two cuvettes was monitored spectrophotometrically at 340nm using a Perkin-Elmer split beam spectrophotometer.

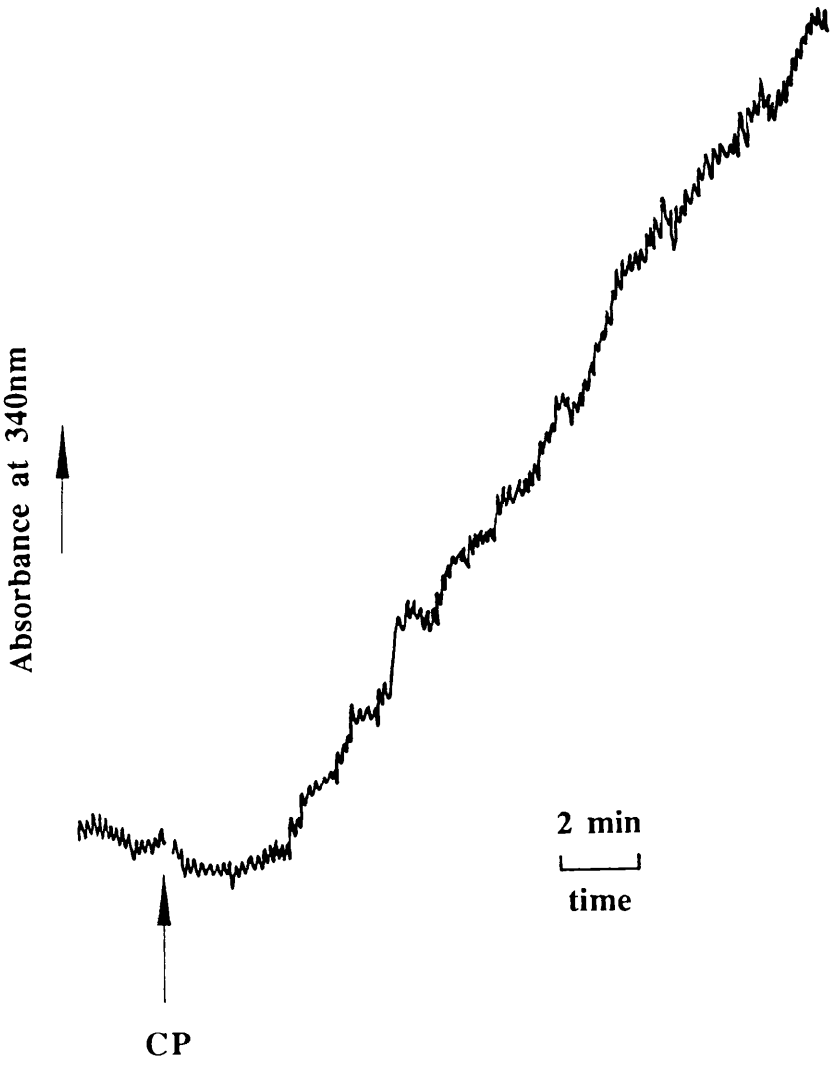


Fig. 6.6

Fig. 6.7. ***ADP inhibits cyclophilin stimulated uncoupled respiration in a dose dependent manner.*** Sub-mitochondrial particles (0.7-1mg protein/ml) were suspended in medium (pH 7.0) containing 10mM HCl (Tris salt), 1.2mM $MgSO_4$, 100μM $CaCl_2$, fumarase (4 units), malic enzyme (1 unit), 0.25mM NADP and 3mM succinate. ADP was added at the (μM) concentrations indicated in parentheses. The incubate was split between two 3ml glass cuvettes. Cyclophilin (CP; 100 pmol/ml) was added as indicated and the difference in absorbance between the two cuvettes was monitored spectrophotometrically at 340nM using a Perkin-Elmer split beam spectrophotometer.

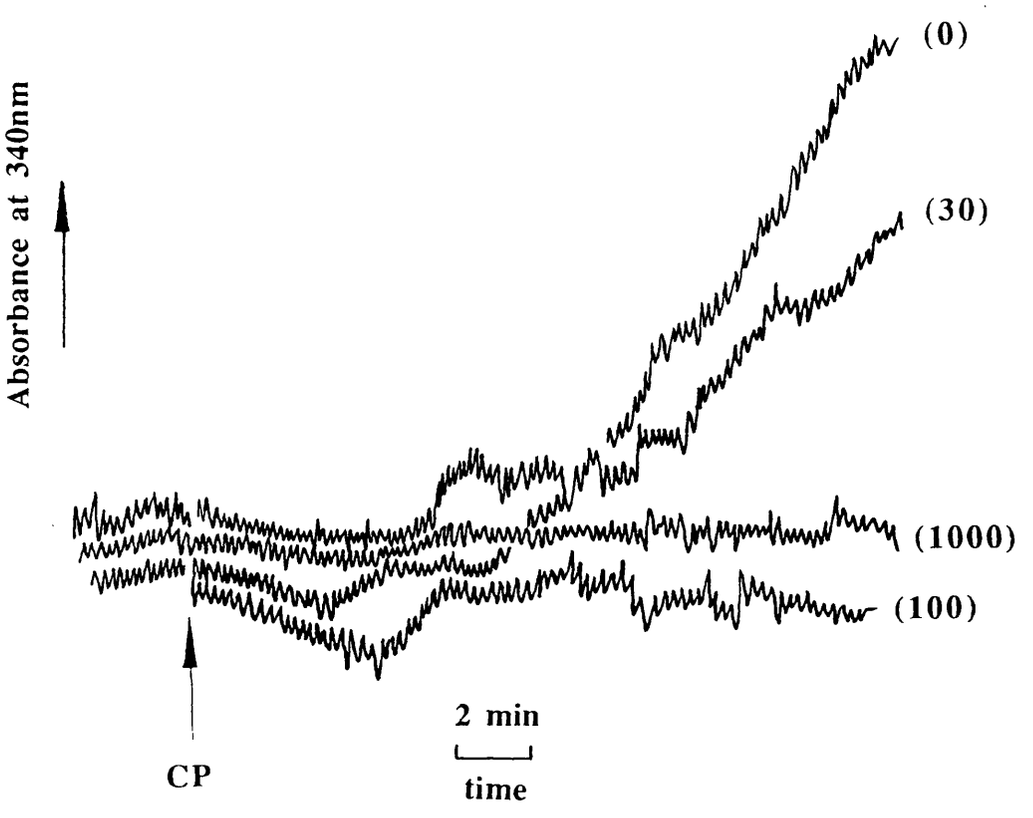


Fig. 6.7

CHAPTER 7: DISCUSSION.

[7.1] *The sensitivity of mitochondria to buffered extramitochondrial free Ca^{2+}.*

It is evident from this study that isolated respiring rat liver mitochondria *in vitro* are highly sensitive to changes in buffered extramitochondrial Ca^{2+} above a critical concentration of around 2μM . To appreciate the significance of the sensitivity of isolated mitochondria to the free extramitochondrial [Ca^{2+}] *in vitro*, it is instructive to consider what happens to matrix free Ca^{2+} in response to a rise in the steady-state free cytosolic [Ca^{2+}] *in vivo*. The flux of Ca^{2+} ions across the inner membrane is a tightly regulated process mediated by at least three distinct carriers [reviewed by Crompton (1985b)] as shown in Fig. 7. 1. As none of the carriers are gated continuous Ca^{2+} cycling occurs across the membrane. The steady-state Ca^{2+} gradient established when influx and efflux are equal, is determined by the relative kinetic properties of the Ca^{2+} carriers. There is a limiting upper cytosolic [Ca^{2+}] compatible with steady-state recycling. The limiting cytosolic [Ca^{2+}] is that yielding a uniporter activity equal to the V_{max} for the Na^{2+}-Ca^{2+} carrier. If cytosolic Ca^{2+} rises above this upper limit mitochondria accumulate Ca^{2+} until the cytosolic [Ca^{2+}] is again reduced to a level compatible with steady-state recycling. Thus mitochondria effectively act as buffers of cytosolic Ca^{2+}. Nicholls (1978) first drew attention to this behaviour showing that at 30°C and pH 7.0 isolated rat liver mitochondria maintain a steady-state extramitochondrial [Ca^{2+}] of 0.8μM. Becker (1980) obtained very similar results and also showed that with the inclusion of 1mM Mg^{2+} the steady-state value of extramitochondrial Ca^{2+} ranged from 1-2μM. Thus, one would expect that rises in extramitochondrial Ca^{2+} above 0.8-2μM would result in a pronounced uptake of Ca^{2+} by mitochondria.

As pointed out in Chapter 3 (section 3.2), although the rate of "permeabilization" does not show any threshold dependence on matrix free Ca^{2+} [Al Nasser and Crompton (1986)], the studies presented in this study clearly show a threshold dependence on external free Ca^{2+}. While exposure of respiring liver mitochondria to $<1\mu$M Ca^{2+} induces little activation of the pore, exposure to 5.6μM Ca^{2+} induces maximal pore activation within 6 min. As the rate of "permeabilization" is far from saturated at 25μM matrix free Ca^{2+}, one would conclude that it is a pronounced uptake of intramitochondrial Ca^{2+} which causes pore opening in this study. The threshold behaviour presumably reflects the dependence of matrix free

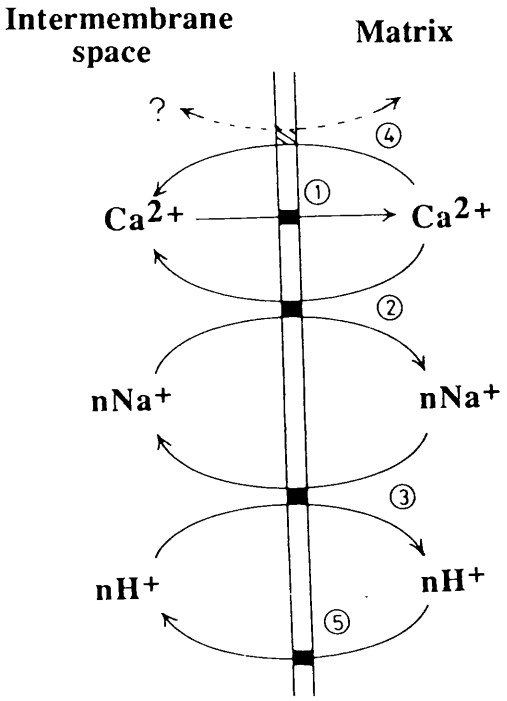


Fig. 7.1

Ca^{2+} cycling across the inner mitochondrial membrane and its interaction with the chemiosmotic H^+ circuit. The transport systems are designated as follows: 1, Ca^{2+} uniporter; 3, Na^+-H^+ antiporter; 4, Na^+-independent process of Ca^{2+} efflux; 5, H^+-pumping complexes of the respiratory chain.

2, Na^+/Ca^{2+} exchange

Ca^{2+} on external free Ca^{2+} i.e. that the increase in matrix free Ca^{2+} with increasing external Ca^{2+} due to the buffering effect described above becomes progressively steeper as external Ca^{2+} is raised.

[7.2] ***Pore activity could contribute to cellular reperfusion injury***.

In these studies both Pi and *oxidative stress* (created by increasing the oxygen tension) acted synergistically with a minimal extramitochondrial buffered free $[Ca^{2+}]$ of 5.6μM to induce pore opening. This is analogous to the situation previously examined where "permeabilization" is induced by an unbuffered $[Ca^{2+}]$ of 35nmol/mg of protein and either Pi or *oxidative stress* imposed by the addition of *tert*-butyl hydroperoxide. The demonstration that "permeabilization" only occurs when ATP is sufficiently depleted and that physiological levels of ATP maintained by an ATP regenerating system actually protect mitochondria from the adverse effect of Ca^{2+}, Pi and oxidative stress extends these investigations. This study lends further support to the proposal by Crompton *et al.*, (1988) that "permeabilization" via the pore may contribute to reperfusion injury. This proposal stems from the observation that pore opening is induced under conditions which would prevail in the cell on reperfusion after an ischaemic period.

In view of its clinical importance ischaemic injury to intact myocardial tissue or to isolated myocardial cells has been most intensely studied. The metabolic status of the cells undergoes key changes during ischaemia in addition to the immediate adverse effects of O_2 deprivation, substrate depletion and waste product accumulation. In myocytes these include an increase in cytosolic Ca^{2+} and Pi [Poole-Wilson, *et al.*, (1984); Kammermeier *et al.*, (1982)] and a depletion of ATP. In heart irreversible injury is associated with a decline of ATP to less than 40% of its physiological level [Humphrey (1985)]. More recently Gasbarrini *et al.*, (1992) shown that anoxia induces a massive increase in cytosolic Ca^{2+} in isolated hepatocytes. Here, ATP levels fell by 66% after 1 hour of anoxia. Cellular reperfusion causes a further rise in Ca^{2+} as well as the generation of oxygen free radicals [Braunwald and Kloner, (1985); Leaf *et al.*, (1983)]. The extent of cell recovery in response to reoxygenation is acutely dependent on the length of the ischaemic period experienced by the cells before reperfusion. Hearse (1977) in his studies on the ischaemic myocardium found that a minimum of 45min ischaemia may be required before reoxygenation induced major damage. He suggested that this

might reflect a requirement for Ca^{2+} to reach a threshold level before irreversible injury ensues. Consistent with this idea, Cobbold and his colleagues have shown in myocytes that provided oxygen was readmitted before cytosolic [Ca^{2+}] exceeded 2-3μM, cells were able to restore Ca^{2+} to resting levels. If the [Ca^{2+}] rose beyond this critical concentration reintroduction of oxygen resulted in cell shortening and subsequent cell death [Allshire *et al.*, (1987)]. However Cobbold's studies [Bowers *et al.*, (1992)] showed that extreme cell contracture due to lack of ATP and structural damage preceded the rise in Ca^{2+}. This is in contrast to results obtained with cultured myocytes in which cytosolic [Ca^{2+}] rose during and after the time of contracture development and preceded irreversible cell injury [Barry *et al.*, (1987)] and it is in contrast to studies in perfused rat heart demonstrating that an increase in cytosolic [Ca^{2+}] precedes lethal myocardial cell injury [Steenbergen *et al.*, (1989)]. Results of *in vivo* measurements in brain using the fluorescent indicator Indo-1 [Uematsu *et al.*, (1988)] also suggest that an increase in cytosolic Ca^{2+} is closely related to the poor recovery observed on reperfusion after an ischaemic period.

Hearse (1977,1978) proposed that reoxygenation after an ischaemic period may lead to a re-energization of the mitochondrial electron transport chain resulting in uncontrollen uptake of Ca^{2+} from the cytoplasm and that this is the primary lesion which predisposes the cell to sudden tissue damage. This would occur due to the buffering effect of mitochondria described in the previous section. Although it is not universally agreed that an increase in cytosolic [Ca^{2+}] occurs before cell damage it is clear from the studies presented in this thesis that at least in isolated liver mitochondria, if ATP is sufficiently depleted a rise in extramitochondrial Ca^{2+} above a threshold value of around 2μM acts synergistically with oxidative stress to induce pore opening. This criti cal concentration of Ca^{2+} could reflect the threshold which must be reached before reperfusion can be induced. Pore opening *in vivo* would not only uncouple oxidative phosphorylation and allow release of low molecular weight metabolites from the mitochondrial matrix but would expose the F_1 ATPase to the cytoplasm allowing reverse hydrolysis of ATP. The consequent rapid depletion of ATP would result in the cell losing its ability to maintain Ca^{2+} homeostasis. Further influx of Ca^{2+} would trigger irreversible cascades , including the generation of oxygen free radicals and the liberation of free fatty acids due to activation of phospholipases, resulting in eventual cell death. The observation that reperfusion of myocytes/ myocardial tissue with Ruthenium Red (which appears to enter heart cells, [Mc Cormack and England (1984)]) an inhibitor

of the uniporter, improves mitochondrial function [Ferrari *et al* (1982)] and myocardial contractility [Smith and Kent (1980)] brings further support to the proposal that mitochondrial Ca^{2+} uptake contributes to defective mitochondrial and myocardial function.

[7.3] ***Possible involvement of perturbations in the membrane lipid phase in permeabilization.***

In these studies there was no change in the free fatty acid content of mitochondria in response to the minimal free [Ca^{2+}] of 5.6μM found to trigger "permeabilization" in the presence of a high [Pi]. This makes it unlikely that uncoupling and "permeabilization" is due to a protonophoric action of free fatty acids, or due to their direct effects on inner membrane proteins. There were already strong indications that any Ca^{2+}-activated phospholipid hydrolysis is unconnected with pore-opening. The rapid reversibility of the process [Crompton and Costi, (1988)] coupled with the fact that the essential cofactors (ATP, CoA) involved in a possible cycle of phospholipid acylation/deacylation would be released from the matrix during "permeabilization" belied the notion that "permeabilization"/resealing involves the operation of such a cycle. Support for the involvement of such a cycle in "permeabilization" came from the discovery that inhibitors of phospholipase A_2 including local anaesthetics and calmodulin antagonists inhibit Ca^{2+} and Pi induced "permeabilization" with an equal potency. In these experiments, "permeabilzation" was assayed by monitoring the increase in absorbance associated with the large amplitude swelling of mitochondria which accompanies the process. However, one would question the reliability of such data, as two groups [Crompton and Costi (1988); and Haworth and Hunter (1976)] have shown that Ca^{2+} induced-swelling of mitochondria can occur in the absence of "permeabilization" as assayed directly by solute entrapment techniques. In addition, support for the lack of involvement of phospholipase A_2 comes from the studies of Malis and Bonventre (1986) on renal mitochondria. By measuring the associated increase in state IV respiration, they showed that the increased permeability of mitochondria induced by the synergisitic action of Ca^{2+} and oxygen radicals is not inhibited by the phospholipase A_2 inhibitor, dibucaine.

Further support for the lack of involvement of phospholipase A_2 activity in permeabilization comes from recent studies of Broekmeier *et al* (1989). These

investigators have shown that 0.5μM CsA, (which was more than sufficient in these studies to inhibit the dissipation of $\Delta\Psi$ and the entry and entrapment of sucrose induced by 5.6μM free Ca^{2+}, Pi and oxidative stress), had *no* effect on phospholipase A_2 activity as compared to 50μM trifluoperazine which was an effective inhibitor of phospholipase A_2 under the same experimental conditions.
Thus one would conclude that "permeabilization" is not due to perturbations in the membrane lipid phase and that Ca^{2+}-induced "permeabilization" reflects the presence of a proteinaceous pore in the inner mitochondrial membrane as originally proposed by Haworth and Hunter (1976).

[7.4] ***Inhibition of "permeabilization" by cyclosporin A.***

The fungal immunosuppressant CsA inhibited "permeabilization" of liver mitochondria induced by a minimal unbuffered [Ca^{2+}] of 5.6μM with high potency. Rather more CsA was needed to completely inhibit uncoupling than to prevent permeability to sucrose. This together with evidence that at low pH the pore becomes permeable to protons before it is fully permeable to sucrose suggests that the pore may exhibit more than one gating state. Indeed patch clamp studies of the 1.3 nano-siemens pore/channel have indicated that substates of the channel with lower conductances may exist [Szabó and Zoratti, (1991)]. Other evidence for the presence of at least two distinct states of the pore come from studies on the ability of CsA to promote pore closure. While it is demonstrated in Chapter 3 that CsA if given sufficient time can restore the impermeability of fully "permeabilized" mitochondria to sucrose it has been shown that CsA (1μM) alone cannot restore $\Delta\Psi$ and impermeability to protons under these conditions unless ADP (100μM) is also added [Novgorodov *et al.*, (1991)]. Although this data may be indicative of two gating states of the same pore by which ADP facilitates a conformational change allowing CsA to restore permeability to protons, one could envisage two separate pores: a 20Å pore which is inhibited by CsA and which is non-selectively permeable to ADP and other low molecular weight solutes; and a smaller pore inhibitable by ADP on the matrix side of the membrane which is selectively permeable to protons. Leakage of ADP through the 20Å pore causes constitutive activation of the smaller pore allowing continued uncoupling, even after the 20Å pore has been blocked by CsA. It may be that depolarization due to uncoupling triggers opening of the 20Å pore. Indeed Hunter and Haworth (1979) have shown that even if NAD is oxidised energization prevents Ca^{2+}-induced "permeabilization" of mitochondria, whereas if

NAD is maintained in a highly reduced state by the addition of β-hydroxybutyrate, the uncoupler FCCP stimulates Ca^{2+}-induced "permeabilization" to low molecular weight species.

In the light of the implication of "permeabilization" in reperfusion injury it is important to evaluate CsA's *in vivo* capacity to protect cells/tissues against reperfusion injury. Preliminary studies in this laboratory have shown that CsA protects populations of myocytes against such injury. In addition it has been shown that pretreatment of dogs with CsA before their livers were subjected to ischaemia was found to increase greatly the chances of complete recovery [Hayashi *et al.*, (1988)].

[7.5] ***Cyclosporin A inhibits "permeabilization" by binding to mitochondrial cyclophilin:***

This study shows that CsA binds with high affinity to two mitochondrial components besides partitioning into phospholipids. It seems most likely that CsA-binding component I is implicated, judging from the amounts of bound CsA required to saturate the "permeabilization" process and component I after the standardized 8 min preincubation period with CsA, and by the Ca^{2+}-dependent decrease in CsA binding to this component.

Component I comprises $<10\%$ of the high-affinity CsA-binding sites of liver mitochondria. A similar proportion is attributable to cyclophilin. Halestrap *et al.*, (1990) have demonstrated using swelling measurements that the inhibitory potency of three CsA analogs on mitochondrial cyclophilin's peptidyl-prolyl-*cis-trans*-isomerase (PPIase) activity parallels their ability to inhibit "permeabilization". In addition these workers showed that the novel immunosuppressant FK506, which binds to and inhibits a quite separate cytosolic PPIase [Harding *et al.*, (1989); Siekierka, J.J. *et al.*, (1989); Schreiber, (1991)] inhibited neither mitochondrial PPIase nor "permeabilization". This raises the question of the involvement of PPIase activity in pore opening. Although the particular roles of prolyl isomerases *in vivo* have not been established, they may recognise and/or interconvert suitably exposed proline residues in proteins [Fischer and Schmid, (1990)]. The resulting redirection of a local protein chain could provide the conformational change necessary for the regulation of a transport channel. In a survey of bilayer spanning

regions of integral membrane proteins, Brandl and Deber (1986) found membrane buried proline residues in nearly all the transport proteins examined whereas those proteins not involved in transport rarely contained such proline residues. However, CsA reversed "permeabilization" excluding the possibility of the isomerase directly catalysing pore closure as if this were so CsA would be predicted to maintain the "permeabilized" state rather than reverse it. This conclusion is supported by a study of the effect of CsA on the rate of EGTA induced resealing examined using the rapid pulsed flow technique developed in this laboratory [Mc Guinness *et al.*, (1990)]. Thus the notion that the isomerase might interconvert surface-exposed proline residues of the pore during transition between open and closed states was not borne out.

One alternative is that component I/cyclophilin is involved in a binding capacity. For example, one might envisage that the component recognises a conformation capable of opening and stabilizes it, i.e.

$$[Ca^{2+}\text{-pore}]\text{cyclophilin} \leftrightarrow \text{cyclophilin} \leftrightarrow \text{cyclophilin}\cdot\text{CsA}$$

This model would account for a number of observations made in this study, namely the capacity of Ca^{2+} to decrease CsA binding to component I, the capacity of CsA to inhibit pore opening and the capacity of CsA to induce pore closure. If pores open and close continuously in "permeabilized" mitochondria [Crompton and Costi, (1990)], cyclophilin might increase permeability by binding to a form disposed to alternate between open and closed states thereby increasing the open time.

This model is clearly hypothetical. It predicts that both CsA and Ca^{2+} removal would be expected to induce pore closure. However whereas Ca^{2+} removal affects rapid pore closure, i.e. within 60ms after chelating matrix Ca^{2+} [Crompton and Costi, (1990)], CsA induced resealing occurred at least three orders of magnitude more slowly [Mc Guinness *et al.*, (1990)]. In terms of the model, this would mean that CsA caused an extremely tight binding of cyclophilin to the pore so that CsA could remove it only relatively slowly. On these grounds one would expect that a high $[Ca^{2+}]$ would depress CsA binding to cyclophilin by providing an alternative binding partner for it, whereas in fact CsA binding to component I was depressed by 30-50% only (Fig. 4.2). This contradiction may be resolved if it is assumed that only

30-50% of component I/cyclophilin may be bound tightly to the pore in this way or, in other words that component I/cyclophilin is in 50-70% excess over the pore so that this percentage was freely available to bind CsA in the presence of high Ca^{2+}. This suggestion is fully supported by the observed sigmoidal relationship between "permeabilization" and [CsA] (Fig, 1.10) since this also indicates that the cyclosporine-A binding component was in excess over whatever component it combined with. Since after the standardized preincubation with CsA, cyclophilin/component I amounted to <5 pmol/mg mitochondrial protein, the model leads to the tentative conclusion that the maximal number of pores available for binding to cyclophilin is <2 pmol/mg mitochondrial protein.

With this interpretation in mind, it has been suggested that the adenine nucleotide translocase may be distorted by Ca^{2+} into an open pore form [Zoccaroto *et al.*, (1981); Le Quoc and Le Quoc (1988)] and, very recently, that cyclophilin may stabilize this form [Halestrap *et al.*, (1990)]. The present study supports this to the extent of implicating cyclophilin, but raises some questions which are more difficult to reconcile. An interaction between the adenine nucleotide translocase and cyclophilin was proposed on the basis of a linear relation between inhibition of swelling and inhibition of cyclophilin, the content of which was estimated to be 110 pmol/mg liver mitochondrial protein from best fitting data to a single (hyperbolic) binding component [Halestrap *et al.*, (1990)]. The existence of two CsA binding components may explain the discrepancy between that value and the amounts of cyclophilin/component I indicated in this study. From the present measurements, the amount of cyclophilin implicated would permit <3% of the adenine nucleotide translocase (150-350 pmol/mg liver mitochondrial protein [Klingenberg, M. (1976)] to be stabilized in a distorted form, whereas, as discussed above cyclophilin is in excess of any putative partner for binding. If the adenine nucleotide carrier is involved, it becomes necessary to consider why only a very small fraction of the pores are capable of acting non-specifically.

[7.6] *ADP-ribosylation is not functionally involved in "permeabilization".*

As already stated in chapter 5 protein ADP-ribosylation in intact mitochondria has not been studied in detail because the inner mitochondrial membrane is impermeable to NAD. One can only obtain indirect evidence for a functional link between ADP-ribosylation and pore opening in these experiments by showing that the agents which

modulate pore opening affect the degree of protein ADP-ribosylation observed in the same way. Previous investigators observed ADP-ribosylation almost exclusively of a protein with a MW of 30kDa in sub-mitochondrial particles prepared from rat liver [Richter and Frei (1988)] and bovine heart [Hilz *et al.*, (1984)]. In this study, incubation of sub-mitochondrial particles under standard conditions with [*adenylate* ^{32}P]-NAD resulted in the radiolabeling of one membrane polypeptide migrating with an apparent MW of approximately 34kDa. If labelling is a reflection of protein modification by ADP-ribosylation, none of the effectors of the pore altered the extent of ADP-ribosylation arguing against its functional involvement in "permeabilization". In addition, CsA did not inhibit hydrolysis of NAD to nicotinamide and ADP-ribose as no decrease in the extent of radiolabeled protein was observed in the presence of the immunosuppressant. This contradicts Richter's claim that CsA inhibits the hydrolysis of NAD (Richter *et al* (1991). In Richter's investigations, rat mitochondrial pyridine nucleotides were labeled *in vivo* and NAD hydrolysis assayed by measuring the subsequent release of nicotinamide from mitochondria. It may be that protein ADP-ribosylation is not linked functionally to "permeabilization", but rather that hydrolysis of oxidised NAD (and subsequent protein ADP-ribosylation) occurs due to its cytoplasmic exposure as a result of "permeabilization". ADP-ribosylation in sub-mitochondrial particles is inhibited by adenine nucleotides [this study; Richter *et al.*, (1985)]. Losses of adenine nucleotides after "permeabilization" might allow NAD hydrolysis to occur subsequently. Thus, pore opening would be associated with the release of nicotinamide. CsA, by inhibiting "permeabilization" would prevent any NAD hydrolysis occurring in the first instance. The lack of any functional involvement of ADP-ribosylation in "permeabilization" is supported by the observation that there is no significant increase in protein bound ribose content [Weis *et al.*, (1992)] or only a transient increase [Frei and Richter (1988)] associated with the process. Those that argue in favour of a functional involvement of ADP-ribosylation in "permeabilization" attribute this to the lack of sensitivity of total ribose measurements combined with the high turnover of protein-bound ribose content [Richter *et al.*, (1983); Kun *et al.*, (1975)]. Support for the functional involvement of protein ADP-ribosylation in "permeabilization" comes from the observation that *meta*-iodo-benzylguanidine (MIBG), a high-affinity substrate for mono(ADP-ribosyl)ating enzymes and a strong inhibitor of intracellular ADP-ribosyltransferases [Smets *et al.*, (1988); Loesberg *et al*, (1989)] inhibits "permeabilization" induced by Ca^{2+} and *tert*-butylhydroperoxide [Richter C., (1990)]. MIBG is taken up by succinate-energized mitochondria.

Clearly, more investigation is needed in order to clarify the role of ADP-ribosylation in "permeabilization".

[7.7] ***Pore activity could not be induced in sub-mitochondrial particles.***

Unlike the case in mitochondria, pore activity could not be induced in sub-mitochondrial particles by simply supplementing them with Ca^{2+} together with Pi, or the oxidised members of redox couples. Sub-mitochondrial particles pump Ca^{2+} out of the matrix on energization with succinate and it may be that Ca^{2+} must be bound to both sides of the inner mitochondrial membrane to induce pore opening. However pore activity could not even be detected in deenergized sub-mitochondrial particles supplemented with Ca^{2+} which would presumably have equilibrated with the matrix space under these conditions.

As described in Chapter 6, mitochondrial cyclophilin was isolated in order to examine its specific effects on membrane permeability.A purification protocol which yielded a 1320 fold purification of cyclophilin was developed. Quantitative analysis estimates the amount of cyclophilin to be 28.98 pmols/mg mitochondrial protein. This is five times the amount of cyclophilin (5 pmol/mg mitochondrial protein) bound to CsA after the standardized preincubation with CsA when "permeabilization" is completely inhibited. This indicates that at least 80% of mitochondrial cyclophilin is redundant as regards an involvement in "permeabilization". Connern and Halestrap (1992) have recently shown that there are two isoforms of mitochondrial cyclophilin, a major form of 18.6 kDa and a minor isoform of 17.6kDa. It may be that only the minor component is involved in inhibition of "permeabilization".

Although the results presented in Chapter 4 implicated cyclophilin in mediating CsA's actions, key questions have not been resolved. This was partly due to the strategy undertaken to investigate cyclophilin's and in fact other soluble matrix component's involvement in pore activity. It was hoped that pore activity could be reconstituted in sub-mitochondrial particles. Sub-mitochondrial particles were supplemented with the concentration of pure cyclophilin normally present in the matrix space for a more precise investigation of its role in pore opening/closure. However, cyclophilin alone did not sensitise sub-mitochondrial particles to the unbuffered concentrations of Ca^{2+} and Pi which induce pore activity in

mitochondria. This again argues against the highly speculative model proposed by Halestrap and Davidson, (1990), in which cyclophilin stabilizes a Ca^{2+}-induced distortion of the adenine nucleotide translocator into an open pore form. If this were simply the case, one would expect Ca^{2+} and cyclophilin to induce "permeabilization" in sub-mitochondrial particles. As described in the Introduction agents which stabilize the adenine nucleotide translocator in the *c*-conformation can act synergistically with Ca^{2+} to induce "permeabilization" [Le Quôc and Le Quôc (1988)]. In addition, the conformational state of the adenine nucleotide translocator has a strong influence over the ability of CsA and ADP to induce pore closure, especially when matrix ADP is substantially depleted [Novgorodov *et al.*, (1990); Novgorodov *et al.*, (1991)]. The adenine nucleotide carrier is one of the most abundant proteins in the mitochondrial membrane being present at a concentration of 150-350 pmol/mg liver mitochondrial protein [Klingenberg, (1976)]. Changes in the conformational state of the adenine nucleotide carrier therefore have gross effects on the morphological state of mitochondria including a drastic reorganisation of the cristae network [Klingenberg *et al*, (1971); Scherer and Klingenberg (1974)]. Thus ADP and bongkrekate cause mitochondria to contract, while atractyloside causes decontraction. It may be that these gross conformational changes make mitochondria more susceptible to the agents which induce "permeabilization" perhaps by exposing masked binding sites as opposed to a distorted form of the adenine nucleotide translocator actually forming the pore itself.

It is possible that cyclophilin binds to a Ca^{2+}/protein complex, which for some reason is not expressed after sonication to induce pore opening. The alternative possibility, that in fact CsA acts as an allosteric effector of cyclophilin, the complex binding to the pore to induce pore closure, is not the case as cyclophilin did not promote CsA binding to sub-mitochondrial particles.

One final possibility is that cyclophilin, say the minor isoform, could be compartmentalised in the intermembrane space acting on the outside of the inner membrane to induce "permeabilization". As the bulk of the sub-mitochondrial particles in the preparations used in this study were in the inside out configuration, this would explain the apparent absence of pore activity observed.

[7.8] ***Cyclophilin stimulates uncoupled respiration in sub-mitochondrial particles.***

Cyclophilin caused a small ($\simeq$ 10%) stimulation of uncoupled respiration. The possibility that cyclophilin is interacting directly with some component of the respiratory chain is intriguing. Furthermore ADP an important modulator of pore activity inhibits the stimulation of respiration conferred by cyclophilin. There may thus be a connection between cyclophilin's effect on redox components and its role in pore activity. The subunits of the respiratory chain are involved in proton translocation/pumping and the pore could represent a distorted state of one of these proton pumps. This possibility clearly merits further investigation.

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