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On the Impermeability of the Outer Mitochondrial Membrane to Cytochrome *c*

II. Studies on Isolated Membrane Fragments

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Summary. Outer mitochondrial membranes isolated by the swelling-shrinkage sonication procedure of Sottocasa *et al.* [19, 20] forms small sealed vesicles. If cytochrome *c* is present during the procedure it is trapped inside these vesicles and can not be washed out nor is accessible to external enzymes, e.g., cytochrome oxidase (EC 1.9.3.1) or succinate-cytochrome *c* reductase present as contamination by the inner membrane, but is fully accessible to rotenone-insensitive NADH-cytochrome *c* reductase of the outer membrane. This indicates the impermeability of the outer mitochondrial membrane to cytochrome *c*.

A modification of the original procedure for the separation of the outer mitochondrial membrane is described.

It has been postulated [7, 10, 13, 15] that the outer mitochondrial membrane is freely permeable to compounds of low molecular weight and impermeable to large molecules. In the previous paper of this series [23] it has been shown that, in intact mitochondria, the outer membrane forms a permeability barrier to external cytochrome *c*, thus preventing it from reacting with enzymes located in the inner membrane, *viz* cytochrome oxidase (EC 1.9.3.1.) and the succinate-cytochrome *c* reductase system. The present report demonstrates that not only the intact outer membrane but also fragments isolated from it are impermeable to cytochrome *c*. Thus, cytochrome *c* enclosed in vesicles formed by these fragments can not be removed by washing and is not accessible to external enzymes.

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Materials and Methods

Isolation of Intracellular Particles

Male albino rats starved overnight were used throughout. Mitochondria were isolated by a standard procedure [4] and washed twice with 250 mM sucrose. Microsomes were obtained by centrifuging the post-mitochondrial supernatant at 105,000 $\times g$ during 60 min.

Separation of Mitochondrial Membranes

This was achieved by modifying the procedure of Sottocasa *et al.* [19, 20] in two points: (1) omission of ATP and Mg^{2+} from the contraction medium; and, (2) increased concentration of sucrose during the sonication. The first modification was introduced because experiments had shown that a high concentration of sucrose alone was sufficient to produce a maximal contraction of hypotonically swollen mitochondria. The aim of the second modification was to obtain a better purification of the outer membrane fraction. It was presumed that the vesicles formed from both the outer and the inner membrane during sonication should contain the same concentration of sucrose as the sonication medium. After the subsequent dilution of the mixture, sucrose should diffuse out of the outer membrane fragments, while it should remain in fragments of the inner membrane which is known to be impermeable to this disaccharide [7, 12, 22]. This was expected to result in further increasing of the density difference between the particles of the inner and the outer membrane and, consequently, in a better separation.

The modified procedure was as follows: Mitochondria from 4 livers (600 to 1000 mg protein) were suspended in 9 ml of Tris-phosphate buffer, 10 mM with respect to P_i (pH 7.4 to 7.5). After 5 to 10 min at 0 °C an equal volume of 1.8 M sucrose was added and the mixture was kept at 0 °C for another 5 min. The suspension was then subjected to sonic irradiation in portions of 3.5 ml for 10 sec in a Branson Sonifier Model S-75, operated at the output of 3.3 amp, the tip of the sonifier probe being placed 2 to 3 mm above the bottom of the test tube containing the sample. A jacket containing water and ice ensured cooling during sonication. The time of sonication, the energy output and the geometrical arrangement of the sonicator probe with the sample were found to be critical for obtaining maximal efficiency and the highest purity of the outer membrane fraction.[1] The suspension was then diluted twofold or more either with 10 mM Tris-P_i or with 150 mM KCl, thus decreasing the concentration of sucrose to 0.45 M or less, and layered on top of a discontinuous sucrose gradient consisting of 5 ml 40% (1.17 M) sucrose and 6 ml 26% (0.76 M) sucrose in 34-ml tubes of the 25 SW rotor of a Spinco preparative ultracentrifuge. The centrifugation was performed at 24,000 rpm ($RCF_{max} = 83,000 \times g$) for 4 hr or at 20,000 rpm ($RCF_{max} = 58,000 \times g$) for 6 hr. The outer membrane fraction was collected by puncturing the bottom of the tube with a syringe needle which was inserted, through the pellet, to the appropriate level.

This procedure routinely yielded up to 5% of mitochondrial proteins as the outer membrane fraction. Specific activity of the rotenone-insensitive NADH-cytochrome *c* reductase, a marker enzyme for the outer membrane, was in this fraction usually 6 to 15 times higher than in whole mitochondria, while succinate-cytochrome *c* reductase, a marker for the inner membrane, was 5 to 10 times lower. A further purification of the outer membrane fraction could be achieved by a second gradient centrifugation.

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1 Using the MSE 60 W sonicator with a 19 mm (diameter) probe the optimum sonication time was found to be 10 sec as well.

For the purpose of the present investigation, cytochrome *c* (final concentration 40 to 100 μM) was added either before or after sonication and the 1.8 M sucrose used to contract the mitochondria was made 300 μM with respect to KCl to prevent the binding of cytochrome *c* to the inner membrane [5]. For the same purpose, sucrose solutions used for the gradient centrifugation also contained 150 mM KCl and 10 mM Tris-HCl (pH 7.4). To keep cytochrome *c* fully in the reduced state, 10 mM succinate was also present in some experiments during the swelling and the sonication. It appeared, however, that this was not necessary, owing to the fact that endogenous substrates provided sufficient reducing power.

Determination of Marker Enzymes

Rotenone-insensitive NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase were determined spectrophotometrically as described before [19]. The medium contained 100 mM P_i (pH 7.4), 50 μM cytochrome *c*, 1 mM KCN, 2 μM rotenone and either 0.15 mM NADH or 2.5 mM succinate.

Measurements of Cytochrome c

Measurements of cytochrome *c* enclosed inside the membrane fragments were made with a dual-wavelength scanning spectrophotometer (Phoenix Precision Instruments Co., Philadelphia, Pa.) either at 550 nm *minus* 540 nm or at 550 nm *minus* 560 nm.

Protein Determination

Because of high sucrose concentration in the outer membrane fraction the biuret method could not be used. Therefore, protein was determined by solubilizing the material in 1.5% sodium deoxycholate or a 0.2% solution of the nonionic detergent Lubrol (I.C.I. Organics, Providence, Rhode Island), and measuring light absorbance at 280 nm, using serum albumin (also dissolved in the same detergent solution) as standard. Because specific absorbances at 280 nm of serum albumin and mitochondrial proteins differ considerably, values obtained for mitochondrial proteins were multiplied by a factor of 0.61 (found experimentally) to obtain true protein as based on the biuret method [3].

Results

Fragments of the outer mitochondrial membrane as obtained by the procedure of Sottocasa *et al.* form small sealed vesicles [19, 20]. It is presumed that these vesicles are sealed during the sonication step of the procedure and it can be expected that they enclose the medium in which sonication is carried out. Thus, they should contain cytochrome *c* if this is present during the sonication.

For each experiment, mitochondria were divided into three equal portions. To the first one cytochrome *c* (40 to 100 μM final concentration) was added after the mitochondria were contracted by hypertonic sucrose and before sonication. To the second portion (control) cytochrome *c* was added

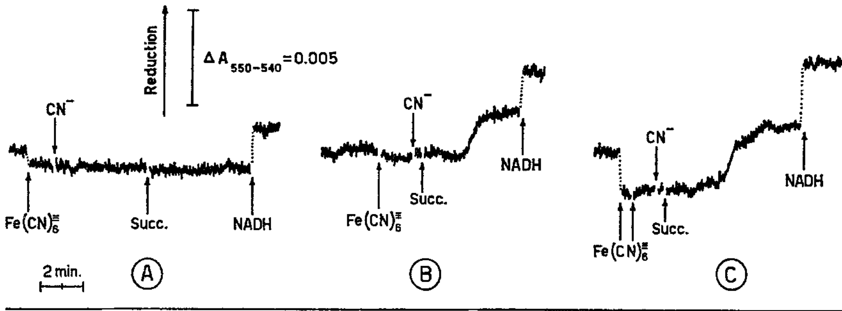

Fig. 1. Enclosure of cytochrome *c* in outer membrane vesicles. Each spectrophotometer cuvette contained 3.0 ml of approximately 0.3 M sucrose + 0.07 M phosphate buffer (pH 7.4) flushed with oxygen, and the outer membrane fractions equivalent to 2.7 mg protein. *A*, outer membrane fraction obtained with no contact with external cytochrome *c* (1st control); *B*, cytochrome *c* added after sonication (2nd control); *C*, outer membrane obtained in the presence of cytochrome *c* (cytochrome *c* present during sonication). Additions to the cuvettes (final concentrations): 1.7 μM ferricyanide (each arrow); 0.3 mM cyanide; 1 mM succinate (Succ.); 50 μM NADH; 1 μM rotenone was added at the beginning of each trace (not indicated). The traces represent absorbance difference between 550 nm and 540 nm

immediately after sonication. The third portion contained no added cytochrome *c* at all.

Added cytochrome *c* became reduced by contact with disrupted mitochondria, the reducing power being supplied either by endogenous substrates or by added succinate. Thus, it should be expected that it was enclosed in the reduced form. However, during the prolonged centrifugation and subsequent collection of the fractions the medium became aerobic and all cytochrome *c* present in a form accessible to cytochrome oxidase, possibly present as a contaminant, could be oxidized. Such an oxidation was insured by saturating with oxygen samples before assay.

Trace *A* in Fig. 1 shows that addition of ferricyanide to control particles obtained in the total absence of added cytochrome *c* causes only a negligible downward deflection at the wavelength pair used. Subsequent addition of cyanide and succinate were without effect, whereas the addition of NADH produced an upward deflection. Trace *B* reported in the same figure shows, on the contrary, that when cytochrome *c* had been added to the particles after sonication and before reisolation succinate was capable to produce an upward deflection, presumably due to a reduction of cytochrome *c* still present in the preparation. It may be noticed, however, that NADH causes the same extent of upward deflection in traces *A* and *B*. The obvious explanation for this finding is that the upward deflection produced by NADH

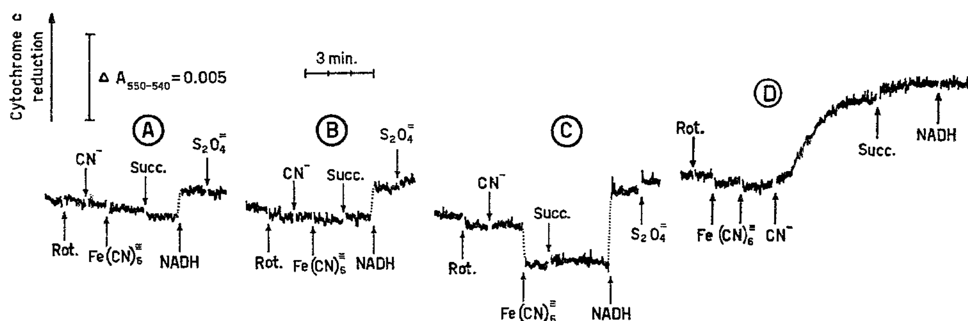

Fig. 2. Same experimental conditions as in Fig. 1 except that the outer membrane fraction was purified by a second gradient centrifugation. Traces *A*, *B* and *C* have the same meaning as in Fig. 1; outer membrane fraction is equivalent to 1.5 mg protein. Trace *D*, inner membrane fraction (7.9 mg protein) obtained by sonication in the presence of cytochrome *c*. Rotenone (Rot), 1 μM; dithionite (S_2O_4), a few crystals

in the two samples is due to the reduction of cytochrome b_5, as this is an integral component of the outer membrane which may contribute at the wavelength pair chosen. Besides, it has been shown previously [19] that upon addition of NADH after the total reduction of the respiratory chain by succinate the only component which can be reduced is cytochrome b_5. A remarkable difference is found when the experiment is carried out with particles sonicated in the presence of cytochrome *c*. Upon addition of ferricyanide a large downward deflection is observed (Fig. 1, trace *C*) indicating that a substantial amount of cytochrome *c* in the preparation was in the reduced state, i.e., inaccessible to cytochrome oxidase. A subsequent addition of KCN and succinate causes a reduction which, in contrast to trace *B*, is biphasic. NADH produces a further reduction considerably larger than in the control experiments shown in traces *A* and *B* of the same figure. This clearly indicates that particles sonicated in the presence of cytochrome *c* contain a portion of this pigment which is reducible only by NADH and not by succinate.

When the membranes were further purified by a second density gradient centrifugation to get rid of the last traces of soluble cytochrome *c* the picture is even clearer (Fig. 2). It may be seen in fact that both when cytochrome *c* was not added at all (trace *A*) or added after sonication (trace *B*) succinate has no effect at all and an upward deflection can be obtained only by addition of NADH. Again the extent of such a deflection is the same in both experiments. It may be noticed in addition that no cytochrome oxidase-inaccessible cytochrome *c* was present in either preparation as indicated by the fact that ferricyanide fails to produce any oxidation of the pigment. In

contrast, when cytochrome *c* was present during sonication, ferricyanide produces a large oxidation (trace *C*). Similarly, to traces *A* and *B*, succinate has no effect, whereas NADH causes a large reduction which corresponds to the sum of the oxidation produced by ferricyanide in the same trace, *plus* the upward deflection observed in the other two traces. This was expected if the upward deflection observed before in the control experiment was caused by the reduction of cytochrome b_5. In all the experiments presented, addition of dithionite virtually did not alter the level of the trace after NADH, indicating a complete reduction of the pigments.

Comparison of Figs. 1 and 2 reveals that purification of the outer membrane fraction by a second gradient centrifugation diminishes or completely removes that portion of cytochrome *c* which is reducible by succinate, but is basically without effect on the portion oxidizable by ferricyanide and reducible by NADH alone. Since these two portions of cytochrome *c* are easily distinguishable a single gradient centrifugation was performed throughout in further experiments.

All the evidence so far presented shows that when outer membrane vesicles are prepared in the presence of cytochrome *c* this pigment is enclosed in such a manner that (a) it cannot be washed out during gradient centrifugation, (b) it is not accessible to cytochrome oxidase, and (c) it can be readily reduced only by added NADH. Such properties are displayed by cytochrome *c* enclosed in the outer membrane and not in the inner membrane vesicles, as shown by trace *D* of Fig. 2 which reports a similar experiment performed with inner membrane particles obtained by the same procedure. It should be added that when the same procedure is applied to isolated liver microsomes (not shown in the figures) no NADH-reducible cytochrome *c* was detected. The explanation for this finding may be that either microsomes are more resistant to sonication than the outer mitochondrial membrane and therefore no cytochrome *c* is enclosed, or microsomal cytochrome b_5 has no access to cytochrome *c* enclosed in the vesicles. Whatever the explanation is, this result allows to exclude that the findings presented in Figs. 1 and 2 are to be ascribed to contaminating microsomes.

In the experiments described so far the oxidoreduction of cytochrome *c* was monitored at the classical wavelength pair 550 nm and 540 nm. Under these conditions a certain interference by changes in redox state of cytochrome b_5 was detected as already pointed out. Therefore, we have experimentally chosen a wavelength pair at which such an interference was minimized. The reference wavelength found was around 560 nm and changed slightly from one preparation to another, presumably due to changes in light scattering. Once the contribution by cytochrome b_5 has been abolished,

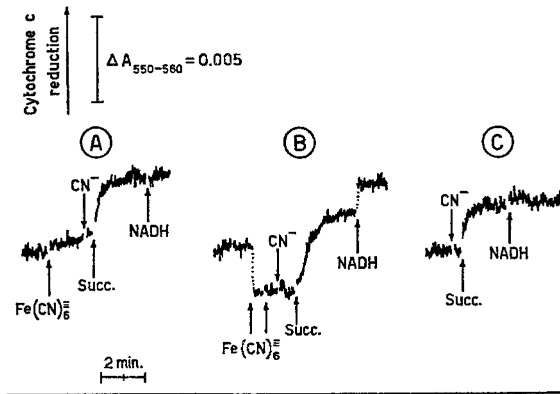

Fig. 3. Same experimental conditions as in Fig. 1. Absorbance difference between 550 nm and 560 nm was recorded. *A*, outer membrane fraction, cytochrome *c* added after sonication (2nd control); *B* and *C*, outer membrane fraction obtained in the presence of cytochrome *c*; in *C* no ferricyanide was added to the cuvette. Each sample corresponds to 3.0 mg protein. Ferricyanide, 0.3 μM; other additions as in Fig. 1

the picture becomes even clearer. All the changes detected are to be attributed only to cytochrome *c* (Fig. 3). Traces *A* and *B* which correspond to the control with cytochrome *c* added after sonication and the experimental sample with cytochrome *c* added before sonication, respectively, show essentially the same features presented in the two previous figures. It should be noted, however, that now an extra upward deflection produced by the addition of NADH could be observed in the experimental sample only (trace *B*).

We previously made the assumption that cytochrome *c* enclosed in outer membrane vesicles should not be oxidized by cytochrome oxidase and should be reduced only by NADH. If this is correct, we expect that without a previous oxidation by ferricyanide no extra reduction by NADH should be detected. This is shown in the experiment reported in trace *C* of Fig. 3. Surprisingly, the amount of cytochrome *c* oxidizable by ferricyanide in trace *B* was substantially larger than the amount which could be reduced only by NADH.

A similar phenomenon could be observed also in trace *C* of Fig. 1 after subtraction of the contribution by cytochrome b_5. It was observed, however, that whereas in the control experiments the reduction of cytochrome *c* by succinate was always monophasic, in the experimental sample the reduction by succinate often proceeded in a biphasic manner (Fig. 1, trace *C* and Fig. 3, trace *B*). The sum of the slowly succinate-reducible and NADH-reducible cytochrome *c* accounted precisely for the amount of the pigment

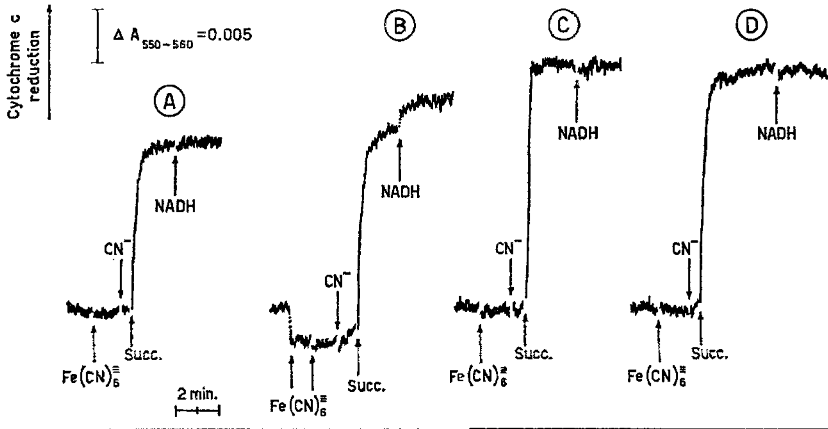

Fig. 4. Release of enclosed cytochrome *c* by sonication and detergents. Experimental conditions as in Fig. 1. Absorbance difference between 550 nm and 560 nm was recorded. Each sample contained an outer membrane fraction corresponding to 16 mg protein. Ferricyanide, 0.4 μM (each arrow). *A*, outer membranes, cytochrome *c* added after sonication (2nd control); *B*, outer membrane fraction obtained in the presence of cytochrome *c*; *C*, same as *B plus* nonionic detergent Lubrol added to final concentration of 0.2%; *D*, same as *B* but sonicated before assay in phosphate buffer for 90 sec

oxidizable only by ferricyanide. A tentative explanation for the slow phase reduction by succinate of enclosed cytochrome *c* will be presented in the Discussion.

If the outer membrane represents the sequestering membrane for that portion of cytochrome *c* in the preparation which is inaccessible to cytochrome oxidase and reducible only by NADH, then it is expected that such an inaccessibility should be abolished by any membrane-disrupting treatment. The effects of resonication and detergent such as Lubrol have been tested and the results are shown in Fig. 4. Clearly, the effects of ferricyanide and NADH shown in trace *B* are totally abolished in traces *C* and *D* (Lubrol treatment and sonication, respectively) which are indistinguishable from the control experiment (trace *A*). As expected, trace *A* indicates a lower response to succinate due to a smaller amount of cytochrome *c* present. As can be seen in Fig. 4, outer membrane preparation used in this experiment contained a relatively large proportion of cytochrome *c* reducible by succinate. This is, however, irrelevant to the interpretation presented here since only the portion of the pigment oxidizable by ferricyanide and reducible by NADH alone represents cytochrome *c* trapped inside the vesicles.

Assuming that the concentration of cytochrome *c* enclosed inside outer membrane vesicles is equal to the concentration of cytochrome *c* in the medium in which the vesicles have been formed (sonication medium) the

volume of the vesicles can be calculated. A series of experiments gave the values of 1.2 to 2.7 μliter/mg protein, with a mean of 1.84 μliter/mg protein. From this value the average volume and diameter of one vesicle can be calculated.

Assuming the membrane thickness as 7 nm [11], the density as 1.1 [14] and protein content as 55% [14], the value of 47 nm for the average diameter of perfectly spherical vesicles can be obtained. This corresponds to the lower limit of diameter of outer membrane vesicles obtained by Sottocasa *et al.* [19] and agrees fairly well with an average diameter of profiles of flattened vesicles obtained in the present study as measured using the negatively stained specimens.

Discussion

The present investigation supports the view presented earlier [23] that the outer mitochondrial membrane is impermeable to cytochrome *c*. It indicates that this impermeability is not only characteristic to the intact membrane, but is also preserved when the membrane is removed from mitochondria and separated in the form of vesicles. A similar enclosure of cytochrome *c* was not observed with the microsomal fraction, supplying a further evidence for the existence of substantial differences in some properties of the outer mitochondrial membrane and microsomal membranes. This is also an additional argument against the postulation of Green's group (*cf.* [1]) that the procedure of Sottocasa *et al.* [19, 20] developed for the isolation of the outer mitochondrial membrane results, in fact, in the separation of microsomal contaminations.

Inner membrane fragments sonicated in the presence of cytochrome *c* contained a certain amount of this cytochrome (Fig. 2*D*) apparently trapped inside the vesicles. It is, however, fully accessible to both cytochrome oxidase and succinate-cytochrome *c* reductase (*cf. also* [9]) and can be thus easily distinguished from the inaccessible cytochrome *c* sequestrated by the outer membrane fragments.

The fact that the enclosed, "inaccessible" cytochrome *c* could be slowly reduced by succinate was most likely due to a shunt operated by the ferricyanide/ferrocyanide system. Ferricyanide added to the cuvette to oxidize enclosed cytochrome *c* was, upon addition of succinate, subsequently reduced to ferrocyanide by succinate dehydrogenase (EC 1.3.99.1) of the inner membrane contaminations. Re-entering the vesicles, ferrocyanide slowly reduced enclosed cytochrome *c*. Experiments not described in the present report have shown that the rate of this slow-phase reduction is

dependent upon the concentration of ferricyanide added to the medium. Thus, this phenomenon was not an indication of a leakage of cytochrome *c* from the vesicles nor of a trans-membrane transfer of reducing equivalents as once suggested for semi-permeable membranes [2].

There is an analogy between sequestration of cytochrome *c* by the outer membrane fragments and trapping by artificial phospholipid membranes as described by Kimelberg and Lee [6].

The impermeability of the outer mitochondrial membrane to cytochrome *c*, and most likely to other proteins (e.g., trypsin, [8]), supports the idea that the intermembrane space forms a discrete compartment of the cell differing, with respect to enzyme composition, from the cytosol although sharing with the latter the pool of low molecular-weight compounds. Enzymic composition of the mitochondrial intermembrane space is not well known. Only adenylate kinase (EC 2.7.4.3) and nucleosidediphosphate kinase (EC 2.7.4.6) have been found so far [7, 16, 17, 19, 20]. In view of the finding of the present investigation it would be interesting to see whether free cytochrome *c* can also be present between the outer and the inner mitochondrial membranes. Cytochrome *c* is known to be tightly bound to the inner membrane, being released only by salts, e.g. KCl, at concentrations of 150 mM or more [5]. It might be speculated that at the concentration of salts in the cell, which is not much lower than that, a small part of mitochondrial cytochrome *c* is in a free form in the intermembrane space. If this is the case, one can expect that cytoplasmic NADH, easily penetrating the outer mitochondrial membrane [21] may reduce this free cytochrome *c* by the NADH-cytochrome *c* reductase system present in the outer membrane. Reduced cytochrome *c* can be subsequently reoxidized in the inner membrane, thus forming an additional system for the oxidation of cytoplasmic NADH. A similar hypothesis has been recently expressed by Skulachev [18]. However, it needs experimental verification.

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