The rupture of the outer mitochondrial membrane is known to be crucial for cell death, but the mechanism, specifically its redox-signaling aspects, still needs to be studied in more detail. In this work, the external NADH oxidation by rat liver mitochondria was studied under the outer membrane rupture induced by the mitochondria hypotonic treatment or the inner membrane permeability transition. The saturation of the oxidation rate was observed as a function of mitochondrial protein concentration. This effect was shown to result from cytochrome c binding to the mitochondrial membranes. At a relatively high concentration of mitochondria, the oxidation rate was strongly activated by 4 mM Mg$^{2+}$ due to cytochrome c desorption from the membranes. A minimal kinetic model was developed to explain the main phenomena of the external NADH oxidation modulated by cytochrome c and Mg$^{2+}$ in mitochondria with the ruptured outer membrane. The computational behavior of the model closely agreed with the experimental data. We suggest that the redox state of the released cytochrome c, considered by other authors to be important for apoptosis, may strongly depend on its oxidation by the fraction of mitochondria with the ruptured outer membrane and on the cytoplasmic cytochrome c reductase activity.

The external pathway of NADH oxidation in liver mitochondria is known to include NADH specific flavoprotein Fp$_b$ and cytochrome $b_5$ of the outer membrane, the intermembrane electron transport, and cytochrome c oxidase of the inner membrane (1–7). Cytochrome c was suggested to function as an electron shuttle between the outer and inner membranes of mitochondria (5). On the other hand, the rate of external NADH oxidation by the intact liver mitochondria is extremely low (1, 4, 8–11), due to the outward orientation of flavoprotein Fp$_b$ and cytochrome $b_5$ in the outer mitochondrial membrane (OMM)$^1$ (see “Discussion” in Ref. 12) and the impermeability of the OMM to cytochrome c (13, 14).

Under some conditions, the OMM may be ruptured or permeabilized that is a crucial step in molecular mechanisms of cellular necrosis and apoptosis resulted from the activation of caspases by cytochrome c released into the cytoplasm (15–18). The OMM damage also induces the external NADH oxidation by mitochondria (5, 12, 19). The degree of the OMM rupture (19) and activation of the rotenone-insensitive oxidation of external NADH (9) under hypotonic treatment of liver mitochondria was shown to significantly increase with the age of rats. An increase in population of mitochondria with fragile OMM was assumed to be one of the possible mechanisms of programmed aging and cell death (19, 20). The idea that the OMM rupture might relate to the aging mechanisms was also recently suggested by Skulachev (21).

According to some data, the rate of external NADH oxidation by rat liver mitochondria may be stimulated by extramitochondrial cytochrome c (22). The concept of “bi-trans-membrane” electron transport was developed to explain this phenomenon (22–24), assuming its possible relation to the mechanisms of apoptosis (25). These authors suggested that extramitochondrial cytochrome c, reduced on the OMM by external NADH, might be directly oxidized by cytochrome c oxidase of mitochondria, transferring electrons through the intact OMM. It was suggested that “intact (but not damaged) mitochondria are able to promote the oxidation of exogenous cytochrome c” (24). The data obtained by Bodrova et al. (10) and our own results (9, 11) do not support this concept, because the OMM rupture was shown to be an obligatory factor for the rotenone-insensitive oxidation of external NADH by rat liver mitochondria (9–12). Additionally, Mg$^{2+}$ ions significantly stimulate the external NADH oxidation (9–12), which may be explained by cytochrome c desorption from the mitochondrial membranes under Mg$^{2+}$ influence (11).

In this work, it was shown that the rate of external NADH oxidation by rat liver mitochondria with the ruptured outer membrane strongly depends on cytochrome c desorption from mitochondrial membranes modulated by Mg$^{2+}$ ions. A minimal kinetic model was developed to explain these experimental data, and its computational behavior was studied.

**EXPERIMENTAL PROCEDURES**

Materials—Sucrose, d-mannitol, Hepes, Trizma base, EGTA, β-NADH, bovine heart cytochrome c, bovine serum albumin (free of fatty acids, fraction V), rotenone, antimycin A, myxothiazol, carbonylcyanide-p-trifluoromethoxyphenylhydrazone, L-ascorbic acid, MgCl$_2$, and other salts were purchased from Sigma. All chemicals were of analytical grade.

Isolation of Mitochondria—White rats of 180–220 g, starved for 12–14 h, were used. Liver mitochondria were isolated by the standard procedure of differential centrifugation using the medium composed of 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM Hepes-Tris, pH 7.4. Mitochondria were washed and finely suspended in the medium composed of 210 mM mannitol, 70 mM sucrose, 0.02 mM EGTA, 5 mM...
Hepes-Tris, pH 7.4, and 0.3 mg/ml of bovine serum albumin (free of fatty acids). Protein concentration was determined by the biuret method using Na$_2$-cholate, with bovine serum albumin as the standard.

**Catalytic Activity**—NADH oxidation was measured polarographically, using the Cole-Parmer Oxygen Meter connected with the Lineis L250-E recorder to register oxygen consumption, or fluorimetrically (9). The Cole-Parmer oxygen electrode of Clark type was modified to minimize the oxygen diffusion through the membrane from the electrolyte chamber into the mitochondrial suspension. The isotonic medium composed of 210 mM mannitol, 70 mM sucrose, 5 mM Hepes-Tris, pH 7.4, 0.02 mM EGTA (MSHG medium) or the strongly hypotonic medium composed of 5 mM Hepes-Tris, pH 7.4, and 0.02 mM EGTA (HG medium) were used for most experiments. The influence of mitochondria permeability transition on the external NADH oxidation was studied using the following two media: (a) 100 mM mannitol, 150 mM sucrose, 5 mM Hepes-Tris, pH 7.4, 5 mM succinate-Tris, 5 mM phosphate-Tris (MS medium) and (b) 50 mM KCl, 150 mM sucrose, 5 mM Hepes-Tris, pH 7.4, 5 mM succinate-Tris, 5 mM phosphate-Tris (KS medium). All measurements were carried out at 37 °C, constantly stirring the mitochondrial suspensions.

**Spectrophotometric Measurements**—Concentrations of cytochromes c and aa$_3$ were determined by the method of differential spectrophotometry according to Williams (26, 27) with some modifications. Mitochondria, 0.2 ml of the suspension with protein concentration around 60 mg/ml, were solubilized in 0.02 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.4% (w/v) Triton X-100. The sample was divided into two cuvettes. To reduce the cytochromes in one cuvette, 5 mM ascorbate, and after a 2-min interval, 0.5 mM KCN and a few crystals of sodium dithionite were added. The cytochromes in the other cuvette were oxidized by 5 mM ferricyanide. Differential oxidation-reduction spectrum was registered using the double beam spectrophotometer Specord UV VIS.

**Cytochrome c Sorption-Desorption Study**—Cytochrome c binding to the mitochondrial membranes was studied by determining its distribution between the supernatant and the pellet of mitochondria centrifuged at 10,000 × g for 30 min at 4 °C in 2-ml Eppendorf tubes. Before centrifugation, mitochondria (0.2 mg of protein/ml) were incubated for 10 min at 4 °C in 1.5 ml of HG medium supplemented with 1, 2, 4, 6, or 10 μM exogenous cytochrome c. In parallel, the similar experiments were also performed in the presence of 4 mM MgCl$_2$ that was added after 5 min of mitochondria incubation, followed by the remaining 5-min incubation in the presence of Mg$^{2+}$. After centrifugation, the mitochondrial pellets were solubilized in 0.6 ml of 0.4% potassium phosphate buffer, pH 7.4, containing 1.6% Triton X-100, and then mixed with 1.8 ml of HG medium. The supernatant (1.8 ml) of each sample was mixed with 0.6 ml of 0.4% potassium phosphate, pH 7.4, containing 1.6% Triton X-100. Cytochrome c concentration in all obtained samples was measured as described above.

**Software**—Computational analysis of the minimal kinetic model was carried out using the MathCAD 8.0 software (MathSoft, Cambridge, MA) for solving the system of mathematical equations.

**RESULTS**

The addition of NADH to uncoupled rat liver mitochondria incubating in isotonic MSHG medium had almost no effect on the rate of oxygen consumption in the presence of rotenone-antimycin A-myxothiazol, the inhibitors of the respiratory chain (Fig. 1, curve a). An increased rate of NADH oxidation was observed when exogenous cytochrome c was added at relatively high concentration. One order higher rate of external NADH oxidation in the presence of added cytochrome c was observed in hypotonic HG medium (Fig. 1, curve b), as a result of the OMM damage (see Refs. 9–11 and references therein).

Fig. 1 (curves a and b) show that Mg$^{2+}$ ions did not stimulate the external NADH oxidation in the presence of a relatively high concentration of exogenous cytochrome c in both isotonic and hypotonic media, as well as in the absence of exogenous cytochrome c in isotonic medium (Fig. 1, curve c). Moreover, some inhibition of the oxidation rate was observed after addition of 4 mM Mg$^{2+}$ (Fig. 1, curve a). In the HG medium, when the OMM was ruptured, Mg$^{2+}$ ions alone significantly stimulated the rotenone-insensitive oxidation of external NADH, even in the absence of exogenous cytochrome c (Fig. 1, curve d).

Stimulation (Fig. 2, curve a), no effect (Fig. 2, curve b), or some inhibition (Fig. 2, curve c) of the external NADH oxidation was observed after addition of 4 mM Mg$^{2+}$ into the HG medium containing 1 μM exogenous cytochrome c. The effect depended on the mitochondrial protein concentration that was used in the range of 0.075–0.6 mg/ml. Fluorimetric measurement of NADH oxidation was used in these experiments to obtain a higher sensitivity. At mitochondrial protein concentration of 0.6 mg/ml, a significant activation of the external NADH oxidation by Mg$^{2+}$ ions was observed (Fig. 2, curve d). In this case, a maximal stimulation was observed at 4–6 mM Mg$^{2+}$ (Fig. 2, curve e), while no essential activation was registered at 12 mM KCl (Fig. 2, curve f), which has the same ionic strength as 4 mM Mg$^{2+}$. The maximal stimulation was only observed at 48 mM KCN (Fig. 2, curve f), i.e. at four times higher ionic strength than that of 4 mM MgCl$_2$. Some progressive inhibition of the external NADH oxidation by 4 mM Mg$^{2+}$ or higher was observed at relatively small concentration of mitochondrial protein (Fig. 2, curve c). This inhibition was accompanied by a visually observed aggregation of mitochondria, and thus it might be a consequence of cytochrome c oxide masking within the mitochondrial aggregates.

The obtained data confirm the concept that the OMM damage is an obligatory factor for switching on the rotenone-insensitive oxidation of external NADH by rat liver mitochondria (9–11). On the other hand, the external NADH oxidation was also suggested to be realized through the contact sites between the OMM and the inner mitochondrial membrane (IMM) in the intact mitochondria (24). It was assumed that under some conditions, when the concentration of cytoplasmic cytochrome c is increased, the external NADH oxidation by intact mitochondria might be significantly activated due to a bi-trans-membrane electron transport catalyzed by external cytochrome c.
a specific inhibitor of mitochondria permeability transition media (Fig. 3). The stimulation was prevented by cyclosporin A, matically stimulated the external NADH oxidation in both
panied by oxidation of endogenous pyridine nucleotides, dra-
show that mitochondria permeability transition, accom-
myxothiazol.
The oxidation rate on the total concentration of cytochrome c
MgCl₂; CN, 10⁻⁶ M cyanide; Mg, 6 M myxothiazol were added. NADH, 40 µM, was added 1 min after
of Mg²⁺ ions influence the external pathway of NADH oxidation, the dependence of the oxidation rate on the total concentration of cytochrome c in the HG medium was presented in Lineweaver-Burk plots (Fig.
4). The total concentration included both the endogenous (310 ± 23 pmol/mg of protein, n = 6) and exogenous cytochrome c. The obtained results show that the Michaelis-Menten constant $K_m$ diminished from 7.4 µM cytochrome c, in the absence of Mg²⁺, to 1.36 µM cytochrome c, in the presence of 4 mM Mg²⁺. The influence of Mg²⁺ on the apparent value of $K_m$ seems to be through weakening the electrostatic interactions of cytochrome c with the mitochondrial membranes, resulting in an increase of free hemoprotein concentration (11).

The saturation of the rate of rotenone-insensitive oxidation of external NADH versus the concentration of mitochondrial protein in hypotonic medium without Mg²⁺ was observed earlier (28). The data, obtained in the presented work, show that the saturation level depends on cytochrome c concentration in the system (Fig. 5A, curves a and c). In the presence of 4 mM Mg²⁺, the saturated curves at 1 and 4 µM exogenous cytochrome c were transformed into linear functions (Fig. 5A, curves b and d, respectively). To explain these results, the influence of 4 mM Mg²⁺ on cytochrome c interaction with the mitochondrial membranes was studied (Fig. 6A). As one can see, the saturation of cytochrome c binding was observed when its concentration in the medium was changed from 0 to 10 µM (Fig. 6A, curve a). Only after the saturation was reached, the linear increase in the supernatant cytochrome c concentration versus the amount of added cytochrome c was observed (Fig. 6A, curve b). In the HG medium without Mg²⁺, the mitochondrial membranes bind about 2.7 nmol of cytochrome c/0.2 mg of

(25), and it could play an essential role in mechanisms of apoptosis. However, it cannot be excluded that the stimulation of the external NADH oxidation by exogenous cytochrome c, observed by the authors (24, 25), relates to only damaged mitochondria that represent nearly 10% of the total population of isolated mitochondria (11, 13, 14).

Whatever the mechanism of rotenone-insensitive oxidation of cytoplasmic NADH, activation of this process is likely to be important in redox signaling in apoptosis. The rupture of the OMM is known to be caused by mitochondrial permeability transition resulting, for example, from oxidative stress and/or Ca²⁺ overload of mitochondria. It might lead to activation of the cytoplasmic NADH oxidation by damaged mitochondria. To demonstrate this possibility in vitro, the IMM permeability transition of energized mitochondria was induced by Ca²⁺ and inorganic phosphate (Fig. 3). Mitochondria were incubated at two different conditions: (a) in the low ionic strength MS medium, in which 4 mM Mg²⁺ was added where indicated (Fig. 3, curves a and b) and (b) in the relatively high ionic strength KS medium containing 50 mM KCl (Fig. 3, curves c and d). No exogenous cytochrome c was used in these experiments. The data show that mitochondria permeability transition, accompanied by oxidation of endogenous pyridine nucleotides, dramatically stimulated the external NADH oxidation in both media (Fig. 3). The stimulation was prevented by cyclosporin A, a specific inhibitor of mitochondria permeability transition (Fig. 3, curves b and d).

To clarify the mechanism by which Mg²⁺ ions influence the external pathway of NADH oxidation, the dependence of the oxidation rate on the total concentration of cytochrome c in the HG medium was presented in Lineweaver-Burk plots (Fig.
mitochondrial protein (Fig. 6A, curves a and b). According to these data, the number of cytochrome c binding centers was estimated as 13.5 nmol/mg of mitochondrial protein. The measured concentration of cytochromes aa₃ was 395 ± 34 pmol/mg of mitochondrial protein (n = 6) that yields 68 molecules of the bound cytochrome c per one cytochrome c oxidase complex at the saturation state.

To estimate the dissociation constant \( K_N \), the hyperbolic character of cytochrome c binding to the centers in 1:1 ratio was assumed. Thus, the dissociation constant may be expressed as follows,

\[
K_N = \frac{[C] \cdot ([N] - [NC])}{[NC]} \quad \text{(Eq. 1)}
\]

in which [C] and [NC] are concentrations of the free and bound cytochrome c, respectively, and [N], is the total concentration of cytochrome c binding centers in the system that depends on the concentration of added mitochondria (see Equation 8). To estimate \( K_N \) according to Equation 1, the cytochrome c concentration in the supernatant (Fig. 6A, curve b) and the concentration of the centers occupied by cytochrome c (Fig. 6A, curve a) were taken as [C] and [NC], respectively, using the experimental data for 1 \( \mu \)M exogenous cytochrome c in mitochondrial suspension. It was obtained that \( K_N = 0.51 \mu \)M cytochrome c, which is close to 0.4 \( \mu \)M obtained by other authors for the mitochondrial membranes (see Ref. 29 for review).

The main aspects of the Mg²⁺ influence on the external NADH oxidation by rat liver mitochondria with the damaged OMM and on the cytochrome c interaction with the mitochondrial membranes may be described in the framework of a kinetic model (Fig. 7). According to this simplified model, cytochrome c (C) interacts with N-centers on the mitochondrial membranes or with cytochrome c oxidase (E), forming the NC or EC complexes, respectively.

The model also includes two types of Mg²⁺ interactions with the membranes. One of them is the occupation of N-centers by competing with cytochrome c. Two Mg²⁺ ions were assumed to interact with one cytochrome c binding center, taking into account that the cytochrome c binding to the liposomes is characterized by 4–5 negative charges of the outside oriented phospholipids per one molecule of the bound cytochrome c at the saturation state (29). The interaction of two Mg²⁺ ions with the N-center may be expressed as two sequential events, with the equal microscopic dissociation constants,

\[
K_{Mg} = \frac{[\text{Mg}^2⁺] \cdot [N]}{[\text{NMg}^2⁺]} \quad \text{(Eq. 2)}
\]

\[
K_{MC} = \frac{[\text{Mg}^2⁺] \cdot [\text{NMg}^2⁺]}{[\text{NMg}^2⁺]} \quad \text{(Eq. 3)}
\]

in which [N], [NMg²⁺], and [NMg²⁺] are concentrations of the free N-centers, the N-centers with one bound Mg²⁺ ion, and the N-centers with two bound Mg²⁺ ions, respectively. [Mg²⁺] is the concentration of free Mg²⁺. Setting \( K_{Mg} = 0.5 \) mM, a good agreement between the computational behavior of the model and the experimental data relating to the cytochrome c sorption-desorption (Fig. 6) was obtained.

The other type of Mg²⁺ interaction with the mitochondrial membranes relates to the observed mitochondria aggregation, which might explain the inhibition of the external NADH oxidation. The inhibitory action of Mg²⁺ ions may be formally

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Lineweaver-Burk plots presentation of the rate of external NADH oxidation by rat liver mitochondria versus cytochrome c concentration in HG medium without Mg²⁺ (curve a) or with 4 mM MgCl₂ (b). NADH oxidation was registered according to the protocol described for Fig. 2 (curve a) at mitochondrial protein concentration 0.30 mg/ml and different concentrations of added cytochrome c. The total concentration of cytochrome c, exogenous plus endogenous, was used.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Dependence of the rate of external NADH oxidation on mitochondrial protein concentration in HG medium with 1 \( \mu \)M (curves a and b) and 4 \( \mu \)M exogenous cytochrome c (curves c and d), before (curves a and c), and after 4 mM MgCl₂ addition (curves b and d). NADH oxidation was registered according to the protocol described for Fig. 2 (curve a). A, the experimental data (n = 4–6). B, the computational behavior of the minimal kinetic model (continuous lines) and polynomial regression presentation of the experimental data (dotted lines).
FIG. 6. Cytochrome c distribution between the mitochondrial membranes (curves a and c) and supernatant (curves b and d) as a function of the added cytochrome c concentration in the absence (curves a and b) or presence of 4 mM MgCl₂ (curves c and d). The concentration of mitochondrial protein in HG medium was 0.20 mg/ml. Cytochrome c concentration was measured by the method of differential spectrophotometry. A, the experimental data (n = 6). B, the computational behavior of the minimal kinetic model (continuous lines) and polynomial regression presentation of the experimental data (dotted lines).

Fig. 7. The minimal kinetic model of the external NADH oxidation by rat liver mitochondria with the ruptured outer membrane. C, cytochrome c; E, cytochrome c oxidase; EC, cytochrome c and Mg²⁺ oxidase complex; N, cytochrome c and Mg²⁺ binding centers on the mitochondrial membranes. Competitive interactions of cytochrome c and Mg²⁺ with N-centers are shown. Formation of inactive complexes, EΜg²⁺ and ECMg²⁺, are shown to mimic the inhibition of external NADH oxidation due to Mg²⁺-induced aggregation of mitochondria.

It was estimated that \( K_M = 6.5 \) mM. The total concentration of Mg²⁺ in the system, \([Mg^{2+}]_0\), may be described as follows:

\[
[Mg^{2+}]_0 = [Mg^{2+}] + [NMe^{2+}] + 2 \cdot [NMe^{2+}] + [EMe^{2+}] + [ECMe^{2+}] \quad \text{(Eq. 6)}
\]

The total concentration of the cytochrome c binding centers, \([N]_0\), is as follows:

\[
[N]_0 = [N] + [NC] + [NMe^{2+}] + [NMe^{2+}] \quad \text{(Eq. 7)}
\]

On the other hand,

\[
[N]_0 = n \cdot [E]_0 \quad \text{(Eq. 8)}
\]

in which \( n = 68 \), determined above as the number of N-centers per one cytochrome c oxidase complex, and \([E]_0\) is the concentration of cytochrome c oxidase in the system that may be calculated by knowing the mitochondrial protein concentration, \([M_c]\), and cytochromes aa₃ concentration, \([aa₃]\), in mitochondria.

\[
[E]_0 = [M_c] \cdot \frac{[aa₃]}{2} \quad \text{(Eq. 9)}
\]

On the other hand, the total concentration of cytochrome c oxidase is as follows:

\[
[E]_0 = [E] + [EC] + [EMe^{2+}] + [ECMe^{2+}] \quad \text{(Eq. 10)}
\]

Assuming that the NADH-cytochrome c reductase activity of the OMM is not the limiting factor for the external NADH oxidation by rat liver mitochondria (1), and a simple first-order reaction Michaelis-Menten approximation for cytochrome c oxidase, as it would be at high ionic strength conditions (30), the rate of the external NADH oxidation may be described as,

\[
v = \frac{k \cdot ([E] + [EC]) \cdot [C]}{K_m + [C]} \quad \text{(Eq. 11)}
\]

in which \( K_m \) is simply the dissociation constant,

\[
K_m = \frac{[C] \cdot [E]}{[EC]} \quad \text{(Eq. 12)}
\]

that is \( K_m = k_{-1} / k_{+1} \). It was estimated that \( K_m = 1.36 \) μM (Fig. 4, curve b), assuming for simplicity a complete desorption of cytochrome c from the N-centers in the presence of 4 mM Mg²⁺ (Fig. 6A, curves d and c), like that observed at high ionic strength conditions (8, 31). We have to set \( K_m = 1.36 \) μM at any Mg²⁺ concentration, regardless of a very different apparent
value determined in the absence of Mg$^{2+}$, specifically 7.4 μM (Fig. 4, curve a). This discrepancy is explained by sorption of cytochrome c on the mitochondrial membranes in the low ionic strength medium without Mg$^{2+}$ that leads to a significant diminishing of the free concentration of cytochrome c. Only free cytochrome c seems to be available for the intermembrane electron transport to oxidize the external NADH. To estimate the rate constant $k$ for Equation 11, the value for $V_{max}$ was estimated in the absence of Mg$^{2+}$ (Fig. 4, curve a), and the concentration of cytochrome c oxidase was determined (198 ± 17 pmol/mg of protein) for used mitochondria. It was estimated that $k = 2300$ min$^{-1}$.

The total concentration of cytochrome c, [Cl], may be described as follows,

$$[C]_t + 1.56 \cdot [E]_t = [C] + [NC] + [EC] + [ECMg^2+] \quad (\text{Eq. 13})$$

in which [Cl], and 1.56[E], are the concentrations of the exogenous and endogenous cytochrome c, respectively. The coefficient 1.56 is the relation of endogenous cytochrome c (310 ± 24 pmol/mg of protein) to cytochrome c oxidase (198 ± 17 pmol/mg of protein) concentration.

The computational behavior of the model (Figs. 5B and 6B), described by the system of the mathematical Equations 1–13, was similar to the experimental data related to the external NADH oxidation versus the mitochondrial protein concentration in the HG medium (Fig. 5A) and to the cytochrome c binding to the mitochondrial membranes versus concentration of added cytochrome c (Fig. 6A). This model allows including more details, but even in the simplified form it adequately describes the main aspects of the Mg$^{2+}$ and cytochrome c influence on the external NADH oxidation by rat liver mitochondria with the ruptured OMM.

**DISCUSSION**

The external pathway of NADH oxidation is normally not active in intact liver mitochondria (8–12), and the conditions for switching it on have not been established, except the OMM rupture or its permeabilization for cytochrome c (9–12). Physiological electron acceptors for the OMM electron transport system in the cell are also unknown. It is not excluded that some small redox molecules may serve as electron transporters between the OMM and the IMM executing direct oxidation of cytoplasmic NADH by mitochondria. External cytochrome c, which may be reduced by the intact liver mitochondria, is normally not present in the cytoplasm. Even in the presence of external cytochrome c, the OMM integrity is a limiting factor for external NADH oxidation (9–12). Mitochondria with the ruptured OMM are able to oxidize the external NADH, and Mg$^{2+}$ ions were shown to significantly stimulate this oxidation (9–12, 28), coupled to the proton-motive force generation by complex IV of the respiratory chain (5, 10–12).

However, Mg$^{2+}$ ions do not activate the external NADH oxidation by mitochondria at a relatively high concentration of exogenous cytochrome c in the medium (Fig. 1, curves a and b). A significant activation by Mg$^{2+}$ ions was only observed when exogenous cytochrome c was not added or was present at a relatively small concentration, and the OMM was ruptured by hypotonic treatment (Fig. 1, curve d, and Fig. 2, curves e and f) or by mitochondrial permeability transition (Fig. 3, curve a). The stimulatory effect of Mg$^{2+}$ ions on the external NADH oxidation was phenomenologically revealed in diminishing of the Michaelis-Menten constant $K_m$ for cytochrome c (Fig. 4). That may be mainly explained by cytochrome c desorption from the mitochondrial membranes in the presence of Mg$^{2+}$ (Fig. 6A), suggesting that the liberated cytochrome c becomes available for the intermembrane electron shuttling between the OMM and IMM (5) under the external NADH oxidation by mitochondria with the ruptured OMM (Fig. 5A).

The analysis of cytochrome c binding to the mitochondrial membranes, as a function of the cytochrome c concentration in the HG medium, allowed estimation of the number of cytochrome c binding centers on the mitochondrial membranes (Fig. 6A, curves a and b), as well as the dissociation constant (using Equation 1 and the data in Fig. 6A, curves a and b, for 1 μM cytochrome c). These data were required to develop a minimal kinetic model (Fig. 7) for explaining the main aspects of the Mg$^{2+}$ and cytochrome c influence on the external NADH oxidation. A very good agreement between the computational behavior of this model and the experimental data was obtained for both the external NADH oxidation (Fig. 5) and the cytochrome c binding to the mitochondrial membranes (Fig. 6).

Electrostatic interaction is the main factor for cytochrome c sorption on the mitochondrial membranes, because almost all (8) or at least 90% of cytochrome c (31) were desorbed at physiological ionic strength. The influence of Mg$^{2+}$ on cytochrome c interaction with the mitochondrial membranes was more specific than it would be expected taking into account only the ionic strength value (Fig. 2). Therefore, a discrete character of the charge distribution on the mitochondrial membranes and on the cytochrome c seems to be a prevalent factor.

The number of cytochrome c binding centers of the hypotonically treated mitochondria was estimated as 68 per one cytochrome c oxidase. These centers seem to be mainly formed by negatively charged phospholipids. Mitochondria contain about 150 μg pf phospholipid/mg of protein, where 19% is cardiolipin (32). That yields about 21 nmol of cardiolipin/mg of protein or 106 molecules per one cytochrome c oxidase complex. At least one molecule of cardiolipin may be included in one cytochrome c binding center on the mitochondrial membranes, because some fraction of this phospholipid faces the matrix side of the IMM. The other two of the assumed four negative charges, which form one cytochrome c binding N-center according to our model, might belong to one-charge phospholipids, as phosphatidic acid, phosphatidylserine, etc. (29).

The mitochondrial permeability transition and the OMM rupture, or its permeabilization, have been recognized as crucial steps in molecular mechanisms of apoptosis (15, 17, 19–21). Earlier we showed that the hypotonic fragility of the OMM of rat liver mitochondria was significantly changed with age and that it was different for male and female aged rats (9, 18). These data suggested that the OMM rupture of a critical fraction of mitochondria may lead to cell death, while the age-dependent increase in the population of mitochondria with the fragile OMM might be one of the possible mechanisms of programmed aging (18, 20).

Strong stimulation of the external (cytoplasmic) NADH oxidation, caused by the OMM rupture, may change the redox potentials of cytoplasmic pyridine nucleotides, glutathione, and cytochrome c, influencing oxidative stress-linked (7) and redox-signaling mechanisms of apoptosis (17, 33–36). It was recently proposed that the redox state of cytoplasmic cytochrome c, modulated by reduced glutathione, might be a key regulator of programmed cell death (34). In this respect, the NADH-dependent electron transport systems of the OMM and endoplasmic reticulum seem to be even more powerful modulators of the redox state of cytoplasmic cytochrome c.

It has been directly shown for two mouse lymphoma cell lines in a cell-free system that apoptotic activity depends on the redox state of cytochrome c, i.e., the reduced cytochrome c was not pro-apoptotic (33). In addition, the concentration of cytochrome c liberated from mitochondria with the ruptured OMM is sufficiently high for a significant stimulation of the cytoplasmic NADH oxidation, taking into account the data in Fig. 3.
Since the NADH-cytochrome c reductase activity of the OMM is significantly higher than cytochrome c oxidase activity of the IMM (1), the released cytochrome c is expected to be maintained in the reduced steady state (5), thus preventing pro-apoptotic activity of this hemoprotein until cytoplasmic NADH is exhausted. Besides, the NAD(P)H-cytochrome c oxidase activity of the endoplasmic reticulum membranes might also reduce cytoplasmic cytochrome c. The role of these and other cytoplasmic redox events in the mechanisms of apoptosis and necrosis have to be elucidated in the future.

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