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Permeability of inner mitochondrial membrane and oxidative stress

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The mechanism of increase in the inner membrane permeability induced by Ca^{2+} plus P_i, diamide and hydroperoxides has been analyzed. (1) The permeability increase is antagonized by oligomycin and favoured by atractyloside. The promoting effect of atractyloside is strongly reduced if the mitochondria are simultaneously treated with oligomycin. (2) Addition of the free-radical scavenger, butylhydroxytoluene, results in a complete protection of the membrane with respect to the permeability increase. (3) Although membrane damage and depression of the GSH concentration are often associated, there is no direct correlation between extent of membrane damage and concentration of reduced glutathione. Abolition of the permeability increase by butylhydroxytoluene or by oligomycin is not accompanied by maintenance of a high GSH concentration in the presence of diamide or hydroperoxides. The membrane damage induced by Ca^{2+} plus P_i is not accompanied by a depression of the GSH concentration. (4) It is proposed that a variety of processes causing an increased permeability of the inner mitochondrial membrane merge into some ultimate common steps involving the action of oxygen radicals.

Introduction

A number of agents or conditions are known to increase the permeability of the mitochondrial inner membrane and cause swelling. In the early 1960s it was proposed that many of these agents were acting by inducing the release of fatty acids, also denoted as U factor (for review see Ref. 1). Also reagents interacting with the thiol groups were identified as potentially swelling agents. Attention was also focused on the role of inorganic phosphate [2–4].

In more recent times, attention has been shifted from the swelling process to that of Ca^{2+} release. A variety of compounds has been reported to induce Ca^{2+} release, among which are inorganic phosphate [5,6], sulphydryl reagents [7,9], atractylate [13], menadione [11], and pyridine nucleotide oxidizing agents [12–16]. However, the nature of the process of Ca^{2+} release is controversial. According to one point of view, Ca^{2+} release and swelling are closely related processes in that Ca^{2+} release follows increase of inner membrane permeability and collapse of membrane potential [16–17]. According to another point of view, Ca^{2+} release is not related to increase of inner membrane permeability but rather to activation of an H^+/Ca^{2+} exchange carrier [18,19].

Extensive work supporting a close correlation between Ca^{2+} release and increase of inner membrane permeability has been carried out particularly by the group of Pfeiffer [5,6,16,17]. The

Abbreviations: BuOOH, butylhydroperoxide; TMPD, tetramethyl *p*-phenylenediamide; GSH and GSSG, reduced and oxidized glutathione; BHT, butylhydroxytoluene.

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suggestion has been advanced that the permeability increase follows activation of a Ca^{2+}-dependent phospholipase A with formation of fatty acids and particularly of lysophosphatides. Pfeiffer has also suggested that the action of phospholipase A could be reversed by the mitochondrial acyl transferase after activation of polyunsaturated fatty acids by acyl-CoA synthase and intramitochondrial ATP. The generally observed protecting function of ATP would then be due to removal of fatty acids and lyso compounds and to resynthesis of phospholipids.

Due to the large variety of agents or conditions leading both to Ca^{2+} release from mitochondria and to increase of inner membrane permeability, the question arises, however, as to whether all these agents act by a similar mechanism. For example, while earlier work suggested a direct link between oxidation state of pyridine nucleotides and Ca^{2+} release [12], it has subsequently been shown that pyridine nucleotide oxidation does not always result in Ca^{2+} release [13]. On the other hand, the hydroperoxide-induced Ca^{2+} release has been suggested to involve the activity of the intramitochondrial glutathione reductase, as indicated by the finding that inhibitors of the mitochondrial glutathione reductase also cause Ca^{2+} release [20,21]. It has therefore been suggested that the release process is dependent on the GSSG/GSH ratio and not on the redox state of the pyridine nucleotides. It has also been further proposed that the GSSG/GSH ratio regulates the lysophospholipid acyl transferase activity by controlling the redox state of critical sulphydryl groups [21].

Generation of H_2O_2 and of oxygen radicals by mitochondria has been recognized to be a significant reaction both with respect to the amount of radicals generated and to the physiological role of the radicals [22]. There are indications of an oxidative stress in causing mitochondrial damage both in past [23–25] and in more recent [26] investigations. What is not known, however, is to what extent such a reaction also plays a role during all those processes generally known to result in changes of inner membrane permeability and Ca^{2+} efflux. Recently it has been reported that the SH-crosslinking reagent, phenylarsine oxide, induces an increase of inner membrane permeability by a mechanism which is inhibited by the free-radical scavenger, butylhydroxytoluene [27].

In the present study we will report that a large variety of agents and conditions, all leading to Ca^{2+} release and increase of inner membrane permeability, act by means of a mechanism which is sensitive to two kinds of inhibitor, namely butylhydroxytoluene and oligomycin, while it is apparently independent from the concentration of GSH. On the basis of the effects of these two inhibitors, we will propose that the process of Ca^{2+} release is essentially a consequence of an increase of the inner membrane permeability accompanied by decline of the membrane potential and that such a permeability increase apparently involves the formation of oxygen radicals. The view will be discussed that the processes leading to inner mitochondrial membrane damage act either by decreasing the mechanism involved in the removal of the oxygen radicals or by increasing the electron leak from the respiratory chain and then the formation of oxygen radicals. The combination of these factors has dramatic consequences for the permeability properties of the inner mitochondrial membrane. A preliminary report has already been presented [28].

Materials and Methods

Rat liver mitochondria were prepared in 0.25 M sucrose/10 mM Tris-HCl (pH 7.4)/0.1 mM EGTA, according to Massari et al. [29]. The last washing was carried out in an EGTA-free medium and the final resuspension was made in a bovine serum albumin supplemented medium. Mitochondrial protein was assayed with the biuret method, with bovine serum albumin as a standard. The incubation media are specified in the legends.

$\Delta\psi$ was determined [17] by monitoring the Ph_3MeP^+ concentration in an open thermostatically controlled and stirred vessel with a Ph_3MeP^+-sensitive electrode (final volume 2 ml, 30°C). Medium Ph_3MeP^+ concentrations were calculated from calibration plots of electrode output vs. log Ph_3MeP^+ determined for each individual experiment. The slope of the plot was always very close to 60 mV/decade.

The respiratory rate was measured from the decrease in oxygen concentration of the medium which was followed polarographically with a Clark electrode (Yellow Springs) in closed, thermostatically controlled vessel with magnetic stirring [30]. The Teflon membrane of the electrode was maximally stretched for fast electrode response. The half-time of electrode response after reduction of the oxygen present in the medium with an excess of $Na_2S_2O_4$ was about 3 s. Oxygen concentration was taken as 485 nmol O/ml at 25°C.

ATP concentration in the matrix was determined by a sampling technique [30]. Incubations or samples taken from incubations of phosphorylating mitochondria were quenched with perchloric acid at the desired times and the ATP content of the quenched samples was determined by enzymatic methods [31] after neutralization with triethanolamine-KOH.

The concentration of total free SH groups, mostly constituted by GSH, was determined on the same samples as used for the ATP determination. After quenching with perchloric acid and centrifugation, the samples were supplemented with EDTA and 5,5′-dithiobis(2-nitrobenzoic) acid up to the final concentrations of 0.1 μM and 0.05 mM, respectively. The concentration of the *p*-nitrothiophenol anion was then assayed spectrophotometrically at 412 nm.

Results

The combined effect of Ca^{2+}, hydroperoxides, diamide and phosphorylated compounds

Fig. 1 summarizes some of the effects which will be analyzed in detail in the subsequent figures. A large concentration of butylhydroperoxide such as 80 μM was unable to cause a pronounced $\Delta\psi$ decline when mitochondria were supplemented with EGTA and incubated in a medium devoid of exogenous P_i. The same peroxide concentration resulted in a slightly more marked $\Delta\psi$ decline when the P_i concentration was increased to 10 mM. Endogenous Ca^{2+} had a critical role, since omission of EGTA resulted in a large $\Delta\psi$ decline. Finally, it is seen that no decline of $\Delta\psi$ was observed when 50 μM Ca^{2+} was added to mitochondria supplemented with P_i, provided that oligomycin was also present in the medium.

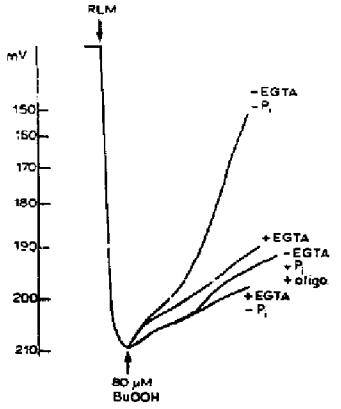

Fig. 1. Effect of EGTA and P_i on hydroperoxide-induced decline. The incubation medium contained 200 mM sucrose, 10 mM Tris-Mops (pH 7.4), 5 mM succinate-Tris, 2 μM rotenone, 1 mg/ml bovine serum albumin, 1 mg/ml mitochondria and 5 μM Ph_3MeP^+ at 25°C. The traces show the pattern of $\Delta\psi$. When indicated, 0.1 mM EGTA, 0.5 mM P_i and 2 μg oligomycin (oligo.).

The permeability increase caused by the hydroperoxide was quantitatively dependent on the Ca^{2+} concentration. The decline of $\Delta\psi$ was always biphasic with a first, rapid, phase related to the activation of the transhydrogenase reaction and a second, slow, phase accompanying the increase in membrane permeability. The extent of decline in $\Delta\psi$ was proportional to the amount of hydroperoxide. At low hydroperoxide concentrations the permeability increase after reaching a maximum tended to diminish. On the other hand, at the higher hydroperoxide concentration, no restoration of $\Delta\psi$ was observed.

Fig. 2 shows that the $\Delta\psi$ decline caused by the combined effect of Ca^{2+} plus hydroxyperoxides was counteracted by the presence of dithioerythritol. Partial protection was observed at 1 mM and complete protection at 3 mM dithioerythritol.

Oligomycin, atractyloside and ATP had a marked effect on the transmembrane potential of liver mitochondria following addition of 20 μM Ca^{2+}. The process of slow $\Delta\psi$ decline was markedly sensitive to ATP, oligomycin and atrac-

tyloside. Oligomycin abolished almost completely the spontaneous slow $\Delta\psi$ decline. Also, exogenous ATP inhibited the process of $\Delta\psi$ decline markedly and the effect of ATP was abolished by the subsequent addition of atractyloside. Furthermore oligomycin was capable of inhibiting the process of slow $\Delta\psi$ decline, even if added after the process had already been initiated or when the process followed the addition of atractyloside.

Both rates and extents of Ca^{2+}-dependent, hydroperoxide-induced $\Delta\psi$ decline, were strictly dependent on the P_i concentration in the range between 0.25 and 0.75 mM P_i.

In Fig. 3, 50 μM diamide was added to mitochondria which had been supplemented with 20 μM Ca^{2+}. Diamide initiated a slow process of decline, the rate and extent of which increased with the increase of the P_i concentration in the range between 0.25 and 0.75 mM P_i. Again as in the case of the hydroxyperoxide-induced $\Delta\psi$ decline, the diamide-induced $\Delta\psi$ decline was also

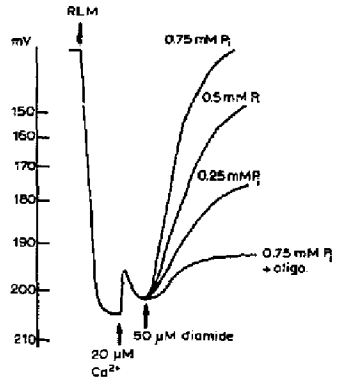

Fig. 3. Effects of oligomycin and P_i on the diamide-induced $\Delta\psi$ decline. The experimental conditions were as in Fig. 1. The traces indicate the Ph_3MeP^+ fluxes. 0.25, 0.5 or 0.75 mM P_i in the absence or presence of 2 μg oligomycin (oligo.) was added to the medium before the mitochondria.

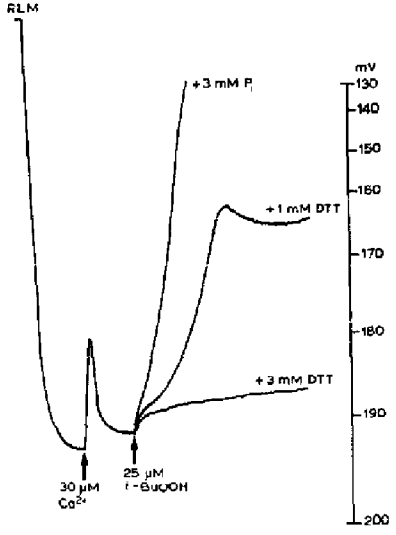

Fig. 2. Effect of dithioerythritol (DTT) on hydroperoxide-induced $\Delta\psi$ decline. The incubation medium was as in Fig. 1, but supplemented with 3 mM P_i; Dithioerythritol was added at the indicated concentrations.

completely abolished when oligomycin was present in the incubation medium.

Membrane damage during operation of the redox pump at site III

It is generally thought [22] that oxygen radicals may be formed by univalent oxygen reduction at the level of the redox pumps both at site 1 and at site 2 of the respiratory chain. Little is known, on the other hand, about the generation of oxygen radicals at the level of site 3, the cytochrome oxidase proton pump. Fig. 4 shows that addition of very low Ca^{2+} concentrations, such as 15 μM, was already capable of inducing a large decline of $\Delta\psi$ if the mitochondria were treated with rotenone and antimycin and supplemented with ascorbate and 60 μM TMPD to leave only the proton pump at site 3 in operation. Since under the conditions of the experiment of Fig. 4 an amount of at least 70 μM Ca^{2+} was needed to cause membrane damage if the mitochondria were oxidizing succinate, it appears that operation of the proton pump at site 3 creates conditions whereby either mitochondria are more susceptible to free-radical damage or there is a faster rate of free-radical generation. Fig. 4 also shows that rate and extent of $\Delta\psi$ decline increased with the increase of the

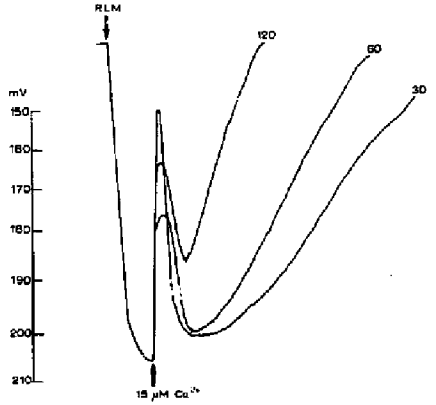

Fig. 4. Effect of TMPD concentration in presence of Ca^{2+} on the $\Delta\psi$ decline. The incubation medium was as in Fig. 1; 3 mM P_i, 1 mM ascorbate and 0.2 μg antimycin were added to the medium before the mitochondria. The concentrations of TMPD were 30, 60 and 120 μM.

TMPD concentration, although the decline always remained sensitive to the protective effect of butylhydroxytoluene. The dependence of the membrane damage on the TMPD concentration may be taken as an indication that the rate of oxygen radical formation is proportional to the rate of operation of the site 3 proton pump. It is to be emphasised that the above conclusion is valid only for the process activated by Ca^{2+} plus P_i and not, for example, for the process activated by hydroperoxides. In fact, when the experiment shown in Fig. 4 was repeated in the presence of hydroperoxides, no dependence of the process on the TMPD concentration was observed. The different sensitivity to the TMPD concentration of the Ca^{2+} plus P_i- and of the peroxide-activated damage indicates that the nature, at least of the initial steps, of the processes leading to the permeability increase is different for the two agents. This point will be supported by additional evidence in forthcoming papers.

The effect of free-radical scavengers

In view of the reported effect of butylhydroxytoluene on the membrane damage induced by SH reagents [27], we have tested the effect of free-radical scavengers on a variety of agents or conditions, all leading to an increase in the inner membrane permeability. Fig. 5 shows the effect of butylhydroxytoluene on the process of $\Delta\psi$ decline induced by hydroperoxides, by diamide and by Ca^{2+} and P_i, with mitochondria either oxidizing succinate or TMPD. It is seen that, under the selected experimental conditions, there was a large $\Delta\psi$ decline in the presence of either substrate. Addition of butylhydroxytoluene at a concentration of 50 μM induced a complete protection against all processes of $\Delta\psi$ decline. Fig. 5 thus shows that, whatever the nature of the agent initiating the process and whatever the substrate, the presence of butylhydroxytoluene insured the maintenance of a low permeability of the inner mitochondrial membrane. The higher sensitivity of the TMPD-supplemented mitochondria means that a larger permeability increase is observed at lower concentrations of damaging agents. However, when the damaging process is too extensive, the protection by butylhydroxytoluene may not be complete. Thus, to compare the protecting effect of butylhydroxytoluene it is necessary to adjust the concentrations of Ca^{2+} plus P_i, diamide and peroxides in order to produce comparable rates of permeability increase.

Fig. 6 shows a titration with butylhydroxytoluene as carried out with mitochondria which had been treated with Ca^{2+} and hydroperoxides. It is seen that a concentration of butylhydroxytoluene as low as 10 μM caused a large inhibition of the respiratory stimulation. An effect of butylhydroxytoluene was observed also when the respiratory stimulation was induced by Ca^{2+} plus diamide or by Ca^{2+} plus phosphate. A number of other agents capable of acting as free-radical scavenger were also tested. A more detailed account of the effects of the different classes of scavengers will be reported in a subsequent paper.

Comparison of the effects of oligomycin and butylhydroxytoluene on the membrane damage

In Figs. 7–9 we have carried out a comparison of the effects of oligomycin and butylhydroxytoluene on the membrane damage induced by peroxides, diamide and Ca^{2+} plus phosphate. Fig. 7 shows the pattern of the respiratory stimulation obtained at increasing peroxide concentrations but

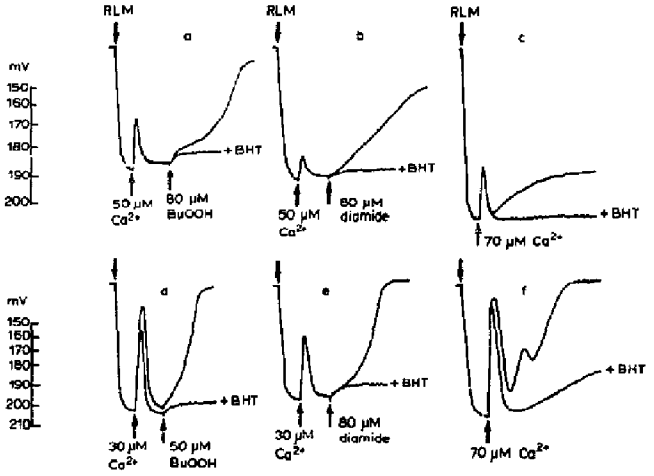

Fig. 5. Protection by butylhydroxytoluene (BHT) of hydroperoxide-, diamide-, and Ca^{2+} plus P_i-induced $\Delta\psi$ decline in mitochondria oxidizing succinate or TMPD. Experimental conditions as in Fig. 1 except that P_i was 0.5 mM in a, b, d and e, and 3 mM in c and f. Substrate was succinate in a, b, c, and 60 μM TMPD, 1 mM ascorbate in the presence of 0.2 μg antimycin in d, e, f. When indicated, 50 μM butylhydroxytoluene.

in the presence of a constant concentration of Ca^{2+} and phosphate. It is seen that butylhydroxytoluene was able to prevent the respiratory stimulation. The prevention was complete below 100 μM peroxides, while above this concentration the damaging effect of the peroxides was still slightly present. This may indicate that, at the higher peroxide concentration, radicals may be produced at a such high rate that they cannot be removed completely by the scavengers. When, on the other hand, the increase in respiratory stimulation was caused by an increase of the phosphate concentration, at constant peroxide concentration ,there was, at the high P_i concentrations, complete protection by 50 μM butylhydroxytoluene but not by oligomycin. Fig. 8 shows a similar experiment conducted in the presence of increasing diamide concentrations or of increasing phosphate concentrations but at constant diamide concentrations. It is seen that butylhydroxytoluene was able to abolish the respiratory stimulation completely in both titrations. On the other hand, again, as in Fig. 7, oligomycin exerted a smaller protection at the higher phosphate concentrations. Finally Fig. 9 shows that also the respiratory stimulation induced by increasing Ca^{2+} concentrations in the presence of a constant phosphate concentration was completely abolished by butylhydroxytoluene. In conclusion, the titrations of Figs. 7–9 indicate that, except at the higher phosphate concentrations, a fundamental parallelism exists in the ef-

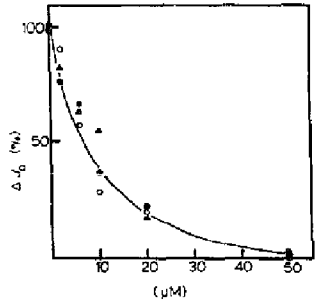

Fig. 6. Effect on respiratory rate of increasing butylhydroxytoluene concentrations. Experimental conditions as in Fig. 1. ΔJ_0 is the difference in respiratory rate, at 5 min, with or without 100 μM tBuOOH (○), 150 diamide (●), 70 μM Ca (▲).

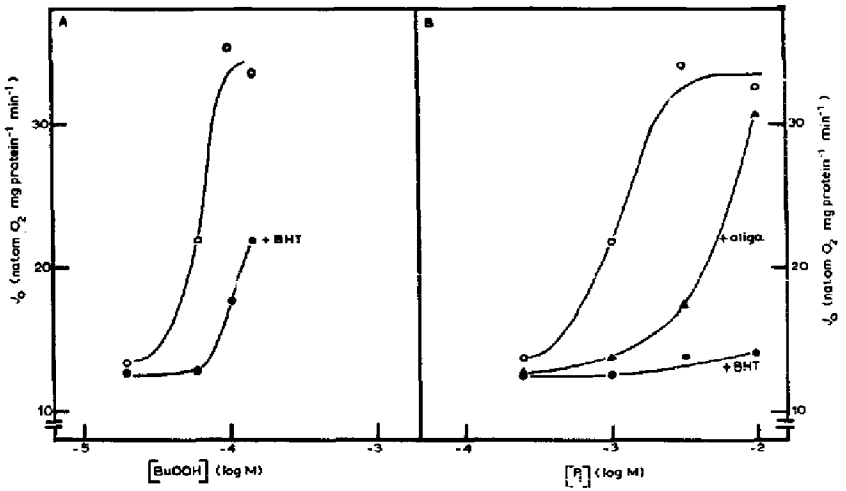

Fig. 7. Effect of oligomycin and butylhydroxytoluene (BHT) on the hydroperoxide-induced stimulation of the respiratory rate at increasing hydroperoxide and P_i concentrations. The incubation medium was as in Fig. 1 except that sucrose was replaced by NaCl. In A, mitochondria were supplemented with 20 μM Ca^{2+}, 1 mM P_i and increasing hydroperoxide concentrations in the absence or presence of 50 μM butylhydroxytoluene. In B, mitochondria were supplemented with 20 μM Ca^{2+}, 60 μM peroxide and increasing P_i concentrations in the absence or presence of 2 μg of oligomycin or 50 μM butylhydroxytoluene.

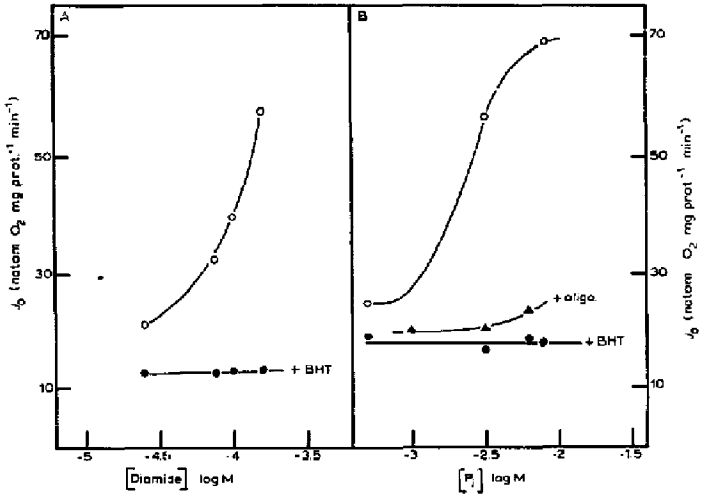

Fig. 8. Effect of oligomycin and butylhydroxytoluene (BHT) on the diamide-induced stimulation of the respiratory rate at increasing diamide and P_i concentrations. Experimental conditions were as in Fig. 7. In A, mitochondria were supplemented with 20 μM Ca^{2+}, 1 mM P_i and increasing diamide concentrations in the absence or presence of 50 μM butylhydroxytoluene. In B, mitochondria were supplemented with 20 μM Ca^{2+}, 75 μM diamide and increasing P_i concentrations in the absence or presence of 2 μg oligomycin or 50 μM butylhydroxytoluene.

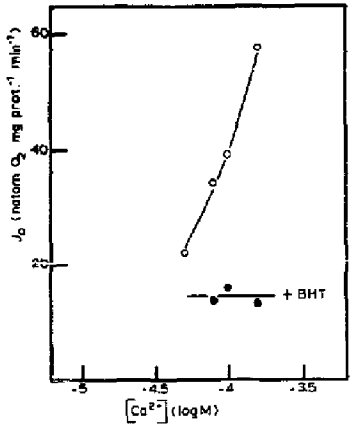

Fig. 9. Effect of butylhydroxytoluene on the Ca^{2+} plus P_i-induced stimulation of the respiration rate. Experimental conditions were as in Fig. 7. Mitochondria were supplemented with 5 mM P_i and increasing Ca^{2+} concentration in the absence or presence of 50 μM butylhydroxytoluene.

fects of the two compounds, i.e., the ATP synthesis inhibitor oligomycin causes protection almost to the same extent as the free-radical scavenger, butylhydroxytoluene.

Reduced glutathione and membrane permeability

It has been proposed that the GSSG/GSH ratio, by controlling the state of critical sulphydryl groups, also regulates the ability of mitochondria to remain impermeable upon activation of phospholipase A_2 [20]. Table I shows the effect of butylhydroxytoluene and oligomycin on the level of GSH under conditions where addition of Ca^{2+} plus P_i, of diamide and hydroperoxides caused increase of the inner membrane permeability. From Table I a close correlation appears to exist between the respiratory rate and the intramitochondrial ATP level. Except in the presence of oligomycin, all conditions leading to a high respiratory rate also resulted in a low intramitochondrial ATP concentration. The decline of the ATP level took place after some minutes, indicating that it was presumably the consequence and not the cause of the increased permeability of the inner membrane, presumably due to a decline of the membrane potential to reach a $\Delta\tilde{\mu}_H$ level where the ATPase proton pump is shifted from the synthetic to the hydrolytic mode. As could be predicted, the ATP concentration was always low in the presence of oligomycin, independent of whether the respiratory rate was low or high.

TABLE I

RELATIONSHIP BETWEEN RESPIRATORY RATE, ATP AND GSH CONCENTRATIONS

The incubation medium was as in Fig. 1. Expt. 1: 2 mg/ml mitochondria, 100 μM butylhydroxytoluene, 8 μg oligomycin, 150 μM Ca^{2+}, 5 mM P_i, 150 μM diamide in presence of 40 μM Ca^{2+} and 1 mM P_i. Expt. 2: 4 mg/ml mitochondria, 200 μM butylhydroxytoluene (BHT), 120 μM Ca^{2+}, 2 mM P_i, 300 μM BuOOH. The determinations of ATP and of GSH were carried out on aliquots taken, at the indicated times, from the same incubation.

Additions	J_o (natom·mg^{-1}·min^{-1})	ATP (nmol·mg^{-1})			GSH (nmol·mg^{-1})		
		3 min	6 min	9 min	3 min	6 min	9 min
Expt. 1							
None	8.2	5.1	5.1	5.6	8.7	9.4	9.8
Ca^{2+} + P_i	46.6	7.6	2.2	2.6	9.8	9.4	9.0
Ca^{2+} + P_i + BHT	8.2	5.5	5.4	5.7	8.7	8.6	8.7
Ca^{2+} + P_i + oligomycin	10.2	2.1	2.2	2.2	9.4	–	9.4
Ca^{2+} + diamide	23.0	5.8	2.6	2.0	6.2	6.3	6.0
Ca^{2+} + diamide + BHT	4.8	7.0	5.9	5.9	6.2	6.3	6.2
Ca^{2+} + diamide + oligomycin	12.4	2.1	1.6	1.4	6.2	–	6.2
Expt. 2							
None	–	6.2	5.9	6.1	7.2	7.2	7.2
Ca^{2+} + BuOOH	–	11.4	3.9	2.1	4.3	4.4	4.3
Ca^{2+} + BuOOH + BHT	–	9.2	8.5	9.2	4.3	4.0	4.0

Table I also shows that diamide and hydroperoxides caused a 30% decrease of the GSH concentration, while Ca^{2+} plus P_i left the GSH concentration unaltered. Most striking was the lack of correlation between the extent of membrane damage, as indicated by the ATP concentration and the respiratory rate, on one side and the GSH concentration on the other. First the increase of respiratory rate caused by Ca^{2+} plus P_i was not accompanied by a decline (in some cases even by a small increase) of the GSH concentration. Second, the marked effects of oligomycin and butylhydroxytoluene on the respiratory rate were not accompanied by significant changes in the GSH concentration, i.e., oligomycin and butylhydroxytoluene abolished the effects of peroxides and diamide on the membrane permeability, but not those on the GSH concentration. This suggests that the decline of the GSH concentration is not an essential feature of the process and that occurring in the presence of diamide and peroxides is due to the specific metabolic effects of these two compounds on the SH groups rather than an expression of the role of these groups on the inner membrane permeability.

Discussion

According to Pfeiffer et al. [20] the increase of inner membrane permeability produced by the agents which increase the oxidation of pyridine nucleotides or inhibit the glutathione reductase is essentially due to interference with the reduction state of some critical SH groups which control the lysophospholipid acyl transferase activity.

The present study reports three major observations. The first is that the increase of inner membrane permeability is promoted or antagonized by compounds which facilitate or inhibit, respectively, the accumulation of phosphorylated high energy compounds. Oligomycin, which inhibits (i) the P_i–ATP and the P_i–oxygen exchange and (ii) the synthesis and hydrolysis of ATP [32], antagonizes the increase of inner membrane permeability (cf. also Refs. 3 and 4). Atractyloside, which inhibits the AdN translocator [33,34], thereby favouring accumulation of ATP in the matrix, promotes the increase of inner membrane permeability. The atractyloside-induced permeability increase is strongly reduced if the mitochondria are simultaneously treated with oligomycin. This indicates that the promoting effect of atractyloside requires the endogenous synthesis of ATP or of phosphorylated high-energy compounds. The effect of oligomycin and atractyloside is observed also in the presence of Ca^{2+} and SH reagents, hydroperoxides, etc. It would therefore seem that accumulation of ATP, or of some phosphorylated compounds, within mitochondria promotes the permeability increase, while depletion of ATP antagonizes it. Such an effect of ATP is clearly in contrast with the view that ATP is needed to form an acyl-CoA compound subsequently utilized by the acyltransferase activity to remove the lysophospholipids.

The second observation concerns the behaviour of the SH compounds during the inner membrane permeability increase (cf. also Refs. 5, 8, 21, 35, 36). We find no relationships between the level of reduced glutathione and the extent of respiratory stimulation. On one side, in the presence of Ca^{2+} plus phosphate, there is a marked respiratory stimulation without an apparent decrease of the GSH concentration. On the other side, in the presence of diamide or peroxides, there is a decrease of GSH concentration which is not related to the permeability increase of the inner membrane. In fact, abolition of the permeability increase by butylhydroxytoluene or oligomycin does not require restoration or maintenance of the normal GSH concentration. Thus, in the presence of butylhydroxytoluene or oligomycin a low GSH concentration is not accompanied by a significant permeability increase. This observation is in contrast not only to the proposal that some critical SH groups control the activity of the lysophospholipid acyl transferase but also with other proposals attributing to the GSH concentration a critical role in controlling the permeability of the inner membrane. Since addition of peroxides or of diamide also induces a marked depression of the steady-state concentration of nicotinamide adenine nucleotides, the same conclusion applies also to the hypotheses that these compounds control the inner membrane permeability.

The third and perhaps more important observation is the protection by butylhydroxytoluene of the permeability increase induced by Ca^{2+} plus

phosphate, by diamide and by peroxides. An inhibitory effect of butylhydroxytoluene on the increase of inner membrane permeability by phenylarsine oxide has already been reported [27]. Hunter and co-workers [23–25] reported in the early 1960s that the swelling process induced by ascorbate, glutathione and iron was accompanied by the formation of peroxidized lipids and was inhibited by α-tocopherol. The group of Orrhenius [26] reported that the Ca^{2+} release induced by menadione was accompanied by the reduction of cytochrome *c* and inhibited by the addition of superoxide dismutase. We have shown that the protective effect by butylhydroxytoluene acts on the increase of permeability induced by different agents. Since butylhydroxytoluene is a well-known free radical scavenger, its protective effect suggests that presumably several so-called swelling agents act by inducing the formation of oxygen radicals. Thus, the common denominator of a variety of inner membrane damaging processes may be constituted by the oxygen radicals. The more marked membrane damage during TMPD oxidation than during succinate oxidation may be due either to the presence of antimycin, which may cause a higher rate of radical production in view of the higher extent of ubiquinone reduction, or to a direct production of radicals in the ascorbate-TMPD system.

Several questions arise. The first concerns the pathways through which the various damaging processes merge into the final steps involving Ca^{2+} and the oxygen radicals. A tentative scheme is indicated in Fig. 10. It is suggested that the increase in membrane permeability is due to an oxygen radical attack of the lipids and proteins of the membrane. The consequences of such an attack is the creation of both specific (through the ATPase) and unspecific water channels with relative increases of the membrane permeability. There is now increasing consensus on the concept that a univalent electron leak is a common event at the level of sites 1 and 2 of the respiratory chain. This explains the presence in the mitochondrial matrix of the superoxide dismutase which disposes of the superoxide anion formed during the univalent reduction of oxygen. The H_2O_2 formed by the superoxide dismutase reaction is then metabolized in the matrix by the glutathione peroxidase by means of GSH utilization. The concentration of oxygen radicals in the mitochondria in the stationary state therefore depends on one side on the rate of the electron leak causing the univalent oxygen reduction and on the other on the rate of the glutathione-dependent reactions causing the elimination of the oxygen radicals and avoiding the initiation of the free-radical reaction chain.

We propose that peroxides and diamide act by decreasing the rate of removal of the oxygen radicals, either directly or indirectly by changing the concentration of the reducing SH groups, while the Ca^{2+} plus P_i act by increasing the rate of formation of the oxygen radicals via an enhancement of the electron leak. In either case the final result is an increase of the steady-state level of oxygen radicals. The scheme of Fig. 10 would thus explain the crucial role of Ca^{2+} and P_i as supported by the observations that all the processes studied here and always protected by butylhydroxytoluene depend on the presence of a minimal Ca^{2+} and P_i concentration. The increase of electron leak may be a consequence of a Ca^{2+} plus P_i-induced activation of endogenous phospholipases as envisaged by Pfeiffer et al. [5,6,16,17]. In the scheme of Fig. 10 the GSH concentration affects the oxygen radicals' steady-state level but it does not control directly the inner membrane permeability. The scheme of Fig. 10 explains also the protective effect of dithioerythritol, which affects the level of the oxygen radicals by maintaining reduced different types of SH group. On the other hand, the more extensive damage in the presence of ascorbate and TMPD is attributed to a higher rate of production of radicals in the presence of these substrates.

A final question concerns the mechanism by which P_i and phosphorylated compounds enhance

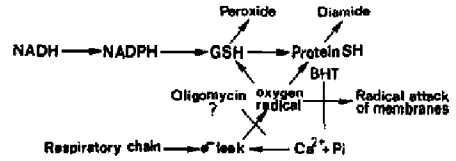

Fig. 10. A scheme for the oxidative stress in mitochondria. The damaging effect of hydroperoxides, diamide and Ca^{2+} plus P_i and the protection by oligomycin, butylhydroxytoluene and dithioerythritol.

a process of membrane damage essentially based on the formation of oxygen radicals. The proposal has already been made [27] that the role of P_i is that of facilitating the action of hydrophilic SH reagents, i.e., rendering accessible to these reagents hydrophobic membrane sites. The inhibitory effect of oligomycin, however, suggests that the effect of P_i is somehow related to the intramitochondrial ATP level in that a high matrix concentration of ATP promotes the electron leak from the respiratory chain and then the initiation of the free radical reaction chain.

References

1 Chappell, J.B. and Greville, G.D. (1963) Biochem. Soc. Symp. 23, 23–39.
2 Lehninger, A.L., Carafoli, E. and Rossi, C.S. (1976) Adv. Enzymol. 29, 259–320.
3 Azzi, A. and Azzone, G.F. (1965) Biochim. Biophys. Acta 105, 253–264.
4 Azzi, A. and Azzone, G.F. (1966) Biochim. Biophys. Acta 113, 438–444.
5 Beatrice, M.C., Palmer, J.W. and Pfeiffer, D.R. (1980) J. Biol. Chem. 255, 8663–8671.
6 Palmer, J.W. and Pfeiffer, D.R. (1981) J. Biol. Chem. 256, 6742–6750.
7 Pfeiffer, D.R., Kaufman, R.F. and Lardy, H.A. (1978) J. Biol. Chem. 253, 4165–4171.
8 Siliprandi, D., Rugolo, M., Zaccarato, F., Toninello, A. and Siliprandi, N. (1979) Biochem. Biophys. Res. Commun. 88, 388–394.
9 Pozzan, M., Bernardi, P. and Di Virgilio, F. (1981) FEBS Lett. 127, 263–266.
10 Asimakis, G.K. and Sordahl, L.A. (1977) Arch. Biochem. Biophys. 179, 200–210.
11 Bellomo, G., Jewell, S.A. and Orrhenius, S. (1982) J. Biol. Chem. 257, 11558–11562.
12 Lehninger, A.L., Vercesi, A. and Bababunmi, E.A. (1978) Proc. Natl. Acad. Sci. USA 75, 1690–1694.
13 Nicholls, D.G. and Brand, M. (1980) Biochem. J. 188, 113–118.
14 Lotscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1980) J. Biol. Chem. 255, 9325–9330.
15 Lotscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1980) Eur. J. Biochem. 110, 211–216.
16 Beatrice, M.C., Stiers, D.L. and Pfeiffer, D.R. (1982) J. Biol. Chem. 257, 7161–7171.
17 Rizzuto, R., Pitton, G. and Azzone, G.F. (1987) Eur. J. Biochem. 162, 239–249.
18 Baumhuter, S. and Richter, C. (1982) FEBS Lett. 148, 271–275.
19 Richter, C. and Frei, B. (1985) in Oxidative Stress (Sies, H., ed.), pp. 221–241, Academic Press, New York
20 Beatrice, M.C., Stiers, D.L. and Pfeiffer, D.R. (1984) J. Biol. Chem. 259, 1279–1287.
21 Pfeiffer, D.R., Schmid, P.C., Beatrice, M.C. and Schmid, H.O. (1979) J. Biol. Chem. 254, 11485–11494.
22 Forman, H.J. and Boveris, A. (1982) in Free Radicals in Biology, Vol. V, pp. 65–90, Academic Press, New York.
23 Hunter, F.E., Gebicki, J.M., Hoffsten, P.E., Weinstein, J. and Scott, A. (1963) J. Biol. Chem. 238, 828–835.
24 Hunter, F.E., Scott, A., Hoffsten, P.E., Guerra, F., Weinstein, J., Schneider, A., Schutz, B., Fink, J., Ford, L. and Smith, E. (1964) J. Biol. Chem. 239, 604–613.
25 Hunter, F.E., Scott, A., Hoffsten, P.E., Gebicki, J.M., Weinstein, J. and Schneider, A. (1964) J. Biol. Chem. 239, 614–621.
26 Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A. and Orrhenius, S. (1982) J. Biol. Chem. 257, 12419–12425.
27 Novgorod, S.A., Kultayeva, E.V., Yaguzhinsky, L.S. and Lemeshko, V.V. (1987) J. Bioener. Biomembr. 19, 191–202.
28 Carbonera, D. and Azzone, G.F. (1988) 5th European Bioenergetic Conference, Aberystwyth.
29 Massari, S., Balboni, E. and Azzone, G.F. (1973) Biochim. Biophys. Acta 283, 16–22.
30 Zoratti, M., Pietrobon, D. and Azzone, G.F. (1982) Eur. J. Biochem. 126, 443–451.
31 Estabrook, R.W., Williamson, J.R., Frenkel, R. and Maitra, P.K. (1967) Methods Enzymol. 10, 474–482
32 Lardy, H.A., Connelly, J.L. and Johnson, D. (1964) Biochemistry 3, 1961–1968.
33 Bruni, A., Contessa, A.R. and Luciani, S. (1962) Biochim. Biophys. Acta 60, 301–311.
34 Klingenberg, M. (1976) in The Enzymes of Biological Membranes, Vol. 3 (Martonosi, A., ed.), pp. 383–438, Plenum, New York.
35 Pfeiffer, D.R., Kouffman, R.F. and Lardy, H.A. (1978) J. Biol. Chem. 253, 4165–4171.
36 Pozzan, M., Bernardi, P. and Di Virgilio, F. (1981) FEBS Lett. 127, 263–266.