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Inhibition of Rat Liver Mitochondrial Permeability Transition by Respiratory Substrates

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The mitochondrial inner membrane can undergo a permeability increase known as "permeability transition" elicited by Ca^{2+} and several other inducing agents. In general, the condition of oxidative stress acts as an inducer, at variance with antioxidants and reducing agents that inhibit the permeability transition. The action of mitochondrial respiratory substrates in preventing the permeability transition induced by Ca^{2+} and phosphate was examined; pyruvate, isocitrate, and glutamate proved to be particularly effective. The effect of substrates was evident also in the presence of an uncoupler, and, in addition, they were able to counteract the swelling stimulated by acetoacetate and *tert*-butylhydroperoxide. In the presence of various pyridine nucleotide-dependent substrates, mitochondria are able to reduce the disulfide 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to an extent far larger than that calculated from the theoretical amount of total mitochondrial thiol groups, indicating the occurrence of a catalytic system. Similarly, the enzymes of the mitochondrial matrix in the presence of either NADH or NADPH are able to reduce DTNB. The results are discussed considering the existence of a close redox communication between pyridine nucleotides and membrane thiol groups, possibly mediated by dithiols such as thioredoxin and lipoic acid.

Key Words: **liver mitochondria; permeability transition; pyridine nucleotides; respiratory substrates; sulfhydryl groups.**

The increase of mitochondrial inner membrane permeability, also known as "permeability transition," is possibly dependent on the opening of an unselective pore and can be elicited, in isolated mitochondria, by Ca^{2+} accumulation and several inducing agents apparently unrelated from the chemical or functional point of view (1). Oxidative stress or, in general, oxidizing conditions appear to act as inducers of the permeability transition. Peroxides, free radical producing agents, and sulfhydryl group reagents are among the inducers (1, 2). Free radical species might be involved possibly through the oxidation of specific SH groups of the inner membrane (3–8). The permeability transition is inhibited by a specific inhibitor, the immunosoppressive cyclic peptide cyclosporin A, and is also inhibited, or decreased, by several agents or conditions such as free radical scavengers and antioxidants (2, 9–11), the reduced state of pyridine nucleotides (12), and sulfhydryl reducing agents (13–16).

We have examined the effect of some mitochondrial substrates in preventing the permeability transition. Their action seems to be mediated by the presence of some essential protein thiol groups, in line with previous observations (3, 8, 17–19), indicating that membrane sulfhydryl groups play a critical role in maintaining membrane integrity.

MATERIALS AND METHODS

Rat liver mitochondria were isolated with differential centrifugation essentially as described by Myers and Slater (20), using a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes[2] (pH 7.0), and 0.5 mg/ml of bovine serum albumin. For the preparation of the mitochondrial matrix, mitochondria were frozen and thawed twice and centrifuged for 1 h at 100,000*g*. The pellet (submitochondrial particles) was discarded and the supernatant was extensively dialyzed against 20 mM Tris buffer (pH 7.4). Proteins were measured

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[2] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); CCP, carbonyl cyanide-*m*-chlorophenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

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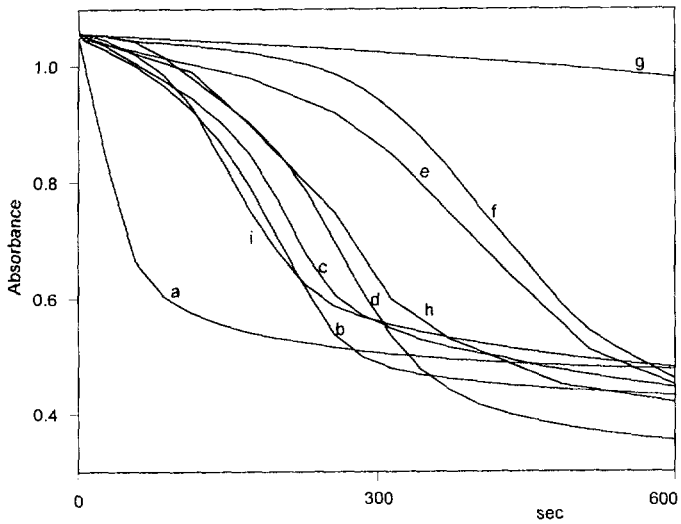


FIG. 1. Effect of respiratory substrates on mitochondrial swelling induced by permeability transition. Rat liver mitochondria (0.25 mg/ml) were incubated at 25°C in 213 mM mannitol/71 mM sucrose, 5 mM Hepes/Tris (pH 7.4), 5 mM succinate, 3 μg rotenone, 4 μg oligomycin, 13 μM EGTA, and the indicated substrates (5 mM) in a final volume of 1.5 ml. Swelling was triggered by the addition of 1 mM phosphate, followed, after 1 min, by 40 μM $CaCl_2$. (a) Control without rotenone; (b) control; (c) L-malate; (d) α-ketoglutarate; (e) pyruvate; (f) glutamate; (g) isocitrate; (h) β-hydroxybutyrate; (i) D-malate.

with the biuret test (21). Mitochondrial swelling was estimated spectrophotometrically by following the decrease of absorbance at 540 nm (22). The reduction of DTNB was followed at 412 nm (23). Reduction of pyridine nucleotide fluorescence was followed as fluorescence increase measured as described by Ernster and Lee (24). Other experimental conditions are described under the appropriate legends.

RESULTS

Rat liver mitochondria incubated in sucrose–mannitol medium and in the presence of succinate as substrate undergo a rapid swelling, indicating the occurrence of a permeability transition, upon addition of Ca^{2+}/P_i. If rotenone is added, the large-amplitude swelling is slowed down, particularly in the first minutes (Fig. 1). Further addition of pyridine nucleotide-dependent substrates strongly prevents the rate and extent of swelling. Isocitrate, glutamate, and pyruvate are particularly effective in protecting mitochondria from swelling. L-Malate exhibits a modest protective effect, while the corresponding D-form is completely ineffective. When the incubation medium contains KCl or NaCl instead of sucrose–mannitol, the protective effect of the various substrates is still evident (not shown). The effect of the various substrates is apparent also in the presence of the uncoupler carbonyl cyanide-*m*-chlorophenylhydrazone (not shown), indicating that energizing conditions are not strictly necessary for the development of this protective effect, even though the presence of the uncoupler markedly stimulates permeability transition (25).

Acetoacetate, when added to respiring mitochondria, brings the pyridine nucleotides to a more oxidized state, leading to a release of the accumulated Ca^{2+} (26). Similarly, *tert*-butylhydroperoxide through the action of glutathione/glutathione peroxidase is able to oxidize pyridine nucleotides (27). As reported in Figs. 2A and 2B, in the presence of Ca^{2+} alone there is a slight swelling, which is strongly increased by the addition of acetoacetate (Fig. 2A (c)) or *tert*-butylhydroperoxide (Fig. 2B (c')). In all of these experiments we avoided adding phosphate, which stimulates the extent of swelling (18). The presence of pyruvate or glutamate appears to prevent the swelling, particularly that induced by acetoacetate; the protective effect is consequently linked to a shift toward a more reduced ratio of the pyridine nucleotides. We therefore tested the rate and

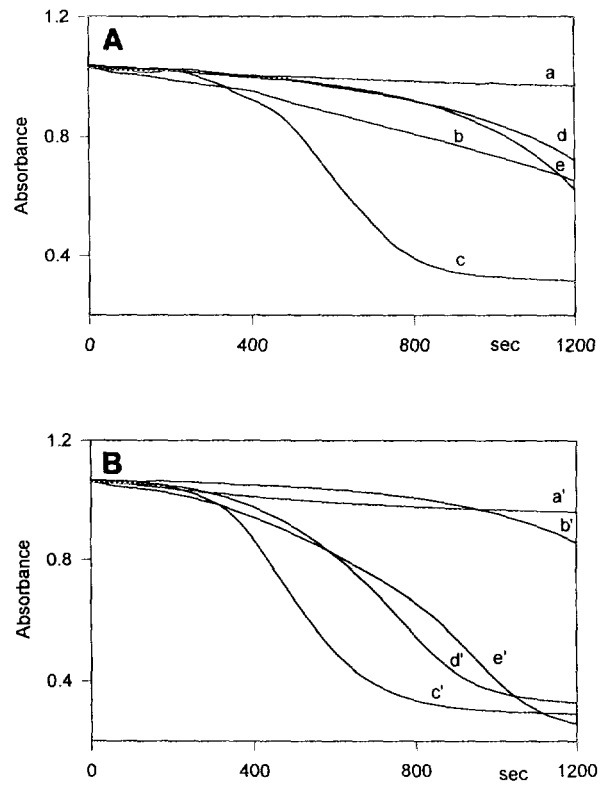
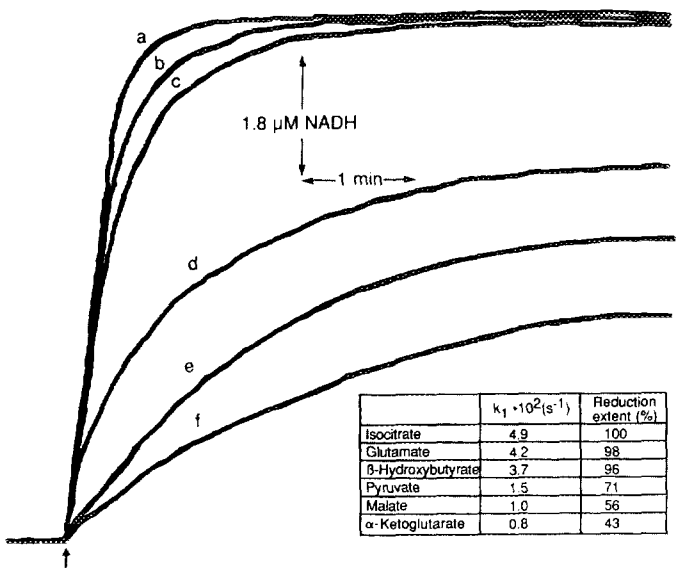


FIG. 2. Effect of pyruvate and glutamate on Ca^{2+}-stimulated swelling in the presence of acetoacetate (A) and *tert*-butylhydroperoxide (B). Rat liver mitochondria (0.25 mg/ml) were incubated at 25°C in 213 mM mannitol/71 mM sucrose, 5 mM Hepes/Tris (pH 7.4), 5 mM succinate, 2 μg/ml rotenone, 3 μg/ml oligomycin, 13 μM EGTA, and, when indicated, 5 mM pyruvate, 5 mM glutamate, 1 mM acetoacetate, and 33 μM *tert*-butylhydroperoxide. In all the experiments (except a and a') swelling was initiated by the addition of 40 μM $CaCl_2$. (a) Control without Ca^{2+}; (b) control; (c) acetoacetate; (d) acetoacetate + pyruvate; (e) acetoacetate + glutamate; (a') control without Ca^{2+}; (b') control; (c') *tert*-butylhydroperoxide; (d') *tert*-butylhydroperoxide + pyruvate; (e') *tert*-butylhydroperoxide + glutamate.



	$k_1 \cdot 10^2 (s^{-1})$	Reduction extent (%)
Isocitrate	4.9	100
Glutamate	4.2	98
β-Hydroxybutyrate	3.7	96
Pyruvate	1.5	71
Malate	1.0	56
α-Ketoglutarate	0.8	43

FIG. 3. Extent of reduction of NAD(P)H in the presence of various pyridine nucleotides-dependent substrates. Rat liver mitochondria (0.66 mg/ml) were incubated in 213 mM mannitol/71 mM sucrose, 1 mM EGTA buffered with 5 mM Hepes/Tris (pH 7.4). After 1 min incubation at 25°C, 0.2 μM CCP was added and, after 6 min, 2 μg/ml rotenone was added. The various pyridine nucleotide-dependent substrates (5 mM) were added 2 min after the addition of rotenone (arrow). The pyridine nucleotides reduction was followed fluorometrically (Ex, 366 nm; Em, 450 nm). The reduction of $NAD(P)^+$ appears to follow a first order kinetic and the relative velocity constants in the presence of the various substrates were obtained from a semilogarithmic plot of the difference between the final fluorescence (F_∞) and the fluorescence at various times (F_t) normalized by F_∞, i.e., $F_\infty - F_t/F_\infty$ vs time. (a) Isocitrate; (b) glutamate; (c) β-hydroxybutyrate; (d) pyruvate; (e) malate; (f) α-ketoglutarate.

extent of reduction of the pyridine nucleotides elicited by the various $NAD(P)^+$-dependent substrates. As can be seen in Fig. 3, in the presence of an uncoupler and rotenone a rapid and large reduction of the pyridine nucleotides is obtained with the substrates isocitrate, glutamate, and β-hydroxybutyrate, while pyruvate, malate, and α-ketoglutarate seem to be less efficient as reducing substrates. Glutamate and isocitrate, on the other hand, show a marked protective effect against swelling induced by Ca^{2+}/P_i (Fig. 1). It should be noted that mitochondrial glutamate and isocitrate dehydrogenase utilize $NADP^+$ in addition to NAD^+ for the oxidation of the corresponding substrates. In the inset of Fig. 3 the rate constants for the reduction of the pyridine nucleotides are also reported. A preventive effect on Ca^{2+} release, swelling, and NAD(P)H oxidation by pyridine nucleotide-dependent substrates was already observed by Moore *et al.* (28).

As reported in Figs. 4A and 4B rat liver mitochondria, in the presence of mitochondrial substrates, are able to reduced the disulfide DTNB. Pyruvate, glutamate, and α-ketoglutarate are particularly effective in reducing the disulfide. The reduction is more evident when phosphate is present, probably because it renders the mitochondrion sufficiently permeable to DTNB. The reduction of DTNB is also slightly stimulated by the presence of rotenone (Fig. 4B).

The supernatant fraction obtained from rat liver mitochondria previously frozen and thawed (mitochondrial matrix) is able to reduce DTNB in the presence of either NADPH or NADH; NADPH is more efficient than NADH (Figs. 5A and 5B). In order to rule out the participation of liberated or contaminating iron ions, the NAD(P)H-dependent reduction of DTNB was tested, in addition to EDTA, also in the presence of other chelators (diethylenetriaminepentaacetic acid and desferrioxamine) and insignificant differences were observed (not shown). Lipoic acid, in its oxidized form, increases the rate of the NADH-stimulated reduction (Fig. 5A), while it is ineffective in stimulating

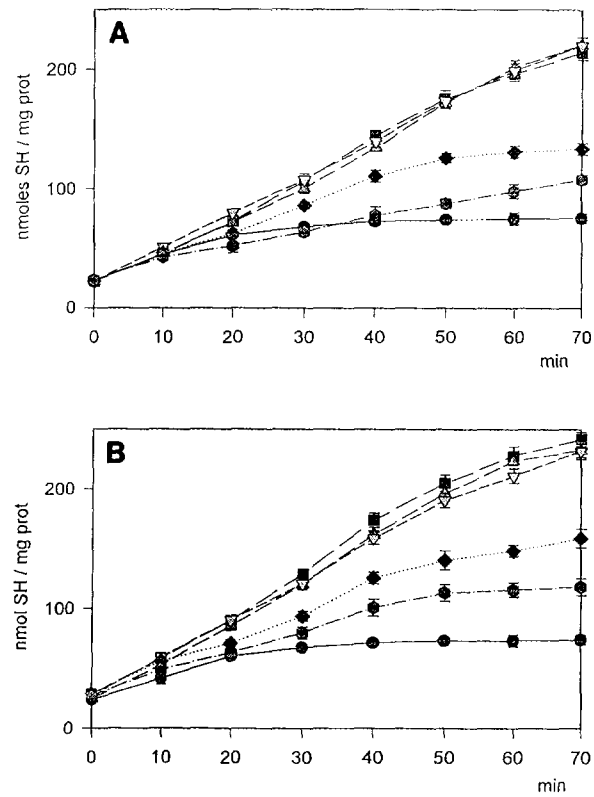


FIG. 4. Reduction of DTNB by rat liver mitochondria in the presence of respiratory substrates. Rat liver mitochondria (1 mg/ml) were incubated at 25°C in 23.4 mM NaCl, 52.2 mM KCl, 5 mM $MgCl_2$, 11.7 mM K_2HPO_4, 2.7 mM KH_2PO_4 (pH 7.4), without (A) and with (B) 3 μg of rotenone. The various added substrates were 5 mM. The reaction was started with 5 mM DTNB and carried on for the indicated times. Afterward, mitochondria were centrifuged at 14,000*g* and thiol groups determined as indicated under Materials and Methods. (○) Control; (●) succinate; (♦) L-malate; (▽) α-ketoglutarate; (△) glutamate; (▩) pyruvate. The reported results are means ± SD of three to six experiments.

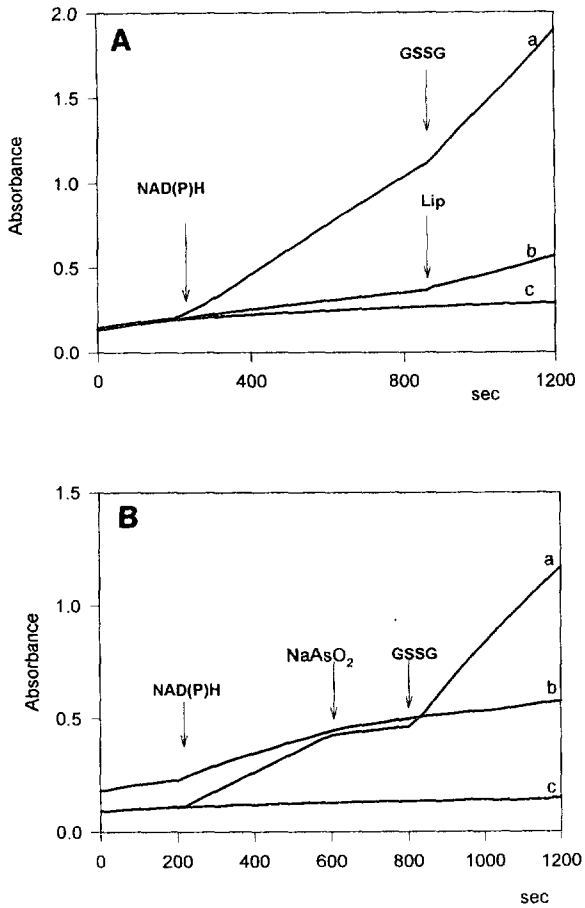


FIG. 5. Reduction of DTNB in the supernatant of submitochondrial particles. Supernatant fraction of rat liver mitochondria frozen and thawed (mitochondrial matrix, 0.3 mg protein/ml) was incubated in 0.2 M Na^+/K^+ phosphate buffer (pH 7.6) containing 1 mM EDTA and 1 mM DTNB. When indicated, 0.2 mM NAD(P)H, 1 mM GSSG, 1 mM lipoic acid (Lip), and 0.1 mM sodium arsenite ($NaAsO_2$) were also added. In B (b) protein was 0.6 mg/ml. (a) NADPH; (b) NADH; (c) control, no additions.

the NADPH-dependent reduction (not shown). In contrast, glutathione stimulates the NADPH-dependent reduction (Fig. 5A), while it has no effect on the NADH reaction (not shown). The dithiol reagent arsenite inhibits both the NADH- and the NADPH-dependent reaction (Fig. 5B). Interestingly, the addition of oxidized glutathione after the complete inhibition of the NADPH-dependent reduction of DTNB by arsenite stimulates the reduction of DTNB again. This partial sensibility to the inhibition by arsenite of the latter reaction indicates the occurrence of two independent reactions: a glutathione/glutathione reductase-dependent reaction (not inhibited by arsenite) and probably a thioredoxin-dependent reaction (inhibited by arsenite).

DISCUSSION

Oxidizing conditions determine an increase of the inner membrane permeability of isolated mitochondria, while reducing conditions appear to act in the opposite way (1). In addition, mitochondria are well known to be able to reduce several low-molecular-weight disulfides (29–32). As reported in Fig. 4 pyridine nucleotide-dependent substrates are able to reduce the added disulfide DTNB at different rates and to varying extents. In the presence of glutamate, pyruvate, and α-ketoglutarate, the reduction is linear with time for more than 40 min and the amount of thiol groups measured after 20 min is far in excess (about 230 nmol/mg protein after 1 h in the presence of glutamate) with respect to the total amount of mitochondrial SH groups measured under conditions where all SH groups are being titrated (about 95 nmol/mg protein) (33, 34). This indicates the occurrence of a catalytic system stimulated by pyridine nucleotide-dependent substrates that act as electron donors and bring about the reduction of disulfides to the corresponding thiols. The rate and extent of reduction of the pyridine nucleotides exerted by the various NAD(P)-dependent substrates added are compared in Fig. 3. Three different systems are considered operative in the reduction of disulfides and involve a thiol–disulfide exchange, respectively, with GSH (35, 36), reduced thioredoxin (37–39), and dihydrolipoate (31). In the first two cases the reduction of disulfides utilizes the reducing equivalents of NADPH, is coupled to the reduction of GSSG or oxidized thioredoxin, and is mediated by glutathione reductase and thioredoxin reductase, respectively. In the third case the dihydrolipoate residue is generated from lipoate during the α-ketoacid dehydrogenation or from NADH and free lipoate dehydrogenase (31). All these reducing systems might alter the characteristic of membrane permeability through a thiol–disulfide exchange that leads to a reduction of a protein disulfide to the corresponding dithiol; the

TABLE I

Total Mitochondrial Thiol Groups under Different Experimental Conditions

	nmol SH/mg protein	
	−Succinate	+Succinate
None	92.04 ± 1.06	92.21 ± 0.83
Ca^{2+}, P_i	90.50 ± 1.35	92.39 ± 0.81
tert-Butylhydroperoxide	83.37 ± 1.38	83.50 ± 1.13
tert-Butylhydroperoxide, Ca^{2+}, P_i	83.33 ± 1.70	84.16 ± 1.01
Acetoacetate	91.79 ± 1.56	93.13 ± 3.38
Acetoacetate, Ca^{2+}, P_i	88.88 ± 1.25	90.99 ± 3.27

Note. Rat liver mitochondria (0.7 mg/ml) were incubated in 213 mM mannitol, 71 mM sucrose, 5 mM Hepes/Tris (pH 7.4) and, when present, 30 μM Ca^{2+}, 1 mM P_i, 50 μM *tert*-butylhydroperoxide, and 1 mM acetoacetate. After 15 min of incubation at 25°C with the indicated reagents, the mitochondrial suspension was treated with 1 mM DTNB, 0.8% sodium dodecyl sulfate, and total thiols were measured spectrophotometrically at 412 nm.

dithiol–disulfide transition has a critical role in regulating membrane permeability (3, 8, 17–19). Therefore, the existence of a close redox communication between pyridine nucleotide and membrane thiol groups is apparent and leads to a pyridine nucleotide substrates-dependent prevention of increase of the mitochondrial inner membrane permeability induced by oxidizing agents.

The effect of rotenone that increases the lag time preceding swelling even in the absence of exogenously added pyridine nucleotide-dependent substrates (Fig. 1) indicates that endogenous substrates increase the NAD(P)H/NAD(P)$^+$ ratio and hence the disulfide reducing capability.

It has been observed for a long time that the redox state of pyridine nucleotides is involved in the movements of Ca^{2+} (26, 27) and K^+ (40) across the mitochondrial membrane. The oxidation of NAD(P)H by oxaloacetate or acetoacetate induces a large release of Ca^{2+} (26); similarly, *tert*-butylhydroperoxide and H_2O_2 are able to induce an oxidation of mitochondrial pyridine nucleotides concomitant with release of previously accumulated calcium (27). Under the appropriate conditions, Ca^{2+} release from mitochondria is accompanied by several alterations such as loss of endogenous Mg^{2+} and K^+, swelling, and loss of membrane potential, indicating that Ca^{2+} release might be a result of an increased inner membrane permeability (18, 41, 44). Diamide, a dithiol oxidizing agent, induces the same effect as acetoacetate or hydroperoxides (42), and dithioerytritol is able to prevent or reverse the action of these agents (42). Further, Harris and Baum (43) have found that the leakage of preloaded calcium from heart mitochondria can be prevented by adding a source of thiol groups.

Among the endogenous thiols of mitochondria, thioredoxin and lipoic acid are especially effective in reducing protein disulfides, probably because of their favorable redox potential. As reported in Fig. 5B, NADPH appears to stimulate two different reactions: one independent of arsenite (glutathione reductase) and the other inhibited by this reagent and therefore referable to a thioredoxin-dependent reduction. In fact, thioredoxin, which is completely unable to stimulate the NADH-dependent reduction, inhibits the NADPH-linked reduction (not shown). This is in agreement with the report of Luthman and Holmgren (38), according to which thioredoxin inhibits the thioredoxin reductase-dependent reduction of DTNB by NADPH measured spectrophometrically at 412 nm.

According to Lenartowicz (39) the most active system in the reduction of mitochondrial protein disulfide is the thioredoxin/thioredoxin reductase system, while the glutathione/glutathione reductase system appears to be scarcely involved; in fact, GSH is mostly utilized for the reduction of low-molecular-weight thiols. Furthermore, as reported by Le Quôc and Le Quôc (44), the oxidation of intramitochondrial NAD(P)H in the presence of acetoacetate does not induce a significant decrease of the GSH/GSSG ratio, while some protein thiols are affected. These alterations are in turn responsible for membrane permeability and the consequent swelling.

The reduction of lipoic acid is mediated by α-ketoacid dehydrogenation or by NADH. Interestingly, lipoate might also have a reducing capacity toward thioredoxin; it was in fact shown to be able to reduce thioredoxin (45), therefore indicating that NADH, in addition to NADPH, is active in mitochondrial disulfide reduction.

The role of mitochondrial protein thiols in regulating membrane permeability was studied with the use of several different thiol groups reagents; a direct indication and localization of the thiols involved still need further study. From preliminary results we have observed a small, although significant, decrease of total mitochondrial thiols under conditions that stimulate the oxidation of the pyridine nucleotides (Table 1).

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