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# Protective effect of magnesium and potassium ions on the permeability of the external mitochondrial membrane

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**Abstract**

The data reported are fully consistent with the well-known observation that exogenous cytochrome *c* (cyto-*c*) molecules do not permeate through the outer membrane of mitochondria (MOM) incubated in isotonic medium (250 mM sucrose). Cyto-*c* is unable to accept electrons from the sulfite/cyto-*c* oxido-reductase (Sox) present in the intermembrane space, unless mitochondria are solubilized. Mitochondria incubated in a very high hypotonic medium (25 mM sucrose), in contrast to any expectation, continue to be not permeable to added cyto-*c* even if Sox and adenylate kinase are released into the medium. The succinate/exogenous cyto-*c* reductase activity, very low in isotonic medium, is greatly increased decreasing the osmolarity of the medium but in both cases remains insensitive to proteolysis by added trypsin. In hypotonic medium, magnesium and potassium ions have a protective effect on the release of enzymes and on the reactivity of cyto-*c* as electron acceptor from both sulfite and succinate; results which are consistent with the view that MOM preserves its identity and remains not permeable to exogenous cyto-*c*. This report strengthens the proposal, supported by previously published data that in isotonic medium the exogenous NADH/cyto-*c* electron transport system is catalyzed by intact mitochondria, not permeable to added cyto-*c*.


*Keywords:* Mitochondria; Cytochrome *c*; Mitochondrial membranes permeability; Adenylate kinase; Sulfite oxidase; Succinate/cytochrome-*c* reductase; Respiratory chain; Cytosolic NADH oxidation; Contact sites

Immediately before and at the beginning of 1980 the dilemma if cytochrome oxidase activity was linked to $H^+$ translocation in intact mitochondria, was resolved also with the contribution of experimental data showing that the oxidation of exogenous cytochrome *c* (cyto-*c*)[1] is coupled, at least in a medium at high ionic strength, to $H^+$ translocation [1,2]. In the comment and discussion of the results therein described, the problem of impermeability of exogenous cyto-*c* to mitochondrial outer membrane (MOM) was not considered. In 1985, it was proposed that the rate of exogenous cyto-*c* oxidation by isolated mitochondria should be ascribed exclusively to a small fraction (5–12%) of the mitochondria that apparently has a damaged MOM [3]. This interpretation was taken as a valid support to explain the difficulty found in Mitchell's laboratory [4,5] to observe proton translocation with the oxidation of exogenous cyto-*c*. Data obtained by our research group are consistent with the existence, in rat-liver mitochondria, of a bi-trans-membrane electron transport pathway in addition to the well-known one-trans-membrane system of the respiratory chain. In the presence of a catalytic amount of cyto-*c* outside the mitochondria, this electron pathway promotes the oxidation of externally added NADH molecules, the consumption of molecular oxygen and the generation of an electrochemical proton gradient [6–8]. The activity of the exogenous NADH/cyto-*c* system

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[1] *Abbreviations used:* Cyto-*c*, cytochrome *c*; MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; MIS, mitochondrial intermembrane space; Adk, adenylate kinase; Sox, sulfite oxidase.

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is inhibited by cyanide but not by rotenone, antimycin A or myxothiazol suggesting that cytochrome oxidase but not the remaining complexes of the respiratory chain are involved in the process. This electron transport pathway is activated only when NADH and cyto-*c* are both present outside the mitochondria. The proposal on the existence of the electron transport system catalyzed by the NADH/cyto-*c* system has been ignored and not accepted for long time, owing to the convincement that the physiological localization of cyto-*c* would be exclusively the mitochondrial intermembrane space (MIS). This belief is still maintained even after the discovery that in the early stages of apoptosis cyto-*c* is found in the cytosol, where participates to the formation of apoptosome responsible for the activation of caspases [9]. Results on the increased rate of extra mitochondrial NADH oxidation in the presence and absence of added cyto-*c* and magnesium ions, obtained with mitochondria incubated in hypotonic media, convinced some authors to suggest that the NADH/cyto-*c* activity of isotonic mitochondria is to be ascribed exclusively to swollen or damaged mitochondria present in the mitochondrial suspension [10–13]. The finding that in hypotonic mitochondria the addition of magnesium promotes the oxidation of exogenous NADH even before the addition of cyto-*c* was utilized to introduce the concept of a magnesium-dependent desorption of endogenous cyto-*c* bound to the mitochondrial inner membrane (MIM). The hypothesis to consider cyto-*c*, present in the MIS, as an electron shuttle between outer and inner membrane, formulated in 1969 [14], was revaluated and invoked to explain the oxidation of exogenous NADH [10,12,15]. More recently, this hypothesis has been revisited to overcome the criticism that the shuttle activity of endogenous cyto-*c* should be independent on the presence of exogenous cyto-*c*. It has been suggested that the added cyto-*c* is reduced at the external side of MOM by the activity of NADH/cyto-$b_5$ oxido-reductase complex of intact mitochondria and oxidized by cytochrome oxidase of mitochondria with permeabilized but not ruptured MOM [16].

Other than the activity of the NADH/cyto-*c* electron transport system, two rationales remain to be considered to explain the oxidation of exogenous NADH in the presence of respiratory chain inhibitors and a catalytic amount of exogenous cyto-*c*: (i) the mitochondrial suspension contains completely broken or fragmented mitochondria with cytochrome oxidase molecules freely accessible to reduced cyto-*c* [3]; (ii) the cyto-*c* mediated NADH oxidation is to be ascribed to the presence of mitochondria with MOM freely permeable to cyto-*c* [10–13,16]. The first possibility cannot be considered since it has been extensively documented that the activity of NADH/cyto-*c* system generates an electrochemical proton gradient, dissipated by uncouplers, which is incompatible with the activity of free molecules of cytochrome oxidase [7,8,10–12,17].

The experimental design of this report was to find approaches and methods to evaluate the permeability of MOM to exogenously added cyto-*c*. In our previous papers, we have described two integrity tests [6,8,17]. One has been outlined to measure the permeability of both outer and inner membranes of isolated mitochondria to exogenous NADH. The method consists simply by comparing the rate of added NADH oxidation (in the absence of rotenone) with the value obtained with water-treated mitochondria. Mitochondrial suspensions with permeability towards the exogenous NADH expressed as the rate of its oxidation and not higher than 2% compared to the value of water-treated mitochondria, have been routinely utilized. The second method is based on the original observation of Kuylenstierna et al. that trypsin, being impermeable to MOM, is unable to hydrolyze adenylate kinase (Adk) present in the intermembrane space unless the enzyme is released outside [18]. A decrease in the total activity of Adk found in the presence of trypsin, at the end of an incubation period, is to be considered as the expression of increased permeability of MOM responsible for the release of the enzyme outside the mitochondria.

Here, we describe some approaches and procedures to measure in isolated mitochondria the permeability of MOM to externally added cyto-*c*. They are based essentially on measuring the activities in iso- and hypotonic medium of Adk, sulfite oxidase (Sox) and succinate/exogenous cyto-*c* reductase.

## Materials and methods

### *Materials*

Horse heart cytochrome *c*, NADH, $NAD^+$, trypsin (EC 3.4.21.4) and EGTA were from Roche Biochemicals (Milan, Italy). Succinic acid, ADP, $NADP^+$, hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), rotenone, myxothiazol, oligomycin, carbonyl cyanide-p-(trifluoromethoxy) phenylhydrazone (FCCP), Hepes (Hydroxyethyl-piperazine ethansulphonic acid), sodium sulfite, β-D-glucose and N-dodecyl-β-D maltoside (laurylmaltoside, LM) was from Sigma Chemicals (St. Louis, MO). Sucrose and mannitol were bought from Baker and potassium ferricyanide (hexa-cyanoferrate) and deoxycholic acid from Aldrich Chemicals. All reagents were of the highest purity available in commerce and solutions were prepared either with bidistilled water obtained from a quartz apparatus or in absolute ethanol, as in the case of respiratory chain inhibitors.

### *Isolation and incubation of mitochondria*

Rat-liver mitochondria were isolated in mannitol–sucrose medium as previously described [7]. Incubations were carried out at 25 °C in a medium at pH 7.4 consisting of either 250 mM sucrose and 20 mM Hepes–Tris (isotonic medium) or 25 mM sucrose and 20 mM Hepes–Tris (hypotonic medium). Oxygen consumption was determined in a closed thermostated chamber equipped with a magnetic stirrer and a Clark-type electrode (Rank Bros., Cambridge, UK). Respiratory control index was obtained in a medium which contained 220 mM Sucrose, 20 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 2 mM Pi and 20 Hepes–KOH, at pH 7.4 and was always higher than 5 with succinate + ADP and not lower than 9 with succinate + FCCP. P/O ratios constantly had a value ranging between 1.9 and 2.1 with freshly isolated mitochondria. NADH oxidation was followed at the wavelength pair 340–374 nm ($E = 4.28\ mM^{-1}\ cm^{-1}$).

Protein was measured by mean of the biuret method and the Bradford protein assay (both using BSA as standard) for samples containing high or low protein concentration, respectively [19].