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The mitochondrial permeability transition

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Abbreviations: ADN, adenine nucleotide; AdNT, adenine nucleotide translocator; ATR, atractyloside; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BGK, bongkrekate; BHT, butylhydroxytoluene; BMV, binding modulation by voltage; BSA, bovine serum albumin; CATR, carboxyatractyloside; CSP, cyclosporin A; DNP, dinitrophenol; DTT, dithiothreitol; EM, electron microscopy; IMAC, inner membrane anion channel; GSH, glutathione; MIBG, meta-iodobenzylguanidine; MMC, mitochondrial megachannel; MW, molecular weight; NEM, *N*-ethylmaleimide; PA2, phospholipase A_2; pCMBS, *p*-chloromercurybenzensulfonate; PEG, poly(ethylene glycol); PEP, phosphoenolpyruvate; PiC, phosphate carrier; Po, open probability; PPIase, peptidyl prolyl isomerase (cyclophilin); PT, permeability transition; PTP, permeability transition pore; PyNu, pyridine nucleotide; RR, Ruthenium red; SR, sarcoplasmic reticulum; *t*-BuOOH, *t*-butylhydroperoxide; VDAC, voltage-dependent anion channel (mitoch. porin); VSMB, voltage sensitivity modulation by binding

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1. Introduction

This review deals with the permeability transition (PT) of isolated mitochondria, a field spawned at the very beginning of mitochondrial research by the observation that the mitochondria soon after isolation begin to swell, lose their ability to phosphorylate and release matrix contents unless Ca^{2+} ions are excluded from the suspension. More than forty years later, the phenomenon might finally be on the verge of being understood in its details, largely thanks to the recent discovery of a high-affinity inhibitor, Cyclosporin A (CSP).

The literature reflects the difficulties bioenergeticists had to face, especially before the advent of the chemiosmotic model. The early reports contain some inconsistencies, a few of which are mentioned, e.g., by Lipsett and Corwin [1,2]. We have attempted to provide access to most relevant papers, apologizing for omissions. A few reviews dealing with the permeability transition [3–9] and the related topic of Ca^{2+} release mechanisms [4,9–14] have appeared.

The next Section presents a brief outline, intended as a guideline for the remainder of the review, in which the various aspects are treated in detail. To facilitate our task as well as the reader's, thereafter we shall assume the reader has kept in mind the schematic description of the outline, and take the liberty of making reference to topics and conclusions not yet covered in detail. Most of Section 2 presents a catalogue of inducers and inhibitors, to which the superficially interested reader might want to refer only as needed.

1.1. Outline

One of the bases of the chemiosmotic model of energy transduction is the impermeability of the inner mitochondrial membrane to solutes not endowed with specific transport systems. Impermeability may be lost by free-radical processes involving the lipid bilayer or via the incorporation of pore-forming compounds. Otherwise, mitochondria become nonspecifically permeable only if loaded with Ca^{2+}, and presented with one of a vast number of 'inducing agents'. This latter phenomenon is the subject of this review. Among inducers, P_i and oxidizing agents figure prominently. In mitochondria exposed to these agents a large (estimated diameter 2–3 nm) proteinaceus pore (the PTP) residing in the matrix-delimiting envelope opens (or not, depending on the details of the experimental conditions). As a consequence, the mitochondria lose matrix components and swell. Opening, or the permanence in the open state(s), is favored by Ca^{2+} binding to two sites on the matrix side of the membrane, a process subject to competitive inhibition by other divalent cations. Activation is also favored by depolarization. On the other hand, physiological transmembrane potentials or protonation of one or more histidine residues cause pore closure, or favor its permanence in the closed state(s). ADP and divalent cations such as Mg^{2+} are also powerful inhibitors. Most of these effects take place at matrix side sites. The cross-linking of SH groups is thought to play an important role in the formation of the pore from (a) precursor membrane protein(s), at least when the PT is induced by oxidizing agents plus Ca^{2+}.

Historically, the field evolved from early unspecific swelling studies through a series of recognizable conceptual stages: (1) the realization that Ca^{2+} overload was at the origin of 'damage' and swelling; (2) the recognition that the phenomenon affected the mitochondria in a given sample in a sequential, all-or-nothing fashion; (3) the eventual prevalence of the model envisioning a proteinaceous pore as the entity responsible for permeabilization.

The molecular identity of the pore has not yet been established. The protein(s) involved might be an unknown, or unsuspected, one, but the currently popular models point to well-known membrane components. The involvement of the adenine nucleotide translocator (AdNT) has long been suspected, on the basis of the effects of AdNT ligands, and one model envisions the exchanger itself forming a large-conductance pore. On the other hand, the electrophysiological properties of a mitochondrial megachannel (MMC) identified as the PTP in patch-clamp experiments suggest that it might consist of a dimer of porin molecules, presumably associated with two molecules of AdNT and possibly with two copies of an 18 kDa peptide, a complex forming the mitochondrial benzodiazepine receptor (mBzR).

The function of the pore, if any, is at present a matter of speculation. Support has been accumulating for the hypothesis that it may play a role in cell damage resulting from anoxia and reperfusion in ischemic tissue.

1.2. Experimental methods

Only a superficial description of the most widely used methods can be given here. Volume changes have been used to follow the permeability transition of isolated mitochondria since the beginning of research on the phenomenon (e.g., [15,16]). Medium components are driven into the matrix space, through the open pores, by a 'colloidosmotic' process [4,16] due to the presence of indiffusible proteins in the matrix. Nephelometric or turbidometric techniques have generally been used to monitor mitochondrial swelling, taking advantage of the fact that the amount of light scattered by a suspension of particles is a decreasing function of the mean particle size, while, correspondingly, the amount of light transmitted is an increasing function of size. The experimental data are generally gathered on a time scale of minutes. In most experiments, the onset of swelling is preceded by a lag time of variable duration. The information obtained, which can be quantitative [17–20,22], obviously concerns the whole sample of mitochondria. EM work [21,22] (for EM

images see also [23–25]) and kinetic studies monitoring the flux of tracers with high time resolution [26] have established that at the level of a single mitochondrion the PT is an all-or-nothing event, taking place on a time scale of hundreds of milliseconds. The kinetics observed in light-scattering experiments, i.e., on a time scale of minutes, reflect therefore the propagation of the transition through the population of mitochondria. A correlation can be established between light scattering and the percentage of mitochondria which have undergone the PT by means of suitable comparisons [22,27] (for reviews dealing with the measurement of mitochondrial volume see [28,29]). The ease of performing swelling-monitoring experiments, which can be conducted in a cuvette using an ordinary spectrophotometer or fluorimeter, the high sensitivity of the procedure, and the possibility to follow the swelling process continuously are at the origin of the popularity of this method, which however does not discriminate against volume or light-scattering changes having other origins.

Coupled, energized mitochondria maintain a transmembrane potential which drives the accumulation of lipophilic, membrane-permeant, cationic species such as phosphonium salts or certain fluorescent dyes. The measurement of the accumulation ratio for these species provides a means to measure the potential itself (review, [28]). Permeabilization leads to the collapse of the voltage gradient, and to the efflux of the probe, which can be followed using specific electrodes or fluorescence measurements. The release of the probe can thus be used to monitor the PT (e.g., [30–32]). The PT-induced loss of transmembrane potential will also lead to the efflux of the Ca^{2+} with which the mitochondria had originally been loaded, which can be followed with a specific electrode or using a fluorescent Ca^{2+} indicator dye and used to monitor the PT itself (e.g., [33]). Mg^{2+} release has also been used as a measure of PT occurrence (e.g., [34,35]). Advantage has also been taken of the fact that the opening of a nonspecific permeation pathway in the inner membrane will allow exogenous, normally impermeant substrates of matrix enzymes to reach the latter (e.g., [23,36]). Non-metabolizable, normally impermeant probe molecules, such as [^{14}C]sucrose, will also diffuse, through the PTP, into the matrix, where they can be trapped upon EGTA-induced closure of the pore (solute-entrapment technique). This approach has been used in pulsed-flow kinetic experiments (e.g., [26]).

The techniques mentioned so far provide data averaged over a large number of particles. With the exception of the solute-entrapment/fast-kinetics experiments, they can provide only limited information at the level of a single mitochondrion or of a single pore. The potential probe release method suffers from the shortcoming that the probe released by the permeabilized mitochondria may be taken up by the organelles still maintaining a potential. Complications may also arise from the relatively slow kinetics of probe permeation. The released Ca^{2+} may also be taken up by still-coupled mitochondria, unless precautions are taken to avoid this (see Section 2.2). Furthermore, a pathway of Ca^{2+} release distinct from the PTP and the calcium uniport may exist (see Section 3).

The application of the patch-clamp technique [37,38] to mitochondria [39–42] has now made it possible to study the PTP at the single-channel level. Patch-clamp provides the most powerful and direct available method to study the PTP as such. On the other hand, the method is laborious, comparative studies may be difficult and subtle effects may be hard to appreciate.

2. Characterization of the permeability transition

This Section provides some historical perspective, and presents information on the most important inducing and inhibiting agents. The early literature has "never been reviewed comprehensively" [4] in the light of current ideas. The studies of the first two decades on mitochondrial aging and swelling eventually led to the identification of factors promoting or counteracting the PT, and were therefore of great relevance for later work. Space limitations prevent a more detailed presentation of this material.

2.1. Early work on mitochondrial aging and swelling

One of the problems the founders of mitochondrial bioenergetics had to face was the lability of the oxidative phosphorylation system (e.g., [43–52]). The earliest research on our topic consisted in the description of the events contributing to the degeneration ('aging') of the isolated organelles, and in the search for conditions which would prevent it. In the absence of an integrated model for the mitochondria, the various phenomena were discovered separately, and considered initially as independent events. Table 1 lists some of the events associated with the PT, and provides references to pertinent early papers.

By the mid- to late-50's it was being recognized [53,54] that the several phenomena (Table 1) were manifestations of the same process, which the mitochondria underwent under identifiable conditions. The need for a comprehensive model which could accomodate the variety of conditions leading to the PT was already being felt [53]. It was also recognized that P_i- and/or Ca^{2+}-induced swelling was different from that caused by decreases in medium osmolarity [55].

It is difficult to single out any given paper as the founding stone of research on the permeability transition. The earliest article to deal with an aspect of the phenomenon might be the one by Potter [56], who identified Ca^{2+} as one of the factors causing a decrease of P_i uptake (i.e., ATP production) in kidney homogenates. The paper establishing that the PT was due to the loss of impermeability by the mitochondrial membrane might be one by Hunter and Ford [53], who also operated the conceptually important distinction between swelling and permeabiliza-

Table 1
A summary of early reports on various aspects of the PT

Observation	References
Swelling in isotonic media	[16,48,57,64–69]
Ca^{2+} causes:	
uncoupling	[46,50,51,53,70–72]
ATPase activity	[51,73,74]
swelling	[60,64,68,75–79]
inhibition of respiration (Site I)	[50,71,80,81]
Protection by EDTA	[50,53,64,71,81–83]
P_i causes:	
uncoupling	[53,81,84]
swelling	[16,52,53,55,60,76,77,85–87]
Permeation by sucrose	[57,88,89]
Loss of K^+	[53,76,84,90]
Loss of nucleotides:	
adenine nucleotides	[49,52,53,66,71,72,76,80,91,92]
pyridine nucleotides	[47,52,53,55,60,80,81,86,91,93–99]
Loss of Krebs cycle intermediates	[100]
Loss of Coenzyme A	[66,80]

tion. In the early times, a distinction was made between 'low amplitude' and 'high amplitude' swelling, based on the extent of the variation in the scattering of light. In his 1962 review [3], Lehninger distinguished also between 'active', or respiration-dependent, and 'passive' swelling. The mechanism(s) underlying these volume changes were not yet clear (despite a well-documented 1958 report [57] that swelling was osmotic in nature). In 1965 Azzi and Azzone [58,59] identified low-amplitude swelling with energy-dependent swelling (reversible by adding uncouplers) and large-amplitude swelling with energy-independent, or passive, swelling. Both were recognized to be osmotic phenomena. In modern terms, we might define active swelling as requiring the presence of a transmembrane electrochemical proton gradient, while passive swelling corresponds to osmotic phenomena which take place without such a requirement.

Hunter and coworkers [55] presented the first EM images of mitochondria subjected to the PT and carried out a prototype resealing and reconstitution study, utilizing ATP, ADP or inorganic triphosphate as a resealing agent, and 'reloading' the mitochondria with variable amounts of pyridine or adenine nucleotides. The possible involvement of SH groups was pointed out already in 1956 [60], as an explanation for the protective effect of anaerobiosis and the destabilizing effect of SH reagents (see Section 2).

By the mid-60's the phenomenological description of the PT had been largely completed [3,54], and attention was focusing on the mechanistic aspects. Hunter and Haworth's landmark work [21,27,61–63], at the end of the 70's, introduced the 'pore' model for the phenomenon, and marked the coming of age of research in the field.

2.2. Induction

Perhaps the only dogma concerning the permeability transition is that it requires the accumulation of Ca^{2+} in the matrix (but see Section 5.4). The amount of Ca^{2+} required (or, in general, the susceptibility to permeabilization) varies depending on the species [50,101,102] or organ [103–112] of origin of the mitochondria (e.g., heart mitochondria are more resistant than liver ones), and also on what compound is used as a coadjuvant.

That the relevant parameter is matrix Ca^{2+} has been established by the observation that the PT can be abolished by RR, which blocks the Ca^{2+} uniport, if the blocker is added before Ca^{2+} [27,110]. The lag time before the onset of permeabilization [113], as well as the rate and final extent of permeabilization, i.e., the percentage of mitochondria in a suspension which have undergone the permeability transition at a given time, has been found to depend on the 'calcium load' [110,114–119]. This implies that isolated mitochondria form an heterogenous population with respect to the induction of the PT [109,110,120]. The best evidence for this statement comes from experiments in which mitochondria are allowed to take up a quantity of Ca^{2+} known to be sufficient to lead to the eventual permeabilization of all the organelles, and RR is then added to inhibit further Ca^{2+} transport by the Ca^{2+} uniport. Under these conditions, only a part of the mitochondria become permeabilized. The currently favored interpretation of this observation is that in the absence of RR the weaker mitochondria become permeabilized, release their Ca^{2+} load, and the released Ca^{2+} is taken up by the more resistent mitochondria, which thereby increase their load until they also must undergo permeabilization. Permeabilization would thus spread in wave-like fashion through the population. RR, by blocking uptake, would 'save' the more resistent organelles [120,121]. Heterogeneity is also evident from observations on sucrose permeation during isolation [88,89]. The heterogeneity of mitochondria has been attributed to variations in their content of PT modulators, such as ADP [22,122] (see Section 2.3).

Haworth and Hunter [21] and Novgorodov et al. [30] have provided evidence, based on the slope of Hill plots, for the involvement of two activating Ca^{2+} binding sites, exhibiting positive cooperation. Ca^{2+}-induced permeabilization can be reverted ('resealing') by the addition of excess EGTA [26,123–130]. If the appropriate experimental conditions are chosen, resealing can be followed by shrinkage to normal volume, with complete recovery of the convoluted membrane structure of the organelles [130].

The addition of Ca^{2+} alone is sufficient to induce the PT at least in liver, heart, kidney and adrenal cortex mitochondria (e.g., [21,23,27,36,61,131–133]), but the presence of an 'inducer' accelerates the process. The classical inducer has long been P_i. Ca^{2+} and P_i display synergism in PT induction [134–136]. A weak acid, phosphate allows the accumulation of enormous amounts of Ca^{2+} (e.g., [137–139]), by providing a mechanism for reducing matrix alkalinization. Above a threshold, hydroxyapatite granules precipitate in the matrix [10,136,140]. The PT-facilitating effect of P_i is not, however, accounted for

simply by the increase in Ca^{2+} uptake. Other weak acids, such as acetate, can also provide counterions for this process, but are much less effective as PT inducers (e.g., [141,142]). The effect of P_i on a population of mitochondria is dose-dependent, i.e., the percentage of mitochondria undergoing the transition is a function of the P_i concentrations, if $[Ca^{2+}]$ and other conditions are held constant [113,118,143]. P_i must enter the matrix [144] to exert its effect, which might be due to its ability to buffer the matrix pH [142] (see Section 2.3.5). Another hypothesis might be that P_i acts by causing a reduction of the matrix [ADP]. Coupled, resting mitochondria are thought to mantain a matrix phosphorylation potential in equilibrium with the transmembrane $\Delta\tilde{\mu}_H$. If these two thermodynamic parameters remain constant, an increase in P_i concentration should cause an offsetting increase in the [ATP]/[ADP] ratio. Evidence has been presented that this indeed happens [145,146]. Since ADP is a better pore inhibitor than ATP (see Section 2.3.3), this should result in a greater ease of activation of the PTP. However, it is not clear that the magnitude of any such changes could account for the observed effects. Furthermore, As_i is as good an inducer as P_i (e.g., [53,61,87]), even though the [ATP]/[ADP] ratio can hardly be expected to increase in its presence. A high concentration of external P_i might also induce depletion of matrix AdNs via operation of the

Table 2
A classification of the major PT inducers

Class	References
P_i and As_i	[53,54,61,87,112–114,119,144,273–281]
Oxidizing agents	
- of pyridyine nucleotides	
acetoacetate and oxaloacetate:	[109,113,117,120,122,258,281–290]
respiration:	[1,2,60,77,81,85–87,291–300]
- hydroperoxides:	
t-butylhydroperoxide:	[25,26,112,119–121,125,147,148,156,158,161,167–170,213,228,275,289,301–307]
cumene hydroperoxide:	[30,165,308]
hydrogen peroxide:	[301,307,309]
- radical-generating species	
menadione:	[119,149]
alloxan:	[160,310,311]
allantoin and uric acid:	[312–316]
adriamycin and derivatives:	[317–319]
nitrofurantoin:	[25,213]
Fe^{2+}, Fe^{3+}:	[177–181,309,320–322]
xanthine and xanthine oxidase:	[188,323–325]
5-aminolevulinic acid:	[326,327]
GSH/GSSG and disulfide hormones:	[177,189,192–195,198,199,295,328–331]
ascorbate:	[193,332]
SH reagents	
- heavy and transition metals and their complexes	
Hg^{2+} and mercurials:	[53,60,270,292,320,332–336]
mersalyl:	[116,279,337]
Cd^{2+}:	[53,226,338,339]
Cu^{2+}:	[53]
Zn^{2+}:	[53,60,86]
- Cross-linkers and disulfide bridge formers	
diamide:	[109,110,112,119,149,166,167,228,275,277–279,283,287,299,340–343]
arsenite:	[53,166]
phenylarsine oxide:	[166,305,344–347]
- NEM	[58,109,115,116,121,220,283,348–351]
- thyroxine:	[3,85,86,97,103,114,116,143,269,270,292,296,333,337,352–362]
- *Adenine nucleotide translocator ligands*	
- atractyloside and carboxyatractyloside:	[27,32,58,102,114,167,241,242,274,276,287,305,363–369]
- pyridoxal-5-phosphate:	[241]
- acyl-CoA's (and acylcarnitines):	[102,109,241,258,283,337,347,370–376]
Agents causing depletion of matrix adenine nucleotides	
- PP_i:	[16,122,263,265,377]
- PEP:	[27,106,220,266–268,378]
Transmembrane potential-reducing agents	
- uncouplers:	[76,77,116,122,220,265,275,285,294,320,379–384]
- lysophospholipids:	[337,370,374,385–387]; see also [388,389]
Fatty acids:	[22,61,82,104,201,232,233,337,373–375,390–394]

P_i/ATP-Mg^{2+} or P_i/$HADP^{2-}$ antiporter (see Section 2.3.3). Direct evidence for such an effect has been recently presented [147].

In addition to P_i and As_i, dozens of other inducers have been found. The reader is referred to published compilations [3,4,8] for a detailed list. We attempt here a classification of these compounds based on their properties and putative mode of action. The subdivision is somewhat arbitrary: different classes of compounds may act by overlapping mechanisms, and the mechanisms of action are (to varying degrees) hypothetical. Table 2 presents examples for each class; it is not intended to be exhaustive.

2.2.1. Oxidizing agents

While in Table 2 we have further subdivided these compounds into subclasses, the mechanisms by which they exert their effect are likely to overlap to a great extent. Even the distinction between oxidizers and SH reagents may be inappropriate, since there is now reason to think that the effect of the various oxidizers results from the conversion of critical SH groups to disulfide bridges (see, e.g., [148,149]). On the other hand, radical processes can induce 'leakiness' and lysis of the mitochondria via peroxidation processes which may accompany, but are distinct from, the PT. The prooxidant-induced Ca^{2+} release attributed to the operation of a specific Ca^{2+} efflux pathway is covered separately below (Section 3). A volume dealing with free-radical damaging processes in biological systems has recently appeared [150].

Oxaloacetate and acetoacetate can be enzymatically reduced to malate and β-hydroxybutyrate by NADPH, leading to oxidation of the NAD(P)H pool. Variations of the redox 'poise' of PyNus presumably also largely account for the early observation that respiration favors swelling.

Hydroperoxides oxidize mitochondrial GSH and PyNus via enzymatic processes involving GSH peroxidase and reductase [151–162] (see also [163] and Refs. therein). Free radicals are involved in PT induction by hydroperoxides or SH reagents such as diamide, as shown by the protecting effect of BHT [164–167] (other authors find only a limited effect [168–170]) and catalase [148] and by spin-trapping experiments [171]. Lipid peroxidation products accumulate in the presence of hydroperoxides [148,163,168–170,172], but not when the PT is induced by Ca^{2+}/P_i [168–170]. Peroxidation involves Cytochrome *P*-450 [163,172–174], and is antagonized by succinate-based respiration [173,175].

Free-radical processes underlie the induction of permeability by metal-based systems (Table 2; review, [163] see also [176]) such as Fe^{2+} [177], Fe^{2+} or Fe^{3+} complexes [148,178–181] (potentiated by Ca^{2+} [181]), NADPH/Fe^{3+}/ADP (e.g., [173,182–185]) or (Fe^{3+})/xanthine/xanthine oxidase (e.g., [186,187]). Takeyama et al. [188] have recently presented evidence that the 'damage' caused by the free-radical producing mixture of xanthine and xanthine oxidase is due to operation of the PTP rather than to lipid peroxidation per se.

The effects of GSH + GSSG and of ascorbate (see references in Table 2), and the similar ones of peptide hormones containing disulfide bridges such as vasopressin, oxytocin and insulin [189] (on hormone-induced swelling of mitochondria see also [190,191]), studied in some detail in the 50's and 60's, can also be ascribed to radical processes. These agents lead to rapid swelling and nearly complete lysis of the mitochondria: the process is quite different from that induced by Ca^{2+}/P_i or other agents. Evidence has been presented that it involves the formation of lipid peroxides and lysophospholipids in the mitochondrial [192–194] as well as in model [192,195] membranes. The action of these agents is believed to depend on the presence of iron ions [163,177], which may derive from mitochondrial stores known to exist [196,197]. Lehninger and coworkers identified a 'C factor' which prevented GSH/GSSG-induced swelling and lysis [3,198], showing that it consisted of a mixture of superoxide dismutase and catalase [199].

It seems clear that permeabilization and lysis processes due to lipid oxidation constitute a process other than the PT. However, practically any free-radical process may be expected to lead to oxidation of SH groups, and thus (see below) to the PT. Whether peroxidation and lysis or the PT take mainly place may be expected to depend on the rate of radical and lipid peroxide formation, as compared to the capabilities of the mitochondrial enzymatic 'defenses'. Peroxidation of lipid membranes can lead to the formation of structures exhibiting channel-like properties, as was proposed by (e.g.) [200] and as many electrophysiologists know by experience.

The ability of CCl_4 to cause the PT [57,201] might be ascribed to its ability to form relatively stable $CCl_3\cdot$ radicals, which would be expected to react preferentially by extracting H · from RSH groups to form RS · radicals of similarly low reactivity.

A few other oxidizing agents might be mentioned: Alloxan (2,4,5,6-tetraoxopyrimidine) oxidizes PyNus directly, and generates radicals by autooxidation [202–205]. Metal ions may be involved in the process [206]. The oxidation of critical SH groups would be the end result [160]. Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) and other dipyridilium herbicides generate H_2O_2 by cyclic reduction by NADPH and reoxydation by oxygen [207]. Paraquat-dependent oxidation of GSH and increase in mixed protein disulfides have been reported [208,209]. Naphthoquinones such as menadione (2-methyl-1,4-naphthoquinone) also appear to work by a mechanism involving oxidation of NAD(P)H. The redox reaction may proceed by two alternative routes: a one-electron transfer, catalyzed by flavoprotein reductases, leading to the quinone radical anion which then generates superoxide radical anions by autooxidation and leads to 'redox cycling', and a two-electron transfer catalyzed by a NAD(P)H:quinone

oxidoreductase, leading to the dianion [159,160,210–212]. Other agents thought to act by similar mechanisms are nitrofurantoin (*N*-(5-nitro-2-furfurylidine)-1-aminohydantoin) [213], divicine (2,6-diamino-4,5-pyrimidinediol) [214] and adriamycin (doxorubicin) and derivatives [215–218] (see also [160,219]).

Rather than directly, the redox state of the NAD(P)H and/or SH pools might be altered by inhibiting enzymes catalyzing redox interconversions, such as GSH reductase and transhydrogenase. Nitrofurantoin has been reported to be an inhibitor of GSH reductase, and its PT-inducing effect has been ascribed to this property [25] (but mediation of radicalic processes [213] seems more likely). Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), another inducer [25,220], inhibits the transhydrogenase [14,221–224] and GSH reductase [25]. As an anthraquinone derivative, rhein would also be expected to be a good redox cycle promoter. Palmitoyl-CoA, AMP, triiodothyronine (whose effects are similar to those of thyroxine) and various SH reagents have also been reported to inhibit the transhydrogenase [225].

2.2.2. Thiol reagents

The literature on PT induction by SH reagents contained a discrepancy concerning the effects of monofuctional SH reagents (essentially NEM). Reports have appeared that NEM induced the PT (see Table 2), while other authors found that this was not the case [61,166,226]. Bernardi's group [149] has confirmed both the protective (at low concentrations) and the inducing (at higher concentrations) effects of NEM, identifying two classes of SH groups. According to these authors' model, cross-linking of SH groups belonging to the first class induces the PT: the process can be inhibiting by modifying the relevant groups with a monofunctional reagent such as NEM, which therefore acts as a protecting agent. Reaction of the cysteinyl residues belonging to the second class with (higher concentrations of) NEM leads instead to the PT. An alternative interpretation of the data might consider the presence of only two cisteinyl residues modifiable by NEM per PTP precursor molecule. Their oxidation to a disulfide linkage would lead to pore formation. One of them would be more accessible, and its modification would obviously prevent reaction with the second thiol residue to give the disulfide, thus accounting for the protective effect. At higher NEM concentrations, the less accessible cysteine would also react. Modification of both cysteines would result in a conformational change leading to pore formation. Be this as it may, the protective effect of NEM confirms that alterations of thiol groups are involved in PT induction by menadione, PhAsO, hydroperoxides and diamide.

Among SH oxidizing agents, the SS-bridge former diamide (diazenedicarboxylic acid bis(*N,N*-dimethylamide)) [227] is one of the most popular (see Table 2). Novgorodov et al. [166] reported that diamide and arsenite have a marked permeabilizing effect only in the presence of P_i. Others have instead observed PT induction in media without P_i [119,228]. Heavy or transition metal divalent cations, e.g., Cd^{2+}, Pb^{2+} or Hg^{2+}, most likely induce the PT via formation of coordination complexes with two SH groups. Unrecognized medium contamination by metal ions may well have been responsible for some of the problems encountered by early researchers [77]. Some thiol reagents, including Cd^{2+}, PhAsO, mersalyl and *p*-hydroxymercuribenzoate, have been reported to induce first a potential-dependent K^+ uptake, followed only later by the permeability transition ([166,226]; see also [229–231] and Section 4.3.1).

2.2.3. Transmembrane potential-decreasing agents

Since the PTP is voltage-modulated (see Section 2.4.2), any agent causing depolarization of the mitochondrial membrane may be expected to favor the onset of the PT. For a given mitochondrion and a given depolarization, whether the permeabilization will take place will depend on the activity of matrix Ca^{2+} and of protective agents such as ADP, matrix protons, Mg^{2+} etc. Bernardi and coworkers [22,142,149] have recently proposed that the effect of many inducers or inhibitors of the PT may actually consist in a modulation of the 'sensitivity' of the pore to voltage (see Section 2.4.2).

2.2.4. Fatty acids

In the late 50's and early 60's Lehninger's group studied a 'U factor' which turned out to consist of enzymatically-generated fatty acids [201,232–234]. Fatty acids act as classical uncouplers by diffusing into the mitochondria as the protonated, electroneutral form, and exiting as anions in an electrogenic process mediated by the AdNT [235–240]. Their PT-inducing effect might be attributed to the consequent decrease in transmembrane potential. On the other hand, Petronilli et al. [22] have shown that the addition of fatty acids causes a shift of the curve relating permeabilization rate to transmembrane potential towards higher potentials: a direct interaction with the PTP seems therefore likely.

2.2.5. Adenine nucleotide translocator ligands

AdNT ligands may have either an enhancing or a restricting effect on the PT. A currently popular model [241–243] is that those ligands which stabilize the 'C' (for cytosol-facing) conformation of the carrier [244,245], such as ATR and CATR [244–247] act as inducers, while those which stabilize the 'M' conformation, such as BGK, have the opposite effect (see Sections 2.3.3 and 5.1).

Acyl-CoA's have been reported to bind to (and inhibit) the AdNT by acting from either the outer or the inner side [248–257]. They may therefore be expected to have opposite effects depending on whether they are added to the suspension or their concentration in the matrix is increased. While the PT-inducing effect of external acyl-

CoA's has been amply documented (Table 2), the effect of matrix acyl-CoA's has not been studied in depth (but see Section 2.3.10).

Bernardi et al. [5] have presented an alternative model, according to which the effect of acyl-CoA's would be accounted for by variations in membrane surface potential. It may also be mentioned that addition of palmitoyl-CoA has been reported to result in the oxidation of mitochondrial NADH [258,259]. The same compound has also been found to inhibit the mitochondrial transhydrogenase [225]. Long-chain acylcarnitines and acyl-CoA's exert a direct activating effect on the Ca^{2+} release channel of the SR [260].

PP_i [122,261–265] and PEP [27,266–268] are both known to deplete the matrix ADN pool, by exchange on the AdNT. Thus, they induce the PT by reducing the level of a protecting agent.

2.2.6. Others

Some inducing agents act by mechanisms more poorly understood than others. One such case is thyroxine (and related compounds), whose effects are very well documented (see Table 2), but mechanistically still uncertain. Thyroxine causes an enhanced production of fatty acids [3,201] (in fact, its action is prevented by BSA). Triiodothyronine has been reported to inhibit the mitochondrial transhydrogenase [225]. An involvement of SH groups is suggested by the DTT-induced decrease of triiodothyronine-induced Ca^{2+} efflux [116]. It may be noted that reportedly thyroxine has little effect on mitochondria from some organs [103,269] and tumors [269], and that mitochondria from hyperthyroid rats swell more readily [270]. Trialkyltin compounds are known to act as Cl^-/OH^- exchanger, but whether their PT-enhancing effects [271,272] may be attributed to this property is doubtful [4].

2.3. Inhibition

This Section presents a commented list of PT inhibitors, classified in Table 3. Several inhibitors of the PT act simply by preventing or counteracting the induction by some agent or condition, in mirror-image fashion. An

Table 3
A classification of inhibitors of the permeability transition

Inhibitors	References
Anaerobiosis and resp. inhibitors:	[1,2,60,76,77,81,85–87,291–300]
Respiratory substrates:	[52,86,282,289,397]
Uncouplers:	[58,76,77,270,295,299,308,352,356,385,478]
Adenine nucleotides	
ADP:	[2,22,26,27,30–32,50,53,58,63,64,133,143,147,168,170,241,242,265,274,285,289,316,352,365–369, 410–412,479–481]
ATP:	[1,15,16,30,45,49,50,53–55,60,64,66,71,76,77,100,102,104,117,137,167,270,274,316,352,366,409, 410,479,481,482]
Ligands of the AdNT	
bongkrekate:	[27,241,242,276,287,289,365,366,369,412]
Oligomycin:	[32,54,58,117,122,165,167,265,285,296,298,408,433,483–485]
Protons:	[16,21,26,68,104,115,122,142,143,270,283,349,369,435–438,486–488]
Divalent cations	
Mg^{2+}:	[21,27,32,36,52,53,61,76,77,81,86,87,97,108,135,143,270,274,385,410,479,486,489–492]
protection or reversal by Mg^{2+} + ATP:	[3,36,46,52,54,110,112,137,273,398,400,401,410,493]
Sr^{2+}:	[36,108,265,441,493–495]
Mn^{2+}:	[36,53,67,71,77,81,86,97,143,270,295]
restoration of coupling by ATP + Mn^{2+}:	[67,71,72,80,91,496]
Ba^{2+}:	[36,270]
Trivalent cations	
La^{3+}:	[21,36,61]
Competitors of Ca^{2+} binding	
phenothiazines:	[35,169,413,447]
local anesthetics:	[35,109,110,283,324,413,444,447,497,498]
Radical scavangers	
butylhydroxytoluene:	[166–168,170,318,323,484]
Polyamines	
spermine and spermidine:	[86,452,455,499,500]
Carnitine and acylcarnitine:	[102,283,347,376,501]
SH-reducing agents	
DTT:	[25,167,170,289,323,343,502]
SH reagents	
NEM:	[149,166]
Cyclosporin A:	[22,30–32,34,127,129,142,169,188,220,242,243,306,317–319,325,346,347,366,413,435–438,452,457, 503,504]
BSA:	[58,81,202,232,273,375,390]

example is the protective effect of ATR [106,267], normally an inducer (see Section 2.2.5), when the PT is induced by PEP.

In several cases (important exceptions being Mg^{2+}, ADP and CSP) information is lacking as to the generality of the protection provided by a particular agent. Vice versa, information is often missing as to what inhibitors act on the PT induced by a given inducer. This type of information is often interesting. For example, LeQuoc and LeQuoc [289] reported that the acetoacetate-induced PT was inhibited by DTT, pyruvate, BGK and ADP, thus suggesting the simultaneous (or concurrent) involvement of SH groups, the NAD(P)H pool redox state and the AdNT. Macedo et al. [170] reported that both ADP and DTT inhibited the *t*-BuOOH-induced PT, while ADP was vastly more effective than DTT if the PT was induced by Ca^{2+}/P_i. The same group [169] found that TFP blocked *t*-BuOOH-induced swelling, while CSP had only a modest effect (but see Broekemeier et al. [220]). The order of effectiveness was reversed if swelling was induced by Ca^{2+} and P_i. Carbonera and Azzone [167] reported that BHT had a protective effect on damage induced by Ca^{2+} plus either hydroperoxides, diamide or P_i, suggesting an at least partial overlap of the mechanism of action of these inducers. On the other hand, low concentrations of nitrofurantoin protected against permeabilization induced by Ca^{2+} + hydroperoxides or diamide, but not against Ca^{2+} + P_i [213]. Some of these observations suggest a multiplicity of PT-inducing processes (see also Section 5.4).

There is solid evidence of synergic effects among at least some of the inhibitors. Thus, ADP, Mg^{2+} or CSP alone were reportedly [30,32,365,395] unable to induce the repolarization of mitochondria which had lost most of their low-MW matrix components following PT induction. However, the combination of ADP + CSP [30,395], ADP + Mg^{2+} [32,365] or Mg^{2+} + CSP [32] was very effective. CATR was able to overcome the effect of these binary inhibitor mixtures, but not that of ADP + Mg^{2+} + CSP, suggesting an interaction between the binding sites of all three effectors [32] (see also [31]). Other examples of cooperativity are mentioned below.

2.3.1. Respiratory substrates and prevention of respiration

The protection by respiratory substrates probably is to be considered as being mediated by the effects on the redox level of the NAD(P)H (and SH) pool. Thus, succinate, often found to protect in comparisons with first-site substrates (e.g., [86,98,134,353,396,397]), presumably acts by keeping the PyNus more reduced than would be the case in its absence. Similarly, the lack of oxygen or the inhibition of respiration have the effect of keeping the PyNus in a more reduced state (see Section 2.4.1).

2.3.2. Uncouplers

Some of the early reports (see Table 3) of a protective effect by uncouplers, which are bona fide inducing agents (Table 2), may be interpreted as a prevention of Ca^{2+} uptake due to the elimination of the transmembrane potential. For other cases, an explanation may be that the presence of an uncoupler prevents uptake of the Ca^{2+} released by the first mitochondria to undergo the PT by the still-functional ones (see Section 2.2). Upon uncoupling, the latter would also release their own matrix Ca^{2+} via the Ca^{2+} uniporter, thus escaping PT induction. Prevention of Ca^{2+} release by RR may also prevent the inhibitory action of the uncoupler [142,308].

2.3.3. Adenine nucleotides

The earliest reports of relevance for a section on inhibition of the PT may be the many observations that the presence of ATP and/or Mg^{2+} was necessary for the uptake of relevant amounts of Ca^{2+} ([3] and Refs. therein; [46,54,137,398,399]) or for 'protection' [52]. The combination of ATP and Mg^{2+} was found to induce shrinkage of PT-swollen mitochondria in saline, respiratory substrate-free media [3,58,59,273,400,401]. Shrinkage is today understood as due to the action of H^+/M^+ antiporters (on which see, e.g., [402–405]), coupled to proton pumping by the ATPases and to anion efflux on IMAC (on IMAC see, e.g., [406,407]. It was also soon evident that ADP was an effective protective agent (see Table 3), while AMP had only a marginal effect and other nucleotides were not effective at all. The protective action of ADP was originally thought by many to be due to its conversion into ATP [1,58,352,353,408]. The view then prevailed that ADP was actually more effective than ATP or even the only relevant nucleotide [26,27,63,118,274,409–411]. Some recent reports indicate that ATP, while inferior to ADP, is also effective [30,395]. Triphosphate also inhibits the PT [55,295], suggesting that the oligophosphate group might be responsible for the inhibitory effect. Inhibition by external ADP (in the 100 μM range) was classified as mixed-type by Haworth and Hunter [63], who interpreted it in terms of a competition with Ca^{2+} for a couple of cooperative binding sites. Lapidus and Sokolove [147] have conversely proposed that Ca^{2+} (plus *t*-BuOOH) may trigger the PT by decreasing the cooperativity of the ADP binding sites. Novgorodov et al. [30] confirmed that the affinity of Ca^{2+} for the activating sites was decreased by ADP. NADH and NAD behaved similarly, but required 200- and 2000-fold higher concentrations respectively. ADP and NADH were found to have a synergic action, suggesting cooperative interactions. ADP and CSP also display synergism [30,31].

In the hands of Crompton and Costi [26] the presence of mM concentrations of ADP increased the rate of EGTA-induced resealing of permeabilized mitochondria about 8-fold. The same authors [128] presented evidence supporting the notion that ADP facilitates the open/closed interconversion of the pores in the absence of Ca^{2+}, i.e., during resealing of permeabilized mitochondria. This effect was not observed in the presence of Ca^{2+}. The electrophysio-

logical evidence available to date (see below, Section 4.3.2), obtained in the presence of Ca^{2+}, is not consistent with a decrease by ADP of the pore mean open or closed times.

Despite the large number of observations, some important aspects of the inhibitory action of ADP and ATP need further clarification. The idea is widespread (see, e.g., the discussion in [32]) that the effect is exerted at the inner side of the mitochondrial membrane. Many researchers believe that the inhibitory action involves interaction with the AdNT (see Section 5.1). The involvement of matrix ADNs was suggested by the fact that agents known to deplete the matrix pool (PP_i and PEP (Section 2.2), and α,β-methylene ADP [412]) have an inducing effect. Hunter and Haworth [27] reported that the rate of propagation of the PT depended on the matrix content of ADNs (see also [147]). That ADP acts on the matrix side has been further confirmed by patch-clamp experiments. In most experiments, however, the nucleotides are added to the mitochondrial suspension. Thus, e.g., Bernardi et al. [413] reported that 30–60 μM external ADP (in the presence of oligomycin) substantially modified the response of the mitochondria to depolarization. Can the protective effects of external ADP at these concentrations be attributed to an increase in the matrix concentration of ADP, or must one invoke an external binding site? Most assessments of the matrix ADN content of rat liver mitochondria fall in the range 10–15 nmol/mg protein [145,414–420]. The proportion consisting of ADP varies depending on the metabolic state and other factors such as P_i concentration, with reported ATP/ADP ratios ranging between 1 and 12 [145,147,414–424]. In oligomycin-treated, succinate-oxidizing mitochondria a larger part of the pool is present as ADP (and AMP) [32,146,420]. Part of the adenine nucleotides is actually bound [146,422,424–426], so that an estimate of the matrix activity of ADP is difficult. Nonetheless, it seems that it might be below 1 mM. If this were the case, and most of the ADP added in the experiments were taken up by the mitochondria, this might lead to important changes in the matrix [ADP], justifying the inhibitory effect. It becomes however necessary to propose an efficient mechanism for net ADN uptake. Such a mechanism might be provided by the recently described P_i/ATP + Mg^{2+} (in liver mitochondria) or P_i/ADP (in heart and kidney mitochondria) antiporter [427–431]. The driving force for transport on this carrier is provided by the P_i gradient, which in turn reflects the transmembrane pH gradient. The carrier needs μM external Ca^{2+} to operate. However, in liver it requires Mg^{2+}, and it transports mainly ATP, with a K_m in the mM range [431] ($HADP^{2-}$ and $(PP_i/Mg)^{2-}$ are poorer substrates [427,431,432]). It remains to be verified whether this mechanism would be kinetically competent, and whether the variations in matrix ADP/ATP concentration it might account for would be large enough to justify the observed effect. It should also be mentioned that during the uptake of Ca^{2+} and P_i, any ADP present in the medium is accumulated into the mitochondria [399].

2.3.4. Oligomycin

The protective effect of oligomycin has been interpreted in two ways: (i) an increase in matrix ADP content, due to a conversion of ATP into the more effective diphosphate [32,147,433] or (ii) an increase in the transmembrane potential of the mitochondria, attributed to an inhibition of the proton leaks through the ATP synthetase [308,434]. Jurkowitz and Brierley [122] have reported that the inhibition by oligomycin of PT-linked Ca^{2+} efflux is steeply dependent on the concentration of matrix nucleotides.

2.3.5. Protons

A protective effect of protons was recognized as early as 1953 by Raaflaub [16], who reported that 'spontaneous' swelling of mitochondria did not take place at pH < 6. Modulation occurs over a relatively narrow pH range, approx. 6.5–8 [21,63,369,435–437]. Haworth and Hunter [21,63] reported that protons inhibited in a Ca^{2+}-competitive manner, the K_m for activation by Ca^{2+} decreasing with increasing pH. Competition was later confirmed by patch-clamp experiments in which the pore channel believed to correspond to the PTP could be inhibited by protons and reactivated by an increase of $[Ca^{2+}]$ [436]. It was also shown that the effect of protons is not due to an inhibition of Ca^{2+} uptake [369], that the relevant parameter is the matrix pH [435], and that inhibition could be substantially eliminated by pretreatment of the mitochondria with diethyl pyrocarbonate, suggesting that histidyl residue(s) are involved [437]. Induction of the PT in many cases is accompanied by variations in matrix pH. Thus, Ca^{2+} uptake is accompanied, even in the presence of weak acids, by alkalinization. When moderately Ca^{2+}-loaded mitochondria are depolarized by addition of an uncoupler, a procedure which would be expected to lead to the immediate onset of the PT (see Section 2.4.2), the pores might not open unless measures are taken to prevent the drop in matrix pH caused by the influx of protons in exchange for Ca^{2+} ions exiting on the Ca^{2+} 'uniporter' [22,142,438]. These measures might be, e.g., inhibition of the uniporter with RR [142], or the previous accumulation of a weak acid acting as a buffer.

The effectiveness of P_i as a pore inducer may thus be explained [142] in terms of its ability to buffer the matrix pH at values close to its first pK_a (7.2). P_i efflux mediated by the P_i/OH^- antiporter [439,440] would counteract the proton influx, limiting the pH excursion [142]. The failure of acetate to behave likewise would be understandable, since its pK_a is 4.8. Whether the effect of P_i can be fully explained in terms of pH buffering remains however to be verified. pH changes might also explain the inhibitory effect of lasolacid-A (X-537A) [303,441], a compound which can exchange K^+ and H^+ in nigericin-like fashion,

and promotes therefore matrix acidification in sucrose media.

2.3.6. Divalent and trivalent cations

It was realized early on that Sr^{2+}, Mn^{2+} and Ba^{2+} could not substitute for Ca^{2+} as inducers of the PT [3,53,134,285]. Tapley [270] found that Mn^{2+} and Mg^{2+} acted as antagonists of Ca^{2+}, while Ernster [80] reported that the inhibitory effect of Ca^{2+} on respiration based on Site I substrates (a consequence of the loss of PyNus) was a function of the Ca^{2+}/Mg^{2+} ratio. That divalent cations behaved as competitive Ca^{2+} antagonists was recognized by Azzi and Azzone [135] and confirmed by later studies [21,61,435,436]. Given the competitive effects, the site of action of these cations may be presumed to be the same responsible for the activating effect of Ca^{2+}, although binding of one or the other of two similar species to the same site might not be expected to produce opposite effects. Mn^{2+} and Sr^{2+} have been shown to exert an inhibitory effect at a matrix-side site [413,441]. In the case of Mg^{2+}, which is not readily transported on the Ca^{2+} carrier, and of Ba^{2+}, evidence pointing in the same direction comes from patch-clamp studies (see Section 4.3.2) [436] and from work utilizing PT-permeabilized mitochondria [32]. Bernardi and colleagues [413] have shown that an inhibitory site for divalent cations (including Ca^{2+}) exists on the cytoplasmic face of the membrane as well. This latter site may be involved in the competitive inhibition of the PT by Mg^{2+} in suspension studies. In the case of those cations which can be transported on the Ca^{2+} uniporter, both the inner and/or outer sites may play a role, depending on experimental details. The two sites can be experimentally distinguished [413,436], and indications exist that binding to either the inner one (patch-clamp experiments; [436]) or the outer one (experiments with Mg^{2+}, which is taken to remain outside the matrix; [61,435]) results in inhibition with Ca^{2+}-competitive characteristics.

Monovalent and trivalent cations are also reported to act as competitive inhibitors of the Ca^{2+}-induced PT, with respectively lower and higher affinities in comparison to divalents [21]. Lanthanides are very effective pore inhibitors in patch-clamp experiments (Szabò and Zoratti, unpublished).

2.3.7. Local anesthetics and phenotiazines

Nupercaine and TFP give Ca^{2+}-competitive inhibition of the PT [413], and have been proposed to work by perturbing Ca^{2+} binding to the internal site [413]. Local anesthetics are known to bind to phospholipid Ca^{2+}-binding sites on biological membranes, displacing Ca^{2+} in a competitive manner [110,442]. Compounds belonging to these classes have rather unspecific effects. For example, TFP is a calmodulin antagonist, and also inhibits the F_0F_1 ATPase [443]. The inhibitory effect of local anesthetics was considered (see Section 4.1) to be related to their ability to inhibit PA2 [444–447]. Since the formation of lysophospholipids is not currently considered to be the major cause of the PT, the mode of action mentioned above seems more plausible.

2.3.8. Radical scavengers

The most used of these is butylhydroxytoluene (BHT). Its protective effect indicates that permeabilization induced, in the presence of Ca^{2+}, by P_i, hydroperoxides, diamide [167] and thiol cross-linking agents [166] involves radical processes. Some authors have reported that this compound prevents PyNu hydrolysis in prooxidant-treated mitochondria, and have suggested that this may be the mechanism of its protective action [448]. The structurally related general anaesthetic Propofol (2,6-diisopropylphenol), which inhibits PT induction by $Ca^{2+} + P_i$, diamide, *t*-BuOOH or FCCP + RR at concentrations having no effect on PA2 activity, may have a similar mechanism of action [275].

2.3.9. Polyamines

The effect of these compounds is well documented (e.g., [147,305,449–452]) but not completely clarified. Inhibition of the PT by spermine has been observed to be more pronounced when permeabilization was induced by Ca^{2+}/P_i than in the case of induction by *t*-BuOOH, PhAsO or CATR [305]. Lapidus and Sokolove have recently reported [147] that matrix ADP and spermine act synergistically and suggested that spermine might act by increasing the affinity of ADP for its inhibitory binding site. Polyamines are known to be taken up by mitochondria in an electrophoretic process [453,454]. Alternatively, the effect might be due to binding of these cationic species to the inhibitory external divalent cation binding site identified by Bernardi et al. [413], or to changes in the surface potential induced by binding to the external side of the inner membrane. This latter hypothesis receives some support from the observation that the effect of spermine is more pronounced in low-salt media [451] and by the old observation that spermine prevents lysis of bacterial protoplasts ([455] and Refs. therein). On the other hand, the effect requires relatively low external concentrations of spermine (40–100 μM [147,452]).

2.3.10. Carnitine and acylcarnitine

A beneficial effect of carnitine has been reported in cases in which the PT was induced by Ca^{2+} + acyl-CoA's. The effect has been ascribed to carnitine providing a 'sink' for acyl groups, with the formation of acylcarnitine [376]. Acylcarnitine may exert its protective effect [289] by transporting acyl groups to the mitochondrial matrix, where they would be converted to acyl-CoA's, exerting, from the inside, a protective effect mirroring the inducing effect of external acyl-CoA's (see above, Section 2.2.5). Data supporting this model have been obtained [456].

2.3.11. NEM

NEM has been reported to act as an inducer, but also to prevent the PT induced by SH cross-linking agents, most likely by protecting crucial SH residues from oxidation [149,166] (see Section 2.2.2).

2.3.12. Cyclosporin A

At the end of the 80's both Crompton's [127] and Pfeiffer's [34,220] groups, following the lead by Fournier et al. [457], reported that CSP, a cyclic endecapeptide used as an immunosuppressive agent [458–460], strongly delayed the PT at sub-μM concentrations (see also [434]). The discovery confirmed that the PT was due to operation of a pore (see Section 4.2) and gave new impulse to research in the field. Inhibition by CSP is used today as the identifying characteristic of the PT.

Inhibition by CSP has been reported for a variety of inducing agents [127,220,319], suggesting the presence of a common mechanism, yet it seems not to be universal. As already mentioned, Nemopuceno et al. [169] reported observing only a modest inhibitory effect on Ca^{2+} + *t*-BuOOH-induced permeabilization. Pastorino et al. [347] reported that CSP did not inhibit the PT caused by the PhAsO + CN^- in 'low-Ca^{2+}' mitochondria. Inhibition was afforded instead by the combination of CSP and butacaine, a local anesthetic and phospholipase A2 inhibitor.

Patch-clamp experiments [436] indicated that CSP exerts its effect in a Ca^{2+}-competitive fashion. Channels corresponding to the PTP that had been inhibited by CSP could be reactivated by the addition of Ca^{2+}, reinhibited by increasing the concentration of CSP, reactivated by adding more Ca^{2+} and so forth, in the course of the same experiment (same patch). Bernardi et al. [435] came to the same conclusion on the basis of swelling experiments, but later reported [413] that the I_{50} for CSP inhibition was independent of the Ca^{2+} load taken up by the mitochondria. McGuinness et al. [129] had reported competition between Ca^{2+} and CSP for binding sites in mitochondria. ADP and ATP (less effective) display a synergic interaction with CSP as pore inhibitors [30,31,345]; CSP increased the affinity of the pore for ADP: the K_i for ADP was reduced from 20 to 5 μM by the presence of 1 nmol/mg protein CSP [30]. In the absence of added ADP, CSP acted as a partial inhibitor of the PT, decreasing the V_{max} (the rate of propagation of swelling) and increasing the apparent K_m for Ca^{2+}, without affecting the Hill coefficient for Ca^{2+} binding. In heart mitochondria depleted of adenine nucleotides, CSP alone could not reverse Ca^{2+} + cumene hydroperoxide-induced permeabilization. Permeabilized liver mitochondria could re-establish a transmembrane potential if treated with CSP + ADP (in the presence of Mg2 +), but not if treated with CSP or ADP alone [31]. Evtodienko et al. [452] have reported that extremely low (0.2 nM) concentrations of CSP are sufficient to dampen Ca^{2+}-induced oscillations of mitochondrial parameters (respiration, transmembrane potential etc.) in permeabilized Ehrlich ascites tumor cells. The oscillations have therefore been proposed to involve the reversible activation of the PTP. The sensitivity to CSP of these oscillations decreased in time; we might speculate that this may have been due to the loss of coadjuvating factors from the mitochondria. Synergism with TFP has also been reported [34]. Novgorodov et al. [345] have reported that the bifunctional SH reagent PhAsO abolished the inhibition by CSP of the Ca^{2+} and P_i-induced PT.

The immediate target of CSP is considered to be a class of molecules known as 'cyclophilins' ([461,462]; Revs., [463–466]), ubiquitous proteins having *cis-trans* peptidyl-prolyl isomerase (PPIase) activity [467,468]. Cyclophilins are present in all tissues and eukaryotic cells as well as in bacteria and in the mitochondria of *Neurospora crassa* [469], yeast [470,471] and of liver and heart [130,242,472–474]. It may therefore be presumed that the mechanism of action of CSP in mitochondria involves the mitochondrial cyclophilin. The idea has been supported by the observation [130,243] that the relative potencies of Cyclosporins A, G and H as inhibitors of PPIase are paralleled by their effectiveness as inhibitors of the PT.

Based on binding studies and titrations of swelling rates, various groups [30,127,220,242,366,474] have estimated the presence of 60–120 pmol/mg protein of binding sites for CSP in liver and heart mitochondria, with K_i values in the 5 nM range. These values were in reasonable agreement with estimates of cyclophilin content and of the K_i for inhibition of isomerase activity by CSP obtained by Halestrap and Davidson [242] and Inoue et al. [474]. Connern and Halestrap [473] purified a mixture of two cyclophilins, with masses of 18.6 (the major form) and 17.5 kDa from rat liver mitochondria. The total amount of PPIase was estimated by these authors at 45 pmol/mg.prot, while a K_i of 3.6 nM was reported for inhibition by CSP. Inoue et al. [474] also reported the presence of two cyclophilins, one presumably being a truncated form of the other.

Crompton's group [129] found evidence for two distinct classes of binding sites, one (class I) with low capacity (< 5 pmol/mg protein) and high affinity ($K_d < 12$ nM), the other (class II) with higher capacity (about 60 pmol/mg protein) and lower affinity (half saturation at approx. 100 nM CSP). Binding to this second class of sites exhibited the characteristics of a cooperative process. While an involvement of the class II sites could not be excluded, pore inhibition correlated more strictly with occupancy of class I sites. Mitochondrial permeabilization and PPIase activity were inhibited by CSP with affinities in the same range as the class I K_d, suggesting that class I sites may correspond to cyclophilin and that the isomerase may be involved in the permeabilization process (a conclusion also reached by Halestrap and Davidson [242]). The addition of CSP (in the presence of Mg^{2+}) to permeabilized mitochondria resulted in pore closing rather than in a stabiliza-

tion of the permeabilized state. This observation, confirmed by numerous observations by other groups, indicates that CSP must act by shifting the position of the open/closed equilibrium for the pore towards the closed state(s), rather than merely by 'taking out' a catalyst of the open/closed interconversion. It is relevant that submitochondrial particles could not be permeabilized [129], even in the presence of purified cyclophilin plus Ca^{2+} [475].

Halestrap and coworkers [8,242,243] and Crompton and coworkers [129] have rationalized the observations on CSP action in terms of a binding model, in which cyclophilin binds to a particular, Ca^{2+}-induced conformation of the pore-forming protein, inducing, or stabilizing, the open state. The cyclophilin-CSP complex would not be capable of such an interaction; CSP would thus cause a shift of the pores to the closed conformation by competing for the available cyclophilin. Halestrap and coworkers also envision a role of P_i and/or PP_i in determining the cyclophilin-sensitive ('pore precursor') conformation of the relevant membrane protein (which these authors propose to be the AdNT) [242,243]. Cyclophilin A, B and C/CSP complexes, but not the cyclophilins or CSP themselves, have been reported to bind and inhibit the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin [476,477]; this interaction is believed to essentially account for the effects of cyclosporin at the cellular level. However evidence for the presence of calcineurin in mitochondria is lacking. Furthermore, Petronilli et al. have shown [130] that a CSP derivative whose complex with cyclophilin does not inhibit calcineurin is as effective as CSP as a PT inhibitor (see also [5]).

Andreeva and Crompton [31] have recently reported the presence of a 10–12 kDa inner membrane CSP-binding protein which might be (part of) the PTP. If this were the case, the mechanism of the PT might not involve cyclophilin at all.

2.4. Modulation

Some of the agents listed in the previous Sections, such as divalent cations, P_i, ADN's, protons and fatty acids, may be considered as particularly significant because they are present in vivo. This Section covers oxidative processes and the transmembrane potential, which are also physiologically relevant and are known to play important roles in the modulation of the PT.

2.4.1. NAD(P)H and SH redox state

Oxidation of the mitochondrial NAD(P)(H) pool has been observed to accompany efflux of calcium and swelling associated with the PT (e.g., [25,27,53,60,63,86,87,108,109,117,155,259,265,282–285, 288,289,300,371,504]). The phenomenon was observed upon induction of the PT with a variety of agents, including P_i (e.g., [27,53,60,86,87]; contra, [144]) suggesting that the oxidation of the the PyNus was a major factor in causing the PT. It was then made clear [108,117,371] that the redox state of the NAD(P)(H) pool was only one of several factors influencing the PT, and that the NADP(H) (not the NAD(H)) redox level was the relevant parameter [259,284,285,288,289,371]. The oxidation state of NAD(H) was much less important, so that a role was also proposed for the voltage-dependent transhydrogenase [14,288]. Other authors concluded instead that the oxidation of the PyNus was a consequence, rather than one of the causes, of the PT. Beatrice et al. [283] argued that the shift to a more oxidized state was due to the acidification of the matrix space caused by the PT, which would act simply by a mass law effect. The same group also argued that oxaloacetate, one of the agents used to modulate the redox levels of the PyNus, might act by inhibiting oxidation or transport of the respiratory substrate (succinate), thus causing a decrease in transmembrane potential and therefore the PT [109]. However, Lehninger and coworkers had shown [282] that the same results were obtained with ascorbate/TMPD as a respiratory substrate, or when the mitochondria were energized by ATP hydrolysis. On balance, the evidence presented supports the notion that oxidation of NADPH favors permeabilization. A partial explanation for the effect of NADPH oxidation may be found in the observation by [63] that NADH acts synergistically with ADP to inhibit the PT, presumably by direct interaction with the PTP, and that NAD is less inhibitory than NADH.

In the literature, as well as, probably, in the actual mechanism of onset of the PT upon treatment with oxidizing agents, the oxidation of PyNus and of SH groups are tightly linked. Organic peroxides are metabolized through the action of the GSH peroxidase/reductase system (see Section 2.2.1). The latter enzyme, which uses NADPH to regenerate GSH, establishes the linkage between the redox state of the mitochondrial GSH pool and that of the PyNus. A transmembrane potential-dependent equilibrium between the redox levels of NAD(H) and NADP(H) is in turn maintained under normal circumstances by the energy-linked transhydrogenase (e.g., [14]). Some authors ([25]; see also [162]) have argued that PT induction may actually be mediated by the redox state of the GSH pool. In experiments in which the redox state of GSH and of the PyNus were changed independently, the occurrence of the PT correlated with the oxidation of GSH [25]. On the other hand, Hoek and Rydström reported that in mitochondria treated with BCNU, an inhibitor of GSH reductase, oxidation of the GSH pool (but not, thanks to the inhibitor, of the NAD(P)(H) pool) did not lead to the efflux of the accumulated Ca^{2+} [14]. Carbonera and Azzone [167] have reported that the peroxide-induced PT can be abolished by BHT or oligomycin, which have no effect on the depletion of the GSH pool. Furthermore, according to the same authors no GSH oxidation accompanied the permeabilization induced by Ca^{2+}/P_i. Novgorodov et al. [166] have presented data suggesting that the SH groups involved in the PT are more accessible to hydrophobic (NEM) than to

hydrophilic (mersalyl) SH reagents, suggesting that they belong to membrane components. The redox level of the GSH pool thus seems not to be in direct control of the PT. However, in the absence of experimental manipulations the redox state of other mitochondrial SH groups is linked to that of GSH [176] and therefore to that of the NAD(P)H pool, so that a correlation exists (see, e.g., [505]). *S*-Thiolation of proteins is well-known to occur in cells exposed to oxidizing agents such as hydroperoxides or menadione (e.g., [506,507]).

Various authors have also proposed that oxidation of crucial SH groups in membrane proteins underlies the development of the PT [25,35,120,343,447] (see also Section 5.1). The idea that tampering with membrane SH groups may facilitate the permeabilization of the mitochondrial inner membrane is supported by many reports on the induction of swelling by SH reagents (see Section 2.2.2). It should again be stressed that models envisioning SH oxidation as a mechanistic centerpiece are not in contrast with those assigning a crucial role to free radicals. SH groups are well known to be eminently susceptible to H · abstraction, and both disulfides and thiols act as radical traps in biological and chemical systems. The coupling of proximal sulfhydryl radicals generates disulfide bonds (e.g., [508–510]).

A recent report [347] raises the interesting possibility that components of the respiratory chain other than NADH dehydrogenase may be involved in modulation of the PT. The authors induced the PT in rotenone- or cyanide-treated mitochondria, using PhAsO or palmitoyl-CoA, and observed an inhibitory effect by CSP or carnitine only when electron transport was blocked by rotenone. The result is unexpected, since the PyNu pool presumably was reduced under both circumstances.

2.4.2. Modulation by the transmembrane potential

An early clue to the effect of 'energization' on the PT was the observation that often swelling was favored by uncouplers such as DNP (see Section 2.2.3). Hunter and Haworth [27] clearly showed that energization of the mitochondria was not a requirement for the PT, by performing their experiments in the presence of FCCP. They further identified 'energization' as one of the factors offering protection against the PT, and recognized that uncoupler-induced Ca^{2+} release from Ca^{2+}-loaded mitochondria involved the PTP [62] (see also [265]). Only recently, however, it was recognized [142,308,413,438] that the transmembrane potential held the PTP at bay, and that depolarization could be the cause, as well as the consequence, of the PT. A single parameter was thus identified which could be modified by several disparate effectors, providing a unifying principle for their action.

Petronilli et al. [22,149] have argued that pore effectors such as Ca^{2+}, Mg^{2+}, ADP and palmitate, as well as conversion of thiol groups to a disulfide linkage, act essentially by modifying the voltage dependence of the pore, i.e., by shifting the curve relating the open probability of the pore to the transmembrane potential. The conclusion is based essentially on the differential effects of doses of FCCP (i.e., of given degrees of depolarization) on the rate of the PT (i.e., on the fraction of mitochondria which have become swollen at any given time) in untreated and in ADP, Mg^{2+} etc.-treated organelles. Ca^{2+}, palmitate and -SS- formation would act by increasing the value of the transmembrane potential at which the pores have a given probability of opening (so that a smaller amount of FCCP would be needed to cause the PT), while Mg^{2+} and ADP would have the opposite effect. The response of the individual mitochondria in a population to a given depolarization would vary depending, e.g., on the amount of ADP or Mg^{2+} in the matrix. The effect was tentatively attributed to a direct interaction of the effectors studied with the voltage-sensing domain(s) of the channel, resulting presumably in a variation of the overall charge of the domain(s) (a variation in a transmembrane electrical field is generally thought to favor the open or closed state(s) of a channel by virtue of the electrostatic attraction or repulsion exerted on a charge-bearing portion - the 'gate' - of the channel [511]). This model, referred to hereafter as 'voltage sensitivity modulation by binding' (VSMB) requires that species with charges of the same sign may have opposite effects. Thus, Ca^{2+} would shift the threshold voltage to higher values, while Mg^{2+} would shift it to lower ones. Palmitate and ADP would behave analogously. This can only be rationalized by assuming that these charged species exert their effect by binding to different sites, situated at different depths in the transmembrane electrical field (presumably on opposite sides of the membrane). Furthermore, both a positive and a negative ion (Mg^{2+} and ADP or Ca^{2+} and palmitate) must be capable of altering the voltage sensitivity of the voltage sensor(s) in the same direction. This again implies that distinct binding sites are involved, in regions of the electrical field with opposite polarity. The voltage sensor(s) would therefore possess a minimum of four binding sites, two for anions and two for cations, with one cation and one anion binding site on each side of the membrane. It follows that if Ca^{2+} exerts its effect on the matrix side, Mg^{2+} must do so by binding to the cytosolic side, and if ADP binds on the matrix side, palmitate must bind on the cytosolic side. The model is supported by the identification of an inhibitory binding site for divalent cations on the cytoplasmic side of the membrane [413]. On the other hand, experimental evidence exists (see Sections 2.3.6 and 4.3.2) that inhibitory site(s) for divalent cations are accessible from the matrix side, as is the case for the activating Ca^{2+} binding sites.

An alternative model, dubbed 'binding modulation by voltage' (BMV), might move from the hypothesis that Ca^{2+}, Mg^{2+} etc. induce, upon binding to their sites, conformational effects resulting in pore opening or closing, irrespective of whether a transmembrane electrical field is

being applied, or of its size. The sites in question would be situated at some depth in the membrane-spanning protein, i.e., they would experience a potential different from those prevailing in the two 'bulk phases' on the two sides of the membrane. In such a situation, the activity of a charged ligand in the vicinity of its binding site, and therefore the probability of finding the binding site occupied, would be determined by the bulk-phase activity, by the diffusional resistence existing between the bulk phase and the binding site, and by the electrical gradient between the bulk phase and the binding site. Rather than the ligands modifying the response of the pore to the applied voltage, the magnitude of the voltage variation would contribute to determine the effective ligand activity. Thus, if Ca^{2+} has access to its binding site from the matrix, its binding site would be at a more positive (with respect to the matrix) potential, and therefore the Ca^{2+} activity at the binding site would be lowered by the presence of a transmembrane field. A decrease in the transmembrane potential (depolarization), at a given matrix bulk-phase [Ca^{2+}], would result in an increase in the Ca^{2+} activity at the binding site, hence in a greater probability of channel opening. Conversely, at a given transmembrane potential, an increase in matrix [Ca^{2+}] would have the same effect. It is clear that a given threshold activity at the binding site may be reached either by lowering the potential or by increasing the concentration. Similar considerations apply to, e.g., ADP (provided it functions as an anionic species). In this case, if the relevant ADP pool is the matrix one, the potential acts to increase the activity of ADP at its site. A higher matrix ADP concentration would mean that a lower transmembrane potential (a stronger depolarization) would have to be applied to maintain a given activity at the binding site. Thus, since ADP inhibits pore opening, the open probability vs. voltage curve would be predicted to shift towards lower transmembrane potential values as ADP increases. The formation of -SS- bridges might affect the diffusion of the various species by steric effects. The response to graded depolarizations predicted by the BMV model is indistinguishable from the predictions of the VSMB model. The BMV scheme implies either (again) the presence of at least four separate binding sites for activating/inactivating anions/cations, or the possibility that occupancy of a given binding site may result in opposite effects on the pore conformation, depending on the occupying species. A distinction between the two models might be based on the following considerations: the BMV model predicts either no saturation of the modulating effect, or that the concentration at which saturation occurs is voltage-dependent; the VSMB model predicts voltage-independent saturation.

Both models discussed above are a priori plausible. While the VSMB model is supported by the presence of an external Me^{2+} binding site, the BMV model is more consistent with electrophysiological [436,512] and swelling (e.g., [413,441]) data, according to which inhibitory divalent cations act also at a matrix-side site, like the activating cation, Ca^{2+}. Furthermore, in electrophysiological experiments, the VSMB model would predict opposite effects of the various effectors at opposite applied transmembrane potentials. For example, if binding of a Ca^{2+} favors, by increasing the positive charge on a particular site of the sensor, the transition to the open state at an applied potential of a given sign, it should have the opposite effect at the opposite voltage. Analogous considerations apply to ADP etc. The available electrophysiological evidence indicates that the effects of Ca^{2+}, ADP etc. do not depend on the sign of the applied voltage: matrix-side Ca^{2+} activates the channel at both positive and negative voltages, and so forth. In biochemical-type experiments it is obviously difficult to impose positive-inside potentials. It is also relevant that uncoupled mitochondria, with no (or a low) transmembrane potential gradient, are still susceptible of inducer-dependent transition from the impermeable to the permeabilized state as well as of 'resealing' or 'protection' by appropriate agents such as Mg^{2+}, as observed also by Bernardi and coworkers themselves [413]. It can be concluded that the effect of the various compounds cannot be confined to a modulation of voltage sensitivity.

As is clear from the literature reviewed in this and preceding Sections, the PTP is regulated by a multiplicity of factors, whose interplay is bound to generate a complicated phenomenology. For example, as already pointed out, depolarization will result in PTP activation, provided that the effect of the potential decrease is not antagonized by the consequent efflux of Ca^{2+} (on the uniporter) and the charge-compensating influx of protons [142]. Depolarization may also result in a counteracting increase in matrix ADP content [308]. The multeplicity of regulatory factors also goes a long way in explaining the variety of inducing agents (e.g., several apparently unrelated inducers might all act by decreasing the transmembrane potential [438]). Furthermore, some of the inducers might exert effects on more than one of the relevant parameters. Ca^{2+}, for example, besides directly activating the pore, as shown by patch-clamp experiments [436], probably acts also at the level of the transmembrane potential, which decreases during the uptake of the cation itself and might also be reduced by Ca^{2+}-dependent PA2 operation (see Section 4.1). Analogously, oxidizing agents, in addition to altering the redox state of PyNus and/or of relevant SH groups, might decrease the membrane potential through lipid peroxidation and leak induction.

3. Prooxidant-induced Ca^{2+} efflux

While the release of pre-loaded Ca^{2+} is certainly one of the consequences of the PT, some authors have described a sodium-independent, RR-insensitive prooxidant-induced Ca^{2+} efflux which they believe to be linked to oxidation of the PyNus and to occur via a pathway other than the PT ([118,156,158–

162,210,214,219,282,287,288,300,301,304,311,513–526]; see also [527,528]) (on a putative $Ca^{2+}/2H^+$ exchanger see [9] and Refs. therein). The experiments performed by these groups often follow the efflux of Ca^{2+} from Ca^{2+}-loaded mitochondria after the addition of EGTA or RR. The prooxidants used include acetoacetate and oxaloacetate (e.g., [282]), hydoperoxides (e.g., [524]), menadione (e.g., [159]), alloxan (e.g., [311]) and divicine (e.g., [214]). Other authors [8,25,62,109,117,119–122,265,283,286,343,529] have come to different conclusions. Several maintain that most of the RR-insensitive Ca^{2+} efflux is related to the development of membrane permeability [8,25,62,109,119–122,265,283,343].

The controversial observations include the following:

- Ca^{2+} efflux has been reported [156,158,519] to precede swelling (under conditions, e.g., omission of RR, allowing swelling to take place). On the opposite side, a nearly perfect correlation has been reported between Ca^{2+} efflux and Mg^{2+} loss, taken to be an indicator of the occurrence of the PT [121,337] (however, Höser et al. [492] reported that P_i-induced swelling did not begin until 70–80% of the mitochondrial Mg^{2+} had been lost).

- Prooxidant-induced swelling of the mitochondria is blocked by EGTA and/or RR (e.g., [156,158]). Proponents of the non-PT efflux mechanism interpret this as showing that excessive Ca^{2+} cycling (i.e. uptake and release via different pathways, blocked by these agents) is at the root of 'damage' to the mitochondrial membrane, an idea which might be traced by an early hypothesis by Rossi and Lehninger [54] (see also, e.g., [108,188,300]). The alternative explanation calls upon the heterogeneity of isolated mitochondria, as discussed in Section 2.2 [8,62,120,121]. The 'Ca^{2+}-cycling' model of mitochondrial damage is much weakened by the observation that uncoupler-treated mitochondria, in which Ca^{2+} cannot cycle because there is no transmembrane potential, still undergo permeabilization and swelling (e.g.: [27,110,438]; see also [121]). Furthermore, Ca^{2+} cycling induced by A23187 does not lead to damage [530].

- Ca^{2+} release alledgedly can take place in the presence of a high transmembrane potential [301,515]. However, the relevant experiments only demonstrate repolarization upon addition of EGTA, which might well act by inducing PTP closure. Furthermore, opponents point to the possibility of artefacts, due to the characteristics of the method (probe distribution) employed to monitor the transmembrane potential [120] (see Section 1.2).

The most important piece of evidence that prooxidant-induced Ca^{2+} efflux from liver mitochondria may occur independently of the PT is that in the presence of EGTA and/or RR complete prooxidant-induced release of calcium can take place without detectable swelling or release of matrix solutes by the mitochondria [161,304,525,531]. On the other hand, it is remarkable that the agents used to induce the efflux from Ca^{2+}-loaded mitochondria also induce the PT, while ATP [514], oligomycin [514], lasalocid-A [303] and CSP [524–526,531–533], all inhibitors of the PT, also inhibit the Ca^{2+} efflux under discussion. Richter and coworkers believe, because of these observations, that induction of the efflux is the first stage of a process which will eventually lead to the PT if it is not controlled by preventing Ca^{2+} cycling [304]. Others believe that only the residual rate of Ca^{2+} efflux in the presence of CSP may be considered as unrelated to the operation of the PTP [534,535].

The mechanism of induction of Ca^{2+} efflux favored by Richter and coworkers envisions oxidation of mitochondrial NAD(P)H, hydrolysis of NAD(P) and ADP-ribosylation of one or more as yet unidentified membrane protein(s), which would then provide the pathway for Ca^{2+} efflux. The mechanism of oxidation of the mitochondrial pyridine nucleotides depends on the prooxidant being used (see Section 2.2.1). The incorporation of NAD-derived ADP-ribose in proteins of the inner mitochondrial membrane has been detected [514,517,525,536,537]. NAD (and NADP) glycohydrolase and ADP-ribosyl transferase activities have been identified in mitochondria [301,537–540]. The former activity was presumed to be the relevant one, generating ADP-ribose moieties thought to react with a 30-kDa protein in a non-enzymatic process [536,537]. Inhibition of NAD hydrolysis, by ATP [514], 4-hydroxynonenal [522] or CSP [524,525,532], or of ADP-ribosylation, by ATP [514] (much higher concentrations were used than those employed to block NAD hydrolysis), or by a competitive inhibitor of ADP-ribosylation, MIBG [521,525], was associated with inhibition of prooxidant-induced Ca^{2+}-efflux (see also [210]). Mitochondrial PyNu hydrolysis and Ca^{2+} release showed the same sigmoidal dependence on the calcium load [541]. The manner by which accumulated Ca^{2+} induces Ca^{2+} efflux via the putative ADP-ribosylation dependent mechanism has not been discussed in detail. High matrix Ca^{2+} caused an acceleration of PyNu oxidation [158], presumably as a consequence of an increased rate of PyNu hydrolysis (which was presumed to take place in the matrix). If the mitochondria had not been loaded with Ca^{2+}, hydroperoxides still induced PyNu oxidation, but hydrolysis did not take place [158]. This mechanistic scheme has however been recently undermined by the report that the NAD glycohydrolase activity associated with mitochondria is actually located outside the mitochondrial matrix, suggesting that degradation of NAD requires first its exit from the matrix space [540].

In summary, the observations suggest that the prooxidant-induced Ca^{2+} efflux and the prooxidant-induced permeability transition may share important portions of the mechanism or may represent separate moments of the same process. It would be worthwhile to check the effects of inhibitors of NADase activity in mitochondria, such as 3-aminobenzidine [539], and of MIBG, on the development of the PT, and of CSP on the occurrence of NAD hydrolysis.

4. Mechanism: membrane 'defect' vs. pore

As all membrane phenomena, the permeability transition could a priori be ascribed either to the lipid bilayer or to the proteins imbedded in it. Both hypotheses have received much attention and support. The former held the upper hand for a long time. The consensus today is that the permeabilization of the mitochondria is due to the operation of a proteinaceous pore.

Before the recognition of the osmotic nature of the swelling, some of the pioneering investigators of the field tended to consider the PT-related phenomena as being closely associated with oxidative phosphorylation (e.g., [1,3,270,294,483,542]). This view was based on the fact that several of the agents which promoted or inhibited swelling were involved in the ATP production process. Thus, during the 50's and early 60's, because of the early recognition of a role of matrix ATP (e.g., [66,100]) the idea was debated that the loss of adenine nucleotides might be the cause, rather than the effect, of the PT (e.g., [66,352,353]).

Other authors emphasized the protective role of Mg^{2+}, attributing the loss of membrane 'tightness' to the release of this ion from its membrane binding sites (presumably because of competition by Ca^{2+}), with consequent disorganization of the phospholipid bilayer [108,299,412,479,480,543]. The idea might have originated from the work of Baltscheffsky [479,489]. Vercesi and coworkers have observed that exposure of mitochondria to Ca^{2+} and oxidizing agents leads to high-molecular weight membrane protein aggregates, most likely via the formation of disulfide bridges by radical processes, and propose that the 'permeability defects' reside in these aggregates [148,228,321,498]. However, the reproducible and rather well-defined characteristics of the PTP do not support the idea that it might arise from the random cross-linking of proteins.

The hypothesis that the permeabilization was due essentially to alterations of the phospholipid bilayer eventually took the form of the sofisticated deacylation/reacylation model, described below.

4.1. The membrane damage hypothesis

After the recognition that the PT-linked 'large amplitude' swelling was an osmotic process [58,59], it was necessary to identify the path taken by the entering osmolites. The origins of the membrane-damage hypothesis might be traced to the association of fatty acid production (the 'U factor') with uncoupling and swelling [201,232], and to the identification of PA2 [544–548] and of lysophospholipid-acyl-CoA-transferase (lysophospholipid acylase) [549,550] activities in mitochondria. The possibility that these two opposing activities might give rise to a cycle, with possible regulatory implications, was considered early on [551]. PA2 turned out to be Ca^{2+}-dependent [552–554], Mg^{2+}-inhibited [553] (see discussion in [555]), fatty acid-activated [548,553] and to have a pH optimum of about 8.5–9.5 [548,553,554]. It was partially purified [546,554] (for more recent work on mitocondrial PA2 see, e.g., purification [556]; sequence: [557]; monoclonal antibodies: [558]) and evidence was presented that it was present mainly in the outer mitochondrial membrane [559,560] (but see [561]). Since the ratio of PA2 activities in the outer and inner membrane fractions was lower than the ratio of the marker enzyme, monoamine oxidase, some of the enzyme was thought to be present in the i.m. [560]. Meanwhile, evidence was accumulating that exogenous fatty acids could cause mitochondrial swelling and uncoupling (see Section 2.2.4). It was also known that phospholipase A from cobra venom could induce swelling of mitochondria [562,563] (see also [564]). Following the suggestion of Wojtczak and Lehninger [201], the proposal was made [24,444,548] that a relationship existed between mitochondrial swelling and 'aging' and PA2 activity. The experimental evidence consisted mainly in the parallelism between activation of PA2 by Ca^{2+} and fatty acids and the induction of swelling by the same agents.

The idea was taken up and developed into an elegant model chiefly by Pfeiffer and coworkers [115,283,348,447]. As envisioned by these researchers, the mitochondrial inner membrane would develop permeability defects upon accumulation of lysophospholipids [283,348], the concentration of which was thought to be controlled by the activities of PA2 on one hand, and of a mitochondrial acyl-CoA-lysophospholipid acyltransferase on the other. The PT would be the consequence of an excessive activation of the former, and/or of an inhibition of the latter. Many experimental observations are consistent with this model. In addition to the similarity of the effects of Ca^{2+} and fatty acids on the PT and on PA2 activity, 1-acyllysophospholipids and free fatty acids have been reported to accumulate in the mitochondria under conditions leading to the PT [278,283,348,565] (contra, [566]). Inhibitors or conditions preventing the PT also inhibited the accumulation of these lysis products [283]. Among the inhibitors, compounds known to ihibit PA2, such as nupercaine [109,119,283,567], tetracaine [278] and *p*-bromophenacylbromide [568] figure prominently. The pharmacological agents trifluoperazine, dibucaine, quinacrine, verapamil and diltiazem have been shown to inhibit PA2 activity and swelling with (qualitatively) the same relative potencies [447]. It was proposed that PT-inducing agents, e.g., NEM and oxidizing agents such as acetoacetate or peroxides, acted by causing inhibition of the acyltransferase [25,120,121,348].

Some observations, on the other hand, could not be readily accomodated. The all-or-nothing nature of the PT [24,27,61] was difficult to reconcile with the gradual development of membrane defects through the operation of a phospholipase. Furthermore, the PT allows even very large

molecules to go through the membrane [569]. *N*-Acylethanolamines are effective inhibitors of the PT [33,447] but do not inhibit PA2 (see also the case of Propofol, Section 2.3.8). While this observation did not result in a change of attitude by the researchers in the field, the discovery that CSP inhibited the PT [127,220,457], but did not affect PA2 activity [220], was convincing evidence that, at a minimum, the PA2-based mechanism was not the only one operating [34]. It has also been recently reported that spermine and Mg^{2+}, good PT inhibitors, actually stimulate PA2 activity in mitochondria, probably by modifying the properties of membrane phospholipids [555]. In 1991 Pfeiffer's group reexamined the presence of acyllysophospholipid transferase activity in mitochondria, and concluded that it was not present: previous reports had been due to microsomal contamination of the preparations [570]. The deacylation-reacylation scheme lost therefore much of its credibility, although the evidence remains good that PA2 becomes activated under many circumstances leading to the PT, and may contribute to the permeabilization of the mitochondrial membrane. In fact, its operation has been found to cause proton (or other ion) 'leaks' [571], and it may thus contribute to a PT-inducing depolarization. Also, the products of its action, fatty acids and lysophospholipids, may interact with the pore in an activating fashion [34,242].

4.2. The PT is due to a pore

The all-or-nothing character of the PT was probably the main reason which convinced Hunter and Haworth that the permeability transition resulted not from a degenerative process of the lipid bilayer, but from the opening of a bona fide pore. They [21,27,61] pointed out that the mitochondria do not become gradually permeable to molecules of increasing molecular weight, but rather admit either basically nothing, or solutes of all sizes [61]. Remarkably, the shrinking process induced by addition of high-MW PEG was also reported to proceed in a quantal fashion, with the mitochondria existing in either a swollen or a contracted state [21]. This observation was taken to reflect the presence of an open/closed equilibrium for the pores, an interpretation which requires, as a corollary, that only few pores are active per mitochondrion or that cooperative effects are present.

The pore hypothesis was greatly strengthened by the work of Crompton's group. Following up on previous work by Hunter and Haworth [21], Crompton and coworkers showed that the permeabilization process could be immediately interrupted by the addition of EGTA [26,123,125,126]. The labeled sucrose, or other compounds [124,572] that had already entered the mitochondria were trapped inside, and no further entry took place, indicating that the still-impermeable mitochondria remained in this state. The observation was difficult to reconcile with the idea that sucrose might be crossing the membrane via 'permeability defects' due to the accumulation of lysophospholipids: in particular, the latter would not be expected to undergo reacylation rapidly, especially after the loss of ATP, CoA etc. from the matrix. A pulsed-flow solute-entrapment technique was used to study the kinetics of the permeation and resealing processes in heart mitochondria [26]. When the PT was induced by Ca^{2+}/P_i, the same extent of [^{14}C]sucrose entry at any given reaction time was measured, irrespective of whether the label was present throughout the permeabilization or was added 4 seconds before resealing [26,127]. The result (also reported, with a lower time resolution, by Hunter et al. [61]), is consistent with the view that each mitochondrion could exist in either an essentially impermeable or an essentially permeable state, with the transition from the former to the latter being rapid. A $t_{1/2}$ of 860 ms was measured for sucrose permeation into the matrix space [26].

Crompton and Costi's kinetic data led these authors to propose that while the Ca^{2+}/P_i-induced permeabilization and the EGTA-induced resealing of the mitochondria were random processes, the pores of each mitochondrion opened or closed with a high degree of synchronization [26,128]. While the nearly simultaneous opening of all the PTP's in one organelle might well be explained by the depolarization induced by the opening of the first pore, an explanation for the cooperative closure is harder to find, unless the mitochondrial membrane is considered to be 'excitable'; the slow kinetics of voltage-induced PTP inactivation in patch-clamp experiments (Section 4.3.2) do not support such an hypothesis. On the other hand, cooperative phenomena might not be required to explain the permeation data, if the large size of each pore is taken into account. The observed behavior might instead be explained by the presence of a single active pore (obviously 'synchronized' with itself) per mitochondrion. The calculation presented in the Appendix indeed suggests that one pore would be enough to account for the kinetics of sucrose permeation. Since sucrose was found to eventually enter all the mitochondria even at intermediate Ca^{2+} concentrations, with a constant population-wide transmembrane potential, Al-Nasser and Crompton [123] argued that the pores existed in dynamic equilibrium between the open and closed state(s), a deduction confirmed by electrophysiological experiments (see Section 4.3.2). Interestingly, when the PT was induced with Ca^{2+}/*t*-BuOOH, the data indicated that [^{14}C]sucrose entry into the 'permeabilized' mitochondria was a much slower process than in the case of Ca^{2+}/P_i. The authors suggested that this was due to a difference in the open probability (Po) of the pores in the two cases: with Ca^{2+} and phosphate the pores would be nearly always open (high Po), thus allowing rapid entry; Ca^{2+} and *t*-BuOOH would still cause pore opening, but the pores would spend a large fraction of their time in the closed state(s) (low Po), and permeation would be correspondingly slower. While this model is reasonable, the

alternative possibility remains that the 'permeability defect' induced by Ca^{2+}/*t*-BuOOH might not coincide with that induced by Ca^{2+}/P_i (see Section 5.4). The point might be verifiable today by an electrophysiological approach, or by solute exclusion measurements.

Definitive evidence for the involvement of a bona-fide pore in the mitochondrial permeabilization process has come from recent electrophysiological work. In 1989 Kinnally et al. [41] and then Petronilli et al. [42] using the patch-clamp technique, independently observed a large, multi-state channel in the mitoplast membrane. The properties of this channel, as observed in electrophysiological experiments, and its identification as the PTP are summarized in Section 4.3.2. The reproducibility of the channel properties, and its sensitivity to low concentrations of inhibitors, strongly suggest that it is indeed a proteinaceous pore, rather than a 'defect' of the lipid bilayer.

4.3. Pore properties

Once the conclusion has been reached that the PT is due to the operation of a proteinaceous pore, its characteristics must be in large part attributed to the protein(s) which form this pore. Thus, we may speak, e.g., of a Ca^{2+}-activated, Mg^{2+}-, proton- and ADP-inhibited, voltage-dependent pore. Obviously, part of the phenomenology of the PT may reflect instead interactions of various agents with (components of) the membrane in which the pore is imbedded. The major known properties of the PTP are summarized in Table 4.

4.3.1. Size

The size of this pore has been estimated from measurements of the Staverman reflection coefficient for various permeant species [573] and from a quantitative comparison of the permeation rates (measured by pulsed-flow experiments) by mannitol, sucrose and Arsenazo III [26,128]. In solute-exclusion (or permeation) studies with PEG, the highest MW of polymers admitted to the mitochondrial matrix were in the 1.5 kDa range [21,63,573] (PT-induced release of mitochondrial proteins has however been reported [325,569]). These various approaches led to estimates of 2–3 nm for the pore diameter.

Halestrap and coworkers [29,366,574–577] have also described a mitochondrial swelling induced by glucogenic hormones in the presence of external Ca^{2+} levels in the low-μM range (depending on Mg^{2+} concentrations; the Ca^{2+} levels were below those needed for PT induction). The swelling was dependent on the presence of P_i in the medium and on Ca^{2+} uptake, and it was enhanced by CATR, reduced by the presence of ATP in the medium, and inhibited by BGK. However, it was insensitive to CSP. The effect was associated with an increase in the mitochondrial PP_i concentration, attributed to inhibition of matrix pyrophosphatase activity because of the formation of a Ca^{2+}/PP_i complex. The pathway for K^+ entry was tentatively proposed to be the AdNT (see below, Section 5.1), which would form a H^+ and K^+-permeable channel upon binding PP_i and P_i instead of adenine nucleotides. At higher matrix Ca^{2+} levels, the AdNT would bind Ca^{2+}, and form the unselective, CSP-sensitive pore. Panov et al. [578] had previously identified an ADP-inhibited K^+ permeation pathway. Since ATR and CATR abolished or reversed the effect of ADP, the involvement of the AdNT had been proposed.

Other authors have envisioned a PTP (not necessarily identified with the AdNT) capable of developing from a relatively selective to a large, unselective pore. Novgorodov et al. [30] reported that the collapse of the mitochondrial transmembrane potential and swelling follow the same time course when the PT is induced by *t*-BuOOH, while the former precedes the latter when $Ca^{2+} + P_i$ is used. The authors proposed that the PTP may exist in two conformations, one allowing permeation only by H^+ and possibly K^+, the other admitting large molecules as well. With Ca^{2+} and P_i as inducing agents, the 'selective' conformation would precede the development of the 'unselective' one. A similar conclusion had been reached also

Table 4
A comparison of the properties of the MMC (electrophysiological experiments) and of the PTP (swelling experiments) supports their identity [a]

MMC	PTP
Activation by sub-mM Ca^{2+} at matrix sites [436,512]	Activation by Ca^{2+} at matrix sites [27,435]
Ca^{2+}-competitive inhibition by Mg^{2+},Mn^{2+},Sr^{2+} and Ba^{2+} [436]	Ca^{2+}-competitive inhibition by the same cations, with similar parameters [21,27,435]. Both external and internal site(s) [413].
Inhibited by lanthanides (Szabò and Zoratti, unpubl.)	Inhibited by lanthanides [21]
Ca^{2+}-competitive inhibition by sub-μM CSP [582]	Ca^{2+}-competitive inhibition by CSP [26,220,435]
Ca^{2+}-reversible inhibition by H^+ on matrix side [436]	Inhibition by H^+ on matrix side [21,369,435,437] Histidine(s) involved [437]
Inhibition by sub-mM ADP on matrix side [512]	Inhibition by ADP on matrix side [27,63]
Inhibition by Amiodarone in the μM range [591]	Inhibition by Amiodarone in the μM range (Bernardi et al., unpubl.)
Fully open at voltages close to zero [587]	Activated by depolarization [142,438]
Data consistent with two cooperating channels [436,587]	Ca^{2+} and ADP have two sites per channel, with cooperative effects [21,63]
	Activated by SH oxidation or acylation [149]
	Affected by AdNT ligands [241]

[a] Only some of the PTP effectors are considered, and only representative references are given.

by Al-Nasser and Crompton [123], who found indications that H^+ permeation precedes sucrose permeation in the presence of Ca^{2+} and P_i, and by Rizzuto et al. [119], according to whom H^+ permeability incresed upon treatment of the mitochondria with various inducers, before the onset of the PT. Andreeva and Crompton [31] have pointed out that while CSP can block the transport of relatively large molecules through the PTP, the presence of ADP is needed to re-establish the transmembrane potential. This has been tentatively explained in terms of a lower-conductance conformation of the CSP-bound PTP, which would only admit small ions, or, possibly, as a steric blockage of the pore by CSP, which, again, would result in the permeation by small species only. The reader will not miss the parallelism between the proposed gradual development of the full-size PTP and the model discussed by Richter and coworkers (Section 3), according to which a selective prooxidant-induced Ca^{2+} efflux pathway may turn into the PTP under the appropriate conditions. A gradual (time-dependent) development of higher conductance states has been described for other channels (e.g., [579]). Electrophysiological approaches have shown that the PTP can adopt a variety of conductance states (i.e., pore sizes) (see Section 4.3.2).

4.3.2. Electrophysiological characterization

The application of electrophysiological methods to the mitochondrial membranes (Revs., [7,580,581]) has provided a new and advantageous viewpoint on the PTP, thanks to its identification with the 'mitochondrial megachannel' (MMC) [435,436,512,582]. This identification was based essentially on a comparison of the properties of the PTP and of the MMC. The relevant information is summarized in Table 4. The electrophysiology of this channel has also been covered in a recent review [7].

Electrophysiological approaches can provide information difficult to obtain otherwise on, e.g., the presence of substates, the kinetics of pore operation (e.g., the mean open or closed times), cooperative effects, the behavior of the pore in unphysiological voltage ranges, the density of active pores in the membrane. Important applications are possible: the possibility of studying excised membrane patches allows complete control on the composition of the media bathing both sides of the membrane; one can thus in principle establish the side from which a given effector acts, and also if matrix components (e.g., cyclophilin) or secondary processes (e.g., Ca^{2+}-induced contact site formation) are involved in the process under study. The properties of pores induced by different methods (e.g., Ca^{2+}/P_i vs. Ca^{2+}/prooxidants) could be compared to establish whether they are indeed the same. The electrophysiological 'fingerprint' of the channel is potentially a powerful tool for its identification in molecular terms, by comparison with the properties of isolated pore-forming proteins.

The MMC was first identified in 1989 [41,42] as a huge

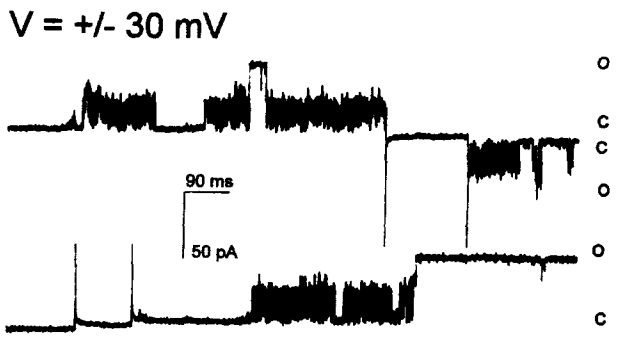

Fig. 1. The 'half-conductance substate' of the MMC. Exemplificative current traces from a patch-clamp experiment on a rat liver mitoplast bathed in symmetrical 150 mM KCl, 20 mM Hepes/K^+, 0.1 mM $CaCl_2$, pH 7.2. Applied voltage (by convention the pipette electrode is assigned 0 mV): ±30 mV. Capacitive current spikes signal voltage changes between +30, 0 and −30 mV (upper segment) or vice versa (lower trace). Filter: 7 kHz. Digital sampling rate: 5 kHz. c: closed; o: open. Notice the frequent transitions between the half-conductance and the closed states, and the absence of closures from the 'fully open' state. The half conductance substate is normally observed for shorter periods and less frequently than in this ad hoc example.

(1.3 nS in 150 mM KCl), substate-rich channel. The MMC may be operationally considered as one channel from an electrophysiological point of view, because it exhibits frequent full-size opening or closing transitions, which are incompatible with the presence of two or more *independent* channels. Lower-than-maximal conductance levels are considered as substates as a consequence. It should be clear that the presence of two (or more) strongly cooperating but separate channels cannot be excluded, and is on the contrary supported, by the patch-clamp records. Indeed, the most often observed substate is one having a conductance of about 1/2 the maximal value [436,583] (Fig. 1). This state is often visited during opening or closing events, a behavior displayed by other channels as well (see, e.g., [584]). This substate appears more frequently at higher salt concentrations (Szabò and Zoratti, unpublished), and it is characterized by a bursting behavior, i.e., by very frequent transitions to and from the closed state(s). Closures are much less frequent when the channel occupies the maximal conductance level (Fig. 1; [436,583]). This behavior strongly suggests, although it does not prove, a binary structure of the MMC, with two pore-forming 'subunits' which can cooperatively stabilize each other's fully open state. On rare occasions, we have observed cooperative behavior by four such subunits, as well as activity by what appear to be single subunits (Szabo' and Zoratti, unpublished results). Mutual reinforcement of the open state by cooperating channels, in analogy to what we observed, has been described in at least one other case [585]. Other channels, e.g., gap junctions [586], have been reported to give rise to activity showing almost a continuum of substate levels.

The MMC is driven to reside in long-lasting closed

state(s) by negative (i.e., physiological) voltages [587]. However, as long as it is open, in the negative voltage range, the channel shows a strong preference for the highest conductance state. The occupancy of lower levels increases with increasingly positive potentials, which also favor frequent transitions between the various levels. The response to abrupt voltage variations is slow: several seconds are required, on average, before the channels open or close. Kinnally and coworkers have observed the same voltage dependence pattern, but have also reported that the megachannel often behaves differently, showing little tendency to close at any voltage, or opening at negative and closing at positive voltages [41,581,588], or exhibiting lower open probability and lower conductance levels in the 0/+40 mV range, returning to high open probability and conductance above +40 mV [589]. These authors also reported that the application of voltages above ±60 mV (either sign), could elicit megachannel activity from silent patches [581,588]. Activation reportedly takes place in stepwise fashion, suggesting either a voltage-induced aggregation process, or a gradual opening of the pore (see Section 4.3.1).

The major positive modulator of the MMC is Ca^{2+} [512,590]. Kinnally et al. [590] found MMC activity only in a minority of patches if the mitochondria had been isolated in the presence of EDTA, whereas it was nearly always observed if the chelator had been omitted. In our hands, MMC activity occurs in about 70% of patches, even though the preparation of mitochondria is carried out in the presence of EGTA (but the standard patch-clamp medium includes 0.1 mM $CaCl_2$). The activity can be elicited in most silent patches by increasing the Ca^{2+} concentration in the bath, i.e., on the matrix side of the membrane patch under examination [436,512]. Besides the activation by Ca^{2+}, the single most compelling piece of evidence in favor of the identification of the MMC with PTP is the strong inhibitory effect of CSP [582]. A competitive interaction, analogous to the one with CSP (see Section 2.3.12) is observed between Ca^{2+}, the positive effector, and divalent cations or protons [436].

A few other compounds affect MMC activity. Antimycin A has been reported to inhibit the MMC at 1–2 μM. Its effect was however overcome by potentials above 40–60 mV, particularly in the negative range. At concentrations above 2–4 μM, Antimycin favored the open state [589]. The cationic, amphiphilic compounds Amiodarone, Propanolol and Quinine behaved in a qualitatively similar manner [581,591]. The inhibitory action of these drugs often involved a stepwise conductance decrease [581], in analogy to the observations by the same group on voltage-induced activation. Recently, Campo et al. [592] have reported that the protonophoric uncouplers FCCP and CCCP increase the open probability of the MMC. However, the effect takes place at much higher concentrations than those needed to fully uncouple the mitochondria, and the effects of these compounds on the PT most probably are not due to a direct interaction of the uncouplers with the pore.

The hypothesis that the MMC might coincide with the mitochondrial benzodiazepine receptor (mBzR) (see below, Section 5.2) has led to tests on the effects of mBzR ligands. Alpidem, a high-affinity ligand of the mBzR [593], elicited high, flickering currents from a high percentage of silent mitoplast patches [583,594]. Kinnally et al. [595] have reported that the benzodiazepine Ro5-4864, a specific ligand of the mBzR, inhibited MMC activity with an IC_{50} of about 70 nM, while Clonazepam and Ro15-1788, which bind specifically to the central Bz receptor, were ineffective. Protoporphyrin IX, a potent inhibitor of mBzR ligand binding [596] also inhibited the MMC at low (8–40 nM) concentrations, but reactivated it at higher concentrations.

The pharmacological properties of the PTP/MMC present some similarities to those of the 'inner membrane anion channel' (IMAC) (reviews, [406,407,597]). For example, the IMAC is activated at alkaline matrix pH (pK_a = approx. 7.8 [598]), and it is inhibited by Mg^{2+} at both an inner and an outer site [599]. Ca^{2+} has been reported to inhibit the IMAC with $K_m = 17$ μM [599], but Selwyn and coworkers [600] have reported an activation in the the 5 μM range. In other respects, the properties of IMAC are however completely different from those of the PTP. For example, Zn^{2+} is a powerful inhibitor [601] (whereas it induces the PT, see Table 2) while CSP [602] and the physiological nucleotides, including ADP and ATP [603], do not inhibit. Overall, the differences between the properties of the IMAC and those of the PTP clearly outweigh the similarities, so that an identification between these species seems unlikely.

5. In search of pore identity

The identity of the PTP-forming protein is not yet known. It might be a new, not-yet-identified protein of the inner mitochondrial membrane. This hypothesis has been strengthened by the recent results of Andreeva and Crompton [31], who reported the presence of a 10–12 kDa inner membrane protein which binds CSP in an ADP-enhanced manner. Since CSP and ADP exert cooperative effects on the PT, this protein is a candidate as the, or a constituent of, the PTP. In these authors' experiments, at least one other protein was photolabelled by the CSP derivative used, but the labeling was ADP-insensitive. This line of investigation may well lead to interesting new findings. Meanwhile, however, the known properties of the PTP point to at least two well-known candidates among mitochondrial membrane proteins: the AdNT and the outer membrane porin, in association with other proteins. These two hypotheses are discussed below.

5.1. The AdNT hypothesis

Ever since the pore hypothesis began to take hold, the AdNT has been considered the most likely candidate as the pore-forming protein [102,170,241,242,289,345,365,366]. Indeed, a currently favored [604–607] structural and mechanistic model for many membrane translocators envisions a pore-forming domain 'closed' by gating domains which may either block or allow access of the substrate to the binding site in the channel. This model was originally developed precisely for the AdNT [245,247,608,609]. Supporting this model, it has been shown [610–613] that at least the AdNT, the P_i carrier, the glutamate/aspartate exchanger and the carnitine carrier of mitochondria can be turned into relatively unselective uniporters (possibly having pore-like structures) by treatment with SH reagents. Whether the characteristics of these unselective putative pores correspond to those of the PTP is however questionable [32].

The AdNT was fingered as being specifically involved in the PT because of the long-standing observation that high-affinity, and, as far as is known, specific ligands of the AdNT, namely ATR, CATR, BGK, as well as ADP, acyl-CoA's and pyridoxal-5-phosphate, also known to bind to the the AdNT, affect the PT (see Section 2.2.5). LeQuoc and LeQuoc [241,289] analyzed the effect of these inhibitors, coming to the conclusion that the conformation of the AdNT is important in determining whether the PT takes place or not in Ca^{2+}-loaded mitochondria. Impermeant AdNT ligands (ATR, CATR, palmitoyl-CoA, pyridoxal-5-phosphate), stabilizing the 'C' conformation [244,245] of the AdNT, would act as PT inducers; vice versa, ligands (BGK, matrix ADP, matrix acyl-CoA's) stabilizing the 'M' conformation would have the opposite effect. The model connects NADH oxidation, the redox state of thiol groups and the conformation of the AdNT. A high NADP/NADPH ratio favored the C conformation [289]. This was proposed to be due to the oxidation of one or more SH groups of the AdNT, since the authors measured a significative decrease of protein SH groups, as well as an increase in the rate of AdNT-catalyzed AdN exchange. DTT prevented the increase in the rate of AdN equilibration (and the PT; see, e.g., [25] and Table 3). SH groups are thought to become 'unmasked' during the C-to-M state transition (or vice versa) [247,614,615], and are generally considered to be important in translocation processes [616]. The cysteins of the beef heart AdNT have been recently shown to exhibit different reactivities towards NEM or eosin 5-maleimide [617], a result consistent with the presence of SH groups of different reactivity in the PTP [149] (see above, Section 2.2.2).

Halestrap and coworkers [8,242,243] have also elaborated a similar model. In their representation, the AdNT adopts an open-pore conformation upon binding Ca^{2+} and cyclophilin. Binding of both Ca^{2+} and cyclophilin, would be required for the conversion of the carrier into the unselective pore. This binding could occur only with the carrier in the C conformation, and would be prevented by CSP binding to cyclophilin (see also Section 2.3.12). Cyclophilin might possibly bind to a proline residue (Pro-61) presumed to be exposed on the matrix side in the C conformation, but 'hidden' in the M conformation [242,243]. Lys-62 is proposed a the possible site of action of pyridoxal-5-phosphate [242]. The model also envisions the permissive binding of P_i.

Novgorodov and coworkers [345] have reported that CATR can reverse the inhibitory action of CSP, provided that the mitochondria have been depleted of matrix ADP by undergoing the PT or by treatment with PP_i. In these authors hands, inhibition by CSP could be reversed by treatment with PhAsO even without AdN depletion. These observations do not contradict an involvement of the AdNT, but constitute an argument against the details of the model presented by Halestrap and coworkers, since they indicate that the PTP may form even if cyclophilin is 'sequestered' by CSP. The same group [30] found CSP to decrease the affinity of the PTP for Ca^{2+}, without altering the cooperativity characteristics of the two Ca^{2+} binding sites, a conclusion, again, not readily reconcilable with Halestrap's model. Fournier et al. [457] reported that CSP had only a limited effect on the rate of State 3 respiration, i.e., it did not significantly affect AdNT operation. Since however the PTP is supposed to correspond to an altered form of the AdNT, this result cannot be considered to militate against its involvement. McGuinness et al. [129] concluded that the component (presumably cyclophilin) binding CSP with high affinity was in 50–70% excess over the PTP, whereas the AdNT is, by all counts, in vast excess over cyclophilin. This difficulty may be circumvented by assuming that only molecules of the AdNT already altered in some way might be capable of binding cyclophilin giving rise to the PTP.

The possibility that the AdNT may give rise to the PTP has been further strengthened by the recent report [618] that the fusion of egg lipid liposomes containing purified AdNT to planar bilayer membranes leads to the observation of high-conductance channels. Channel activity is enhanced by mersalyl treatment. While these observations are very suggestive, a detailed examination of the properties of these channels is clearly necessary before they can be equated with the PTP. Furthermore, caution must be exerted to exclude the possibility of artefacts: according to our experience egg lipid liposomes themselves often give rise to high-conductance, spurious channels in bilayer experiments (Zoratti et al., unpublished).

Novgorodov and colleagues [30,32] have recently argued that the site of action of ADP/ATP must be distinct from the AdNT, since the impermeability of Ca^{2+}-, cumene hydroperoxide- and CATR- treated beef heart mitochondria can be restored by ADP + CSP (but not by ADP or CSP alone). Since the nucleotide binding site of the AdNT is presumably occupied by CATR, and since ADP interacts with both a high-affinity binding site (presumed

to be the AdNT; K_m approx. 5 μM) and a low-affinity (70 μM) binding site [30,395], the authors concluded that the relevant interaction probably concerned another mitochondrial membrane protein. The results of Andreeva and Crompton [31] (see Section 2.3.12) also provide reasons to doubt the identification of the AdNT as the PTP-forming protein. Rottenberg and Marbach [367,368] and Pfeiffer and coworkers [5,32] have suggested that the conformation of the AdNT may have a significative effect on the membrane surface potential, and that the effects of the ADN's, ATR, CATR and BGK on the PT may be mediated by this phenomenon. Alternatively, the AdNT might interact with the actual PTP-forming protein and influence it via conformational interactions (e.g., [32]).

The AdNT is but one member of a vast family of highly homologous 'tripartite' mitochondrial transport proteins (e.g., [619,620]). The possibility must therefore be considered that other members of the family might be capable of forming permeability transition pores. The peculiar role of P_i in the induction of the PT suggests that the P_i carrier might be worthy of special consideration.

5.2. The VDAC hypothesis

In 1985 LeQuoc and LeQuoc [351] reported that mitochondria swollen in isotonic ammonium phosphate or nitrate, and treated with *N*-butylmaleimide or NEM became permeable to PEG with MW up to 6000 Da and released matrix enzymes. The process was inhibited by both ATR and BGK, and was still observable in the presence of EDTA or EGTA. The size of the presumptive pore formed was estimated at some 3 nm on the basis of the solute exclusion measurements. Interestingly, swelling was found to depend on the presence of the outer membrane: no analogous phenomenon could be observed when using mitoplasts. The authors proposed that under the specific conditions they used, the permeabilization might arise from the recruitement of porin molecules from the outer to the inner membrane, possibly via contact sites, and with the participation of the AdNT. Crompton and coworkers [26,129] later mentioned the possibility that the PTP might arise via incorporation of porin into the inner membrane, in part as a way of explaining their inability to obtain pore expression in submitochondrial particles [129]. Haworth and Hunter [63] had speculated earlier on that the PT might "entail fusion with the outer membrane, producing a transient gap junction".

The idea seems now worthy of consideration in view of the observation that the maximal conductance of the mitochondrial megachannel (Section 4.3.2) is twice that expected for VDAC, and that the most prominent of the MMC substates is the 'half-size' one, with approximately the same conductance as the fully 'open' VDAC [42,436,580,587,621–630]. As mentioned above, the patch-clamp records suggest that the channel may be formed by two cooperating 'halves', which might be tentatively identified as porin monomers. The evidence that the AdNT is involved in PTP formation (see above) suggests that while VDAC may form the channel, the AdNT may play a regulatory role, i.e., PTP activity might be due to supramolecular complexes comprising VDAC and the AdNT. Such complexes have long been thought to exist in contact sites [631], and to be possibly involved in their formation. In 1992 McEnery and coworkers reported that the mBzR [632–635] was a supramolecular complex comprising VDAC, the AdNT and an 18 kDa peptide [636,637], thus providing an instant candidate for the PTP. We therefore proposed [6,583,587,594] that the MMC (i.e., the PTP) might comprise two cooperating VDAC molecules as pore-forming components. Furthermore, the MMC/PTP would coincide with the mBzR [583,594,595]. Thus, the two porins would be associated with two AdNT molecules, and presumably with two copies of the 18 kDa mBzR peptide. Table 5 summarizes the available evidence and information supporting this model, which at present seems compatible with the available electrophysiological evi-

Table 5
Evidence supporting the identification of the PTP/MMC with a dimeric form of the mBzR

- The pore sizes of VDAC [621–623,668–674] and of the PTP [26,128,573] are similar (diameter 2–3 nm).
- The MMC may be formed by two cooperating VDAC-size channels [436,587,594].
- The PTP contains couples of cooperating binding sites for Ca^{2+} [21,30] and ADP [63], suggesting a dimeric structure.
- VDAC molecules can form cooperating dimers and higher oligomers in membranes ([583,594,675]; Zoratti et al., unpubl.) VDAC inserts into planar membranes preferably as oligomers [676]. VDAC molecules can be cross-linked in membranes [675]. VDAC in Triton forms dimers [677].
The AdNT functions as a dimer [245,608] or a tetramer [678] [a].
- Both VDAC [580,621,625,629,630,679] and the PTP/MMC [41,42,436,581,582,588] display a variety of conductance substates.
Both VDAC [580,621,668,680,681] and the PTP/MMC [41,512,594] have low and variable selectivity.
- The voltage dependences of VDAC and the MMC/PTP are compatible [583,587].
- VDAC can display fast kinetics [583,594,682], reminescent of the behavior of the 'half-conductance substate' of the MMC [436,587].
- Supramolecular complexes comprising VDAC and AdNT are present in mitochondria [631,633,636,637].
- VDAC and AdNT concur to form the mBzR [633,636,637]. Alpidem [587,594], Ro5-4864 and PPIX [595], which are mBzR-specific ligands affect MMC operation in the nM concentration range, while Clonazepam and Ro15-1788, which are specific ligands of the central BzR, do not [595].
- NADH inhibits the PTP [21,63] and facilitates VDAC closure [683].

[a] This obviously constitutes even stronger evidence for the involvement of the AdNT as such.

dence. It might also be mentioned that Wunder and Colombini [638] have attributed to VDAC the activity observed by patch-clamp upon reconstituting whole mitochondrial membranes from *Neurospora crassa* in liposomes, which is quite reminescent of the MMC. According to the model under discussion, each channel should connect the matrix compartment to the extramitochondrial space. This follows from the maximal value of the conductance of the putative monomeric component, which matches closely that of VDAC. If the channel were in series with another pore (i.e., formed by the AdNT, or another VDAC), the observed conductance would necessarily be lower. Thus, the AdNT would interact in a 'side by side' manner.

The major objections to the model are of topological nature. The MMC/PTP is located in the matrix-enclosing envelope, while VDAC is universally considered to be an outer membrane protein, and there is no doubt that the vast majority of it resides there. However, to our knowledge, no evidence has been provided for its complete lack in the inner mitochondrial membrane. While its presence in inner membrane preparations has been always attributed to contamination, little quantitative work has been performed to verify whether its amount correlates quantitatively with the amount of contamination. In at least one case [639] the fractional amount of VDAC present in a partially purified inner membrane fraction of yeast mitochondria matched exactly the fraction found to be associated with the inner membrane in immunoelectronmicroscopy work. The presence of VDAC in beef heart submitochondrial particles has been reported [640]. It should be stressed that any VDAC present in the inner membrane would be kept tightly closed by the physiological transmembrane potential.

It is also entirely possible that the PTP/MMC might reside in contact sites. Contact sites have been found to be enriched in the AdNT [641,642], VDAC-bound hexokinase and creatine kinase [642–645] and possibly VDAC [643,646]. The abundance of contact sites appears to be under metabolic control, increasing under phosphorylating conditions, and can be reduced by treatment with dinitrophenol, fatty acids or glycerol [641,645,647,648], suggesting a way to investigate PTP location. Interestingly, addition of Ca^{2+} to rat liver mitochondria has been reported to cause an increase in contact sites [631,649,650], and the presence of some may even be necessary for their formation [646]. ADP and ATR are able to induce contact site formation even in deenergized mitochondria, strengthening the hypothesis of a role of the AdNT in contact site formation [641]. Contact sites have been proposed to arise from the localized fusion of the inner and outer membranes [649], but current opinion inclines towards an apposition mediated by proteins. A contact between outer membrane porin and inner membrane AdNT has been widely accepted following the reports that kinases bound to VDAC had preferential access to ATP synthetized in the mitochondria [631,646,651,652] (contra, [653]).

The presence of VDAC and of attached outer membrane fragments in mitoplasts prepared by the osmotic shock method is known [654]. Channel activity suggestive of the presence of the PTP/MMC has been observed in proteoliposomes containing a purified contact site fraction of brain mitochondria [655]. Mitochondria isolated from highly glycolytic tumor cells [107] or from glycogen-treated rats [656] withstand Ca^{2+} loading well, and show few contact sites [657,658].

The available electrophysiological evidence cannot rule out the possibility that the PTP/MMC may comprise not VDAC, but another pore with similar properties. At least one such pore exists, namely the 'peptide sensitive channel' (PSC) [659–667]. We have recently studied this channel by patch-clamping VDAC-less yeast mitochondria (Szabò et al., Biochim. Biophys. Acta, in press). The conductance values and voltage dependence are similar enough to those of VDAC and of the MMC to warrant the hypothesis that it might form the MMC/PTP in stead of VDAC when the latter is absent, or possibly even in its presence in a fraction of cases. However, its selectivity and other electrophysiological and pharmacological properties (Szabò et al., Biochim. Biophys. Acta, in press) argue against an identification of the PSC as the normal channel-forming MMC constituent.

5.3. Help from biochemistry and from lower eukaryotes?

One might hope to gain some insight into the nature of the protein(s) responsible for the PT by matching the known biochemical properties of the PTP with those of the candidates. The task is obviously complicated by the fact that a role is assigned to the AdNT by both models discussed above.

Whatever molecule or supramolecular complex forms the permeability transition pore in RLM must contain at least one histidyl residue accessible to protons from the matrix side [437] and two cysteinyl residues, whose oxidation to form a disulfide link would result in pore formation in a NEM-preventable manner [149]. One or more other SH groups, less reactive with NEM, may or may not be present depending on the interpretation of the results of Petronilli et al. [149] (see Section 2.2.2).

Since the known mammalian AdNT's (bovine, [684]; rat, mouse, [678]; human, [685–687]) and VDAC's [688–693] all contain at least two cysteines and two histidines, it does not seem possible to gain information on whether the PTP is formed by the AdNT or by VDAC (or by a combination of the two) as long as mammalian mitochondria are considered. On the other hand, the sequence of the AdNT from *Neurospora crassa* [694] shows two cysteines, and no histidine, while *Neurospora* VDAC contains five histidines, but no cysteine [695]. If the PT could be induced in *Neurospora* mitochondria using thiol cross-linkers, this would support (although not prove) pore formation by the AdNT itself. Such a PT would not be

expected to be pH-sensitive. If it were, this might be considered to support the involvement of VDAC as well.

Of the three isoforms of yeast AdNT [678,696–698] one contains two histidines and three cysteines, the other two contain four cysteines, but no histidine. Mutants defective in the expression of AdNT genes [697–700], are available. The sensitivity to pH changes of the PT (if it occurs; see Section 7) in appropriate yeast AdNT mutants might therefore establish whether the AdNTs are responsible for pore formation (the yeast porin contains three histidines and two cysteines [701,702]).

Yeast VDAC-less mutants are also available [703–706]. A study of the ability of these mutants to undergo the PT ought to provide additional useful information.

5.4. How many mechanisms for the PT?

The variety of conditions and compounds which enhance the permeabilization of mitochondria, together with the rather unspecific nature of the assays commonly used (swelling, solute efflux) to monitor the PT, raises the question of whether only one mechanism underlies the observed phenomenology. The main arguments against a multiplicity of mechanisms are the general requirement for Ca^{2+} accumulation and the widespread inhibitory effect of CSP and of other agents such as spermine, Mg^{2+} or the transmembrane voltage gradient. These common characteristics suggest that the processes unleashed by the various agents are similar. However, data have been reported which are not fully coherent with these generalizations. CSP-sensitive swelling can be induced in the absence of external Ca^{2+} by at least one SH reagent (PhAsO) [346]. The extent of inhibition by CSP may vary depending on how the permeabilization was brought about (see [220]). CSP and TFP, an inhibitor of phospholipase action [447], as well as of lipid peroxidation and of thiol cross-linking [169], have additive effects on the loss of solutes induced by *t*-BuOOH and Ca^{2+} [34]. As already mentioned (Section 2.3) these same compounds have been reported to affect the PT differently depending on whether it was induced by Ca^{2+}/P_i or by Ca^{2+}/*t*-BuOOH. Palmer and Pfeiffer [109] have reported that in both liver and heart mitochondria P_i antagonizes the permeabilizing effect of SH reagents: under certain conditions, SH reagents (NEM, Diamide) will cause the release of moderate loads of Ca^{2+} in the absence, but not in the presence of P_i.

Other hints at mechanistic differences can be found in the literature and some are mentioned in previous Sections. For example, Palmer and Pfeiffer also reported that release of pre-accumulated Ca^{2+} by heart mitochondria was transient if the releasing agent was oxaloacetate, but not if the releasing agent was palmitoyl-CoA [109]. Since the release of Ca^{2+} was accompanied by that of K^+ and Mg^{2+} and by swelling, the phenomenon presumably reflected the occurrence of the PT. Carbonera et al. [213] reported that nitrofurantoin at low concentrations inhibited the permeabilization induced by peroxides or diamide, but enhanced it if caused by the addition of Ca^{2+}/P_i. Riley and Pfeiffer [35] reported that DTT did not affect swelling induced by clofibric acid and WY-14643, which shows much the same characteristics of the swelling induced by a number of other agents. According to Lapidus and Sokolove [147] depletion of matrix nucleotides dramatically enhanced the ability of P_i to induce the PT, but had no effect on induction by Ca^{2+} or *t*-BuOOH.

The idea [34,144,167,169] that at least two different (besides the massive lipid peroxidation induced by some agents, which cannot be considered as a variety of PT), although overlapping or at least connected, mechanisms may produce the same macroscopic result (i.e., the opening of an unspecific pore in the mitochondrial inner membrane), cannot therefore be lightly dismissed. A clarification of this important point might presumably be achieved by more detailed investigations, e.g., by electrophysiological means, on the nature of the permeability pathways formed under the various circumstances.

6. Physio- and pathological relevance

The physiological role of the PTP, if any, is still a matter of conjectures. The evidence that the pore may function under normal in vivo conditions is limited. Nicchitta et al. [707] found that CSP treatment increased the Ca^{2+} content of the reticular and mitochondrial pools in isolated rat hepatocytes. Altschuld and coworkers [708] reported that CSP increased net Ca^{2+} uptake and reduced efflux from isolated rat cardiomyocites. The effect was traced to the mitochondria, suggesting that Cyclosporin blocked a physiological pathway for Ca^{2+} extrusion from the organelles. Loew et al. [709] have used confocal microscopy to monitor the transmembrane potential of individual mitochondria in neurites; they reported that occasionally the potential of an organelle displayed significant fluctuations, with depolarizations in the range of 20 mV lasting up to 24 seconds. Mannella [710] has suggested that these fluctuations might correspond to opening of mitochondrial channels. Given the limited extent of the depolarization, however, it does not seem likely that the putative channels might be PTPs.

While more evidence would be desirable, PTP operation in the course of normal cell function is a possibility. As pointed out by others [4,8], if the PTP opened only infrequently and for brief periods, its activity would not create survival problems for either the mitochondrion or the cell. A reversible PT might instead provide an energetically convenient way to eliminate excess Ca^{2+} or other undesired solutes from the mitochondrial matrix [4]. The PT might also be useful as a heat-generating mechanism in organisms or tissues which do not possess sufficient brown adipose tissue [4]. Its occurrence has been linked to the Ca^{2+}-dependent activation of respiration of liver mito-

chondria from hibernating squirrels [711]. A role of the PTP [8] or of the prooxidant-induced Ca^{2+} efflux [712] in apoptosis seems possible, since apoptosis is associated with elevation of cytosolic Ca^{2+} and in T cells it may be inhibited by CSP [713–715]. Also, overexpression of one of the genes involved in its regulation, namely *bcl-2* (review, [716]), whose product is found mainly in the mitochondrial outer membrane or possibly contact sites [716], protects cells from death by oxidative stress [717]. Others have suggested as possible roles the formation of gap junction-like connections between mitochondria or the transport of proteins across the mitochondrial envelope system [581]. No evidence supports yet these interesting possibilities. Attempts to inhibit the transport of ornithine carbamoyltransferase into mitochondria with cyclosporin A were unsuccessful [569].

While research into the physiological roles continues, strong evidence (reviews, [8,9]) is emerging that the permeability transition may be important in the processes of cell damage and death which result from oxygen deprivation, the ischemia- and reperfusion-induced injury. It has been known for a long time that the consequences of ischemia depend on its duration, and that much of the damage develops during the reoxygenation stage. In heart [718–720], ischemic contracture develops after 10–20 min, and recovery is possible if reperfusion takes place in its early stages. At the cellular level, ischemia determines creatine phosphate depletion, rise in P_i, Na^+ influx, glycolysis and lactate production and a decrease of cytosolic pH. The return of cytosolic pH to near-neutral values has been identified as a factor in reperfusion damage [721–724]. Prolonged ischemia results in a very large transient increase in cytoplasmic Ca^{2+}, presumably due to SR disruption [719]. Reperfusion (i.e., reoxygenation) also induces a (lower) Ca^{2+} transient, which has been attributed to the operation of the Na^+/Ca^{2+} exchanger of the plasma membrane (e.g., [719]) or to leaks through the plasma membrane (e.g., [725]). Much of the Ca^{2+} in question has been observed to eventually accumulate in the mitochondria [726–729].

Mitochondria themselves become targets for ischemic damage. Studies on mitochondria isolated from infarcted organs show a decrease of NADH dehydrogenase [730–733], AdNT [373,734–737] and ATP synthetase [731,738] activities, and an increase in PA2 activity [739,740]. They also seem to produce more oxygen radicals [733,741], as do also isolated mitochondria upon reoxygenation after incubation under hypoxic conditions [325]. These radicals might be responsible for the damage to the respiratory chain as well as for some of the damage suffered by the cells upon reperfusion (e.g., [742–746] and Refs. therein).

Apart from alterations at the molecular level, the functionality of mitochondria from infarcted organs as integrated energy conservation systems is partly or completely compromised (uncoupling) [373,456,747–752] (contra, [728,753]), and their morphology grossly altered [727,728,730,754,755]. The mitochondria (also in situ) appear as swollen bodies, resembling the isolated organelles which have undergone the PT. They lose a large fraction of their nucleotides ([749,756,757] and Refs. therein) and glutathione [758], and have been reported to lose matrix enzymes in a CSP-inhibitable manner [759] and to accumulate long-chain acyl-CoA's [760–762] and lysophospholipids [163,763].

In view of the observations summarized above, the hypothesis was put forward [26,323,369,481,726,735,764] that the mitochondria might undergo the PT as a consequence of excessive Ca^{2+} uptake, presumably upon re-energization as the hypoxic tissue becomes oxygenated again. The recovery from acidosis and the oxidative processes taking place upon reperfusion presumably coadjuvate Ca^{2+} uptake in inducing the PT. As would be expected on the basis of this hypothesis, isolated mitochondria have been shown to release proteins and to swell in a $[Ca^{2+}]$-dependent, CSP-inhibited manner following reoxygenation after a period of anoxia [325].

The notion that the mitochondria might be subjected to the permeability transition, under pathological conditions, in situ, and that this might contribute to cell death, has been supported by observations of the protective effect of CSP in experiments in which isolated cells were exposed to 'prooxidants' and Ca^{2+} [306,533,765,766] or to anoxia [347,475,767], or whole organs were subjected to ischemia [768–772]. Some caution is in order when evaluating these experiments: for example the immunosuppressants FK506 and azathioprene, which do not antagonize the PT, also had a protective effect in experiments on whole organs [773,774], suggesting that in this case the protection might be due to the prevention of macrophage activation [775]. Synergism between CSP and PA2 inhibitors in protecting cells from anoxic or oxidative death has been reported [306,347,766]. Recent reports by Hoek and coworkers [776] and by Lemasters and coworkers [777] provide the most convincing information available on the occurrence of the mitochondrial permeability transition under conditions mimicking an infarct, in cells. Both these groups followed the permeability properties of the mitochondria in situ. In the case of Lemasters' work, this was done by monitoring the distribution of a fluorescent dye using confocal microscopy.

It seems therefore likely that (a) The PT takes place under pathological conditions (anoxia/reperfusion) in whole cells and (b) the phenomenon contributes to cell and tissue damage. While the extent of this contribution is at present difficult to assess, we would be surprised if it turned out to be nil. The relevance of these conclusions for human health care is evident. The possibility should also be kept in mind that the PT may contribute to cell death caused by oxidizing agents, of natural (e.g., divicine [214]), industrial (e.g., paraquat [209,778,779]) or pharmacological origin (such as quinones (e.g., [211]), nitrofurantoin (e.g., [780]) or adriamycin (e.g., [318,778,781])) (for a

review see, e.g., [782]). The PT has been proposed to be implicated also in Reyes' syndrome [314–316,783].

7. Conclusion

The perspectives of this research field can without exaggeration be qualified as exciting. The protein(s) responsible for PTP formation seem on the verge of being identified. Contributions towards this goal can be expected from both electrophysiological and biochemical approaches. Whether only one or more proteins can give rise to the pore will hopefully be clarified in the course of these investigations. The possibility that mitochondrial permeabilization may play a role in phenomena of great clinical relevance is already stimulating a considerable amount of research. A chance may be at hand of alleviating the effects of interruptions of the blood flow to the heart or brain.

A few of the many points still to be clarified may be mentioned here. One aspect of the PT which has attracted little attention is the lag time which generally preceeds the development of permeabilization after Ca^{2+} uptake. What is its significance? Might it reflect the time needed for assembly of the PTP from components, or for development of a larger pore from an initial smaller one, as suggested by some experiments? Does it instead have to do with some process of membrane reorganization, e.g., contact site formation? Is there a correlation between the rate of propagation of the PT in a mitochondrial suspension and the density of contact sites in these mitochondria? What are the reasons for the different susceptibilities to the PT displayed by mitochondria of various origins? Can these differences be explained only in terms of different contents of protective agents (Mg^{2+}, ADP) and/or different Ca^{2+} uptake capabilities?

The development of research on the PT in an organism amenable to genetic manipulation would be a great bonus to the field. Whether the PT occurs in yeast mitochondria is at present unclear. Our group has obtained some EM evidence pointing to a CSP-sensitive permeabilization process, but has been unable so far to obtain evidence for swelling induced by $Ca^{2+} + P_i$ or oxidizing agents (Zoratti and Szabò, unpublished). If some means could be found to reproducibly induce the PT in yeast, the opportunities for progress would be considerable. For example, it might be possible to disrupt the gene for mitochondrial cyclophilin (which is well known and not essential for yeast life [466,470,471]), and to take note of the consequences. On the other hand, if yeast mitochondria turned out not to undergo permeabilization, this might also offer important clues. For example, do these mitochondria contain a benzodiazepine receptor?

Well-defined questions aimed at precisely identifiable problems can now be asked, and powerful investigating methods are available. Further, decisive progress ought to be rapid.

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Appendix 1

An approximate equation for flux through an open channel, modeled as a cylinder, is given by Hille [511] (Eq. 11-6) as:

$$F = \pi * r^2 * D * C/(l + \pi * r/2) \quad (1)$$

where r is the channel radius, l is its length, D is the diffusion coefficient of the diffusing species and C is its concentration. In the case of sucrose permeation into a mitochondrion, through one channel, if flux coupling phenomena are not taken into consideration the net flux will be given by:

$$F_{net} = \pi * r^2 * D * (C_{out} - C_{in})/(l + \pi * r/2) \quad (2)$$

The net flux is also given by:

$$F_{net} = V_m * dC_{in}/dt \quad (3)$$

where V_m is the matrix volume. Substituting Eq. (3) into Eq. (2), and integrating, assuming C_{out} to be constant, C_{in} to be the same throughout the matrix at all times, considering the mitochondrion to be a sphere with radius 0.5 μm, and taking: $r = 1.0$ nm, $l = 7$ nm, $D = 0.521 * 10^{-5}$ cm^2/s (for sucrose in water at 25° C), the time required to achieve the condition $C_{in} = 0.5C_{out}$ can be calculated as 250 ms. In view of the uncertainties involved, the result of this simple calculation may be considered to be in reasonable agreement with the value of 860 ms obtained by Crompton and Costi [26], suggesting the presence of only one, or very few, active channel(s) per mitochondrion. This conclusion would also be in agreement with our patch-clamp experiments, in which only one, or no, channel per patch is observed in the majority of cases. In those experiments each patch comprises a substantial fraction (20–40%) of the mitoplast membrane.

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