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Arrangement of the Electric Potential-Generating Redox Chain in the Mitochondrial Membrane

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Abstract

The intramembrane arrangement of the respiratory chain generating electric potential difference across the mitochondrial membrane has been studied. The accessibility of various respiratory carriers to the non-penetrating electron donors and acceptors, such as ferri- and ferrocyanide, cytochrome *c*, fumarate and nicotinamide nucleotides has been used as a test for surface localization of the carrier in the membrane of mitochondria and "inside-out" (sonicated) submitochondrial particles. Membrane potential formation was detected by measuring the transmembrane flows of the penetrating anion, phenyl dicarbaundecaborane (PCB^-).

It is shown that ferricyanide reduction can support PCB^- movement if this electron acceptor interacts with intact mitochondria in the region localized on the oxygen site of the antimycin-sensitive point. The same region is accessible for ferrocyanide whose oxidation by O_2 can be also coupled with PCB^- translocation. Added nicotinamide nucleotides cannot be utilized by mitochondria for supporting PCB^- movement.

PCB^- movement in the "inside-out" submitochondrial particles can be supported by reduction of ferricyanide or fumarate by NADH, and of NAD^+ by NADPH, the former process being sensitive to rotenone but not to antimycin. Antimycin-insensitive reduction of ferricyanide or of CoQ_0 by succinate is not coupled with PCB^- transport. Neither ferrocyanide nor ferrocytochrome *c* can be used as electron donors in the particles.

Penetrating electron donors ($TMPDH_2$, succinate) and acceptors (menadione) are effective both in mitochondria and particles.

It is concluded that flavin and transhydrogenase regions of the potential-generating redox chain are localized near the inner surface, cytochrome *c* region—near the outer surface of the internal membrane of intact mitochondria. It means that the redox chain includes at least one act of the transmembrane transfer of reducing equivalents between flavins and cytochrome *c*.

Generation of the membrane potential at the expense of the redox chain-produced energy has been suggested by Mitchell as one of the postulates of the chemiosmotic scheme of oxidative phosphorylation.[1,2] The validity of this postulate was confirmed by experiments demonstrating electrophoretic character of the penetrating ion transport across the membrane of the respiring mitochondria; the ion transfer mechanism proved to be quite non-specific to the structure of penetrating ion, except the sign of charge. The directions of cation and anion flows were opposite.[3–6]

In the experiments described below an attempt was made to verify another postulate

of Mitchell's concept, namely transfer of reducing equivalents across the coupling membrane. It was shown that the mitochondrial redox chain providing energy for generation of the membrane potential includes at least one step of transmembrane hydrogen (electron?) transfer localized in the middle segment of the chain, between flavins and the cytochrome *c* region.

Methods

Isolation of rat liver mitochondria was carried out essentially according Mosolova *et al.*[7] The liver of rat starved for 20–24 h was minced by a metallic press (diameter of holes ≈0·5 mm). The mince was suspended in nine-fold amount of the isolation medium containing 0·25 M sucrose, 1.10^{-3} M ethylene diamine tetraacetate, 4.10^{-4} M Tris-HCl buffer (pH of the mixture 7·5). The suspension was homogenized for 40 sec by glass-Teflon homogenizer at low rate of rotation. The homogenate was centrifuged at 600 *g* for 15 min. The supernatant obtained was centrifuged at 10,000 *g* for 10 min, the sediment was diluted with nine-fold volume of the isolation medium and centrifuged at 200 *g* for 10 min; the sediment was removed, the supernatant was centrifuged at 10,000 *g* for 10 min. The mitochondrial sediment was washed once more by the isolation medium. The final sediment was suspended in the isolation medium (the final protein concentration was 100–120 mg/ml).

Phosphorylating (sonicated) submitochondrial particles were prepared from the "heavy" fraction of beef heart mitochondria[8] according to the method of Hansen and Smith[9] (for details of the procedure see ref. 3).

Phenyl dicarbaundecaborane (PCB^-)* concentration in the incubation mixture with mitochondria or submitochondrial particles was measured using phospholipid membrane as PCB^-—sensitive electrode. Details of this method developed by Liberman and Topali[10] were described earlier.[3]

Ferri- and ferrocyanide concentrations were followed using a Hitachi-356 spectrophotometer (410–510 nm spectral difference was measured).

Results

Studying the problem of the intramembrane arrangement of the redox chain we used as starting points two simple suggestions, namely (1) redox component unable to penetrate mitochondrial membrane being added to mitochondria will interact only with those respiratory carriers which are localized near the outer surface of the membrane, and (2) the point accessible for non-penetrating donors (acceptors) in mitochondria must be inaccessible in the "inside-out" submitochondrial particles and vice versa. Being interested primarily in the mechanism of the membrane potential generation we have studied a parameter which is closely related to the formation of the electric field in the membrane. To this end the PCB^- probe has been applied. As it was shown earlier[3–6] the PCB^- transfer across the coupling membranes is one of the most sensitive tests for the membrane potential. PCB^- anions easily penetrate hydrophobic membranes. Therefore the appearance of the electric potential difference on the membrane always

* Abbreviations: PCB^-, phenyl dicarbaundecaborane anion; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

results in the redistribution of PCB^- across the membrane causing the change in concentration of the free PCB^- in the incubation mixture. Measuring PCB^- concentration changes by very sensitive phospholipid membrane method one can follow the formation of electric potential difference across mitochondrial membrane.[3,4]

PCB^- Transport Coupled with Oxidations—Reductions of Nicotinamide Nucleotides

Experiments with mitochondria and "inside-out" submitochondrial particles showed that added nicotinamide nucleotides can be used for the membrane potential generation in the latter but not in the former system.

As it is seen in Fig. 1A, upper curve, addition of NADH to mitochondria previously equilibrated with PCB^- without available energy does not influence the PCB^- concentration in the extramitochondrial solution. Subsequent addition of the penetrating NAD^+-linked substrates causes the PCB^- efflux from mitochondria measured as an increase in the PCB^- concentration in incubation mixture, the effect testifying to the formation of the electric potential difference across the mitochondrial membrane (the sign "minus" inside the mitochondrion). Inhibition of oxidation of endogenous NADH by rotenone reverses the effect inducing the PCB^- influx.

Addition of NADH to the "inside-out" submitochondrial particles (Fig. 1A, lower curve) results in a decrease in the free PCB^- concentration. In this case the PCB^- uptake is caused by formation of the potential difference across the particle membrane, the "minus" outside the particle. Again, rotenone treatment is inhibitory, inducing the PCB^- efflux.

The amplitude of the PCB^- concentration changes in the case of submitochondrial particles was always much larger in comparison to mitochondria. This difference is due to the fact that PCB^- anions extruded from mitochondria are greatly diluted in the extramitochondrial solution which occupies by far the greater portion of the experimental cell.

Figure 1B demonstrates the responses of the submitochondrial particles to the consecutive additions of four nicotinamide nucleotides. One can see that the treatment with NADPH and NAD^+ results in the PCB^- influx while that with NADH and $NADP^+$ in the PCB^- efflux. These responses are due to the PCB^- movement coupled with operation of the energy-linked transhydrogenase.[3] So, both NADH dehydrogenase and transhydrogenase of the submitochondrial particles are accessible to the added nicotinamide nucleotides.

Effects of Ferri- and Ferrocyanides

Figure 2 shows results of the ferricyanide reduction measurements. It is seen that this process which can be shown both in mitochondria and particles is sensitive to antimycin and rotenone in the former but not in the latter case. These data show that mitochondria and particles under conditions used demonstrate the characteristic difference in the ferricyanide reduction pathways which was firstly observed by Estabrook.[11,12] Further experiments revealed that antimycin and rotenone-sensitive reduction of ferricyanide can support the PCB^- movement. As is shown in Fig. 3A, oxidation of β-hydroxibutyrate by ferricyanide results in the PCB^- efflux from mitochondria, the process being sensitive to antimycin. NADH oxidation by ferricyanide in the particles induces the uptake of some portion of the PCB^- anions. Rotenone treatment reverses the PCB^- accumulation

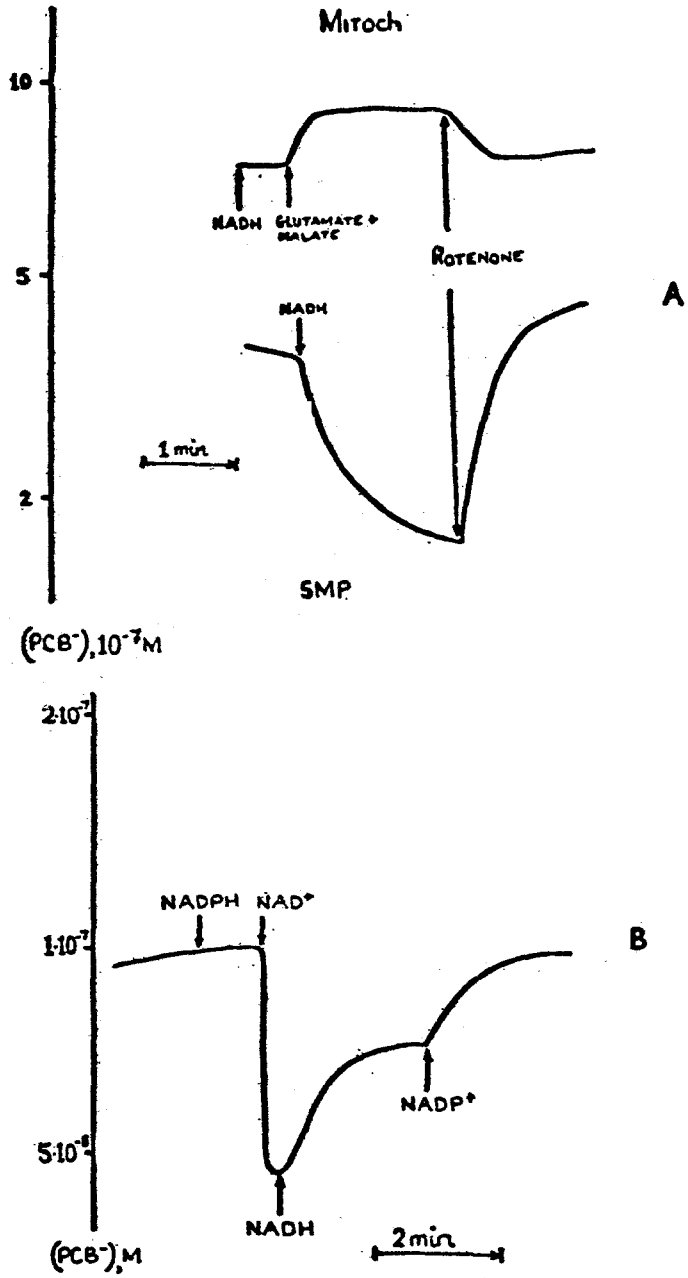

Figure 1. PCB^- transport supported by oxidations—reductions of nicotinamide nucleotides. Incubation mixture: 0·25 M sucrose, 0·05 M Tris-HCl buffer, 6.10^{-3}M $MgSO_4$. Here and below—pH 7·5, room temperature.

A. The upper curve—experiment with rat liver mitochondria (1·8 mg of protein/ml). The lower curve—experiment with beef heart submitochondrial particles (0·6 mg of protein/ul). Additions: 1.10^{-3} M NADH, 5.10^{-3} M glutamate + 5.10^{-3} M malate, 3.10^{-5} rotenone.

B. Incubation mixture with submitochondrial particles (0·6 mg of protein/ml) was supplemented with 0·01 M succinate and 3.10^{-3} M NaCN. Additions: 1.10^{-3} M NADPH, NAD^+, NADH, and $NADP^+$.

process in spite of the high rate of rotenone-resistant ferricyanide reduction. Apparently electron transfer from NADH to ferricyanide taking place in the rotenone-treated particles by-passes the energy coupling sites.

Oxidation of NADH by fumarate was coupled with the PCB^- uptake by particles. Addition of rotenone resulted in the loss of the whole portion of accumulated PCB^- (Fig. 3B).

In another experiment ferricyanide was found to be without any effect on the trans-hydrogenase-supported PCB^- movements in the particles.

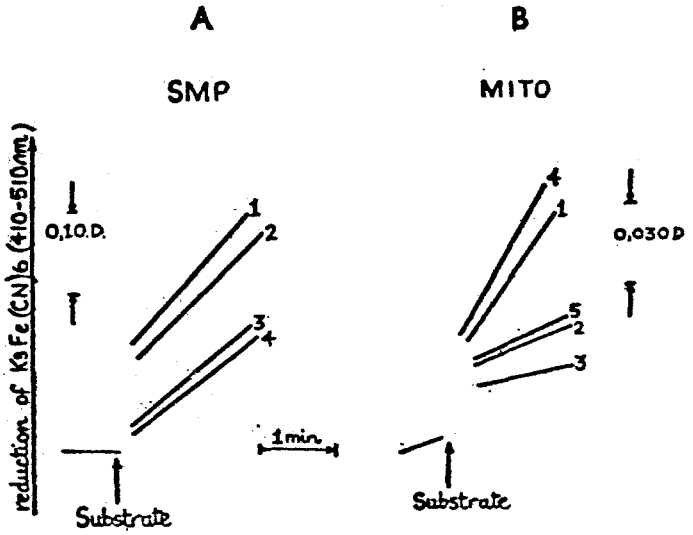

Figure 2. Ferricyanide reduction in mitochondria and submitochondrial particles. Incubation mixture: 0·25 M sucrose, 0·05 M Tris-HCl buffer 1.10^{-3} M $K_3Fe(CN)_6$, $2{\cdot}5.10^{-3}$ M NaCN. The mixture was supplemented with (A) submitochondrial particles (0·6 mg of protein/ml) or (B) with mitochondria (0·8 mg of protein/ml).

Exp. A.: curve 1—1.10^{-4} M NADH, alcohol dehydrogenase (0·1 mg/ml), 0·9% ethanol, 1.10^{-6} M rotenone, 2.10^{-6} M antimycin A; curve 2—rotenone and antimycin were omitted; curve 3—0·01 M succinate; curve 4—0·01 M succinate and 2.10^{-6} M antimycin.

Exp. B: curve 1—5.10^{-3} M glutamate, 5.10^{-3} malate; curve 2—as (1) with 2.10^{-6} M antimycin; curve 3—as (1) with 2.10^{-6} M antimycin and 1.10^{-6} M rotenone; curve 4—0·01 M succinate; curve 5—0·01 M succinate and 2.10^{-6} M antimycin.

Figure 4 demonstrates ferrocyanide oxidation in mitochondria and particles. It is shown that mitochondria rapidly oxidize ferrocyanide *via* the cyanide-sensitive pathway. Particles oxidize only a small portion of ferrocyanide, the process being insensitive to cyanide. Respectively, mitochondria can translocate PCB^- at the expense of ferrocyanide oxidation energy in the cyanide-sensitive fashion while particles cannot. This difference is not the result of a damage in the third site of the energy coupling in the particles since initiation of the electron transfer *via* this site by addition of the penetrating electron donor, $TMPDH_2$, results in the PCB^- influx into the particles (Fig. 5). In another experiment it was shown that ferrocytochrome *c*, like ferrocyanide, does not induce the PCB^- accumulation in the particles.

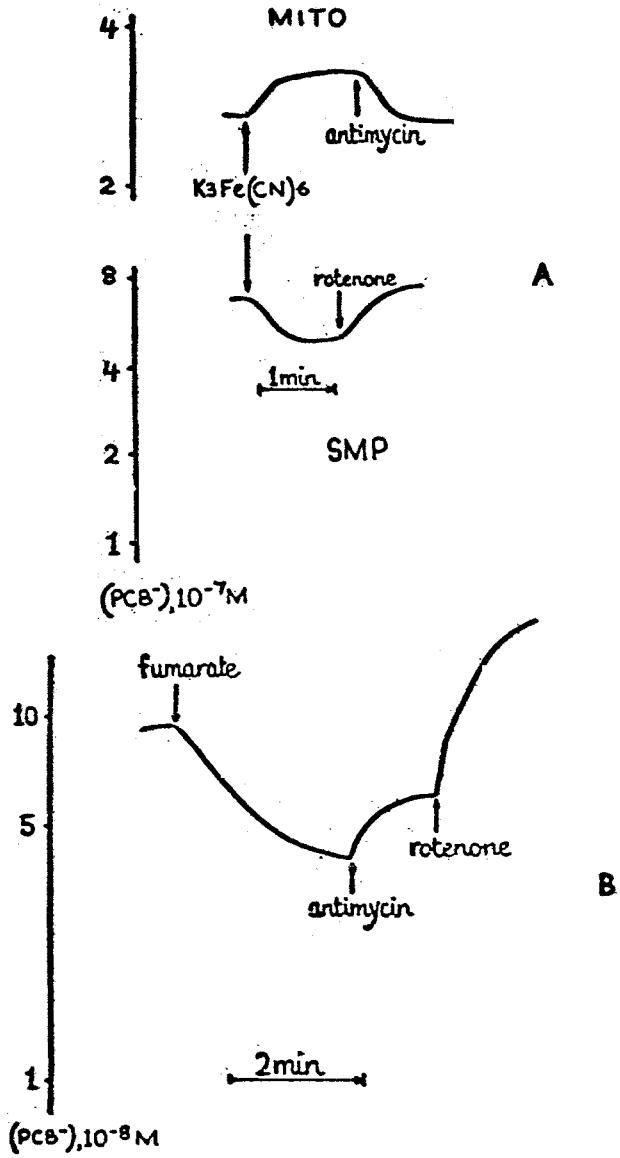

Figure 3. PCB⁻ transport supported by reduction of ferricyanide and fumarate.

A. Incubation mixture (0·25 M sucrose, 0·05 M Tris-HCl) was supplemented in experiment showed by the upper curve with mitochondria (1 mg of protein/ml), 3.10^{-3} M NaCN and $1{\cdot}3.10^{-3}$ M β-hydroxibutyrate, in experiment showed by the lower curve—with submitochondrial particles (0·7 mg of protein/ml), alcohol dehydrogenase (0·1 mg/ml), 0·9% ethanol, 8.10^{-4} M NADH, $2{\cdot}5.10^{-3}$ M NaCN and 1.10^{-4} M antimycin. Additions: 1.10^{-3} M $K_3Fe(CN)_6$, 3.10^{-4} M antimycin, 1.10^{-4} M rotenone.

B. Incubation mixture: 0·25 M sucrose, 0·05 M Tris-HCl, 6.10^{-3} M $MgSO_4$, alcohol dehydrogenase (0·13 mg/ml), 0·9% ethanol, 1.10^{-3} M NADH, 2.10^{-3} M NaCN, submitochondrial particles (0·9 mg of protein/ml). Additions: 0·01 M fumarate, 8.10^{-7} M antimycin, 3.10^{-5} M rotenone.

Effects of CoQ and Menadione

The above described experiment with TMPD (Fig. 5) clearly showed that the difference between mitochondria and particles does not reveal itself if penetrating compounds are used as the electron donor ($TMPDH_2$) and acceptor (O_2). This conclusion was confirmed in the experiments with menadione (Fig. 6). It is seen that addition of menadione to both mitochondria and particles causes the characteristic changes in the PCB^- concentration: the PCB^- efflux from the mitochondria and influx into the particles. In both cases the process was sensitive to rotenone. Antimycin was without effect, the fact suggesting that menadione reacts with the redox chain before the second site of the energy coupling.

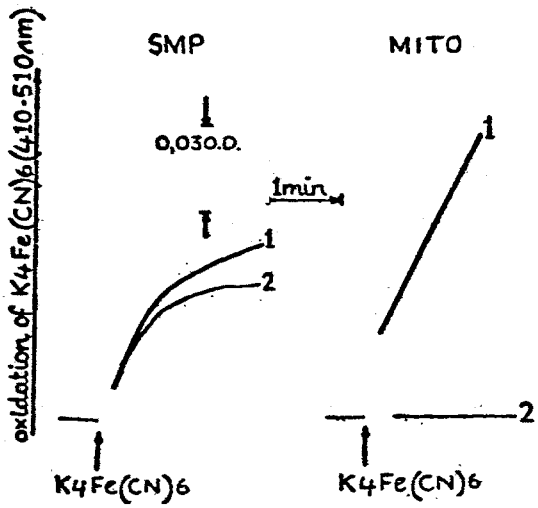

Figure 4. Ferrocyanide oxidation in mitochondria and submitochondrial particles. Incubation mixture: 0·25 M sucrose, 0·05 M Tris-HCl, submitochondrial particles (0·8 mg of protein/ml) or mitochondria (2 mg of protein/ml). Left-hand figure: curve 1—without inhibitors, curve 2—with 2.10^{-3} M NaCN. Right-hand figure: curve 1—with 4.10^{-6} M antimycin, curve 2—4.10^{-6} M antimycin and 2.10^{-3} M NaCN. Additions: 1.10^{-3} M $K_4Fe(CN)_6$.

As was shown in the further experiments (Fig. 7) the point of interaction of CoQ_0 proved to be close to that of menadione. It is seen (Fig. 7A) that oxidation of NADH by CoQ_0 in particles leads to the absorption of PCB^-. Rotenone induces the loss of PCB^- accumulated (Fig. 7A, B). Oxidation of succinate by CoQ_0 does not support the PCB^- influx (Fig. 7A). Spectrophotometric measurements showed that succinate rapidly reduces CoQ_0 in a antimycin- and rotenone-insensitive manner. Reduction of CoQ_0 by NADH measured under the same conditions was partially inhibited by rotenone.

Discussion

The main result of the above experiments consists in that the non-penetrating donors and acceptors of electrons interact with the redox chain of the "inside-out" particles at

levels other than those in intact mitochondria. The cytochrome *c* region of mitochondria proved to be the only site where the non-penetrating donors (acceptors) can get into contact with the redox chain supporting membrane potential generation. This region turned out to be inaccessible in the "inside-out" particles whereas the beginning of the chain (flavin and transhydrogenase regions) can be attacked by non-penetrating donors (acceptors). Penetrating compounds interact with the respiratory chain independently of the membrane orientation.

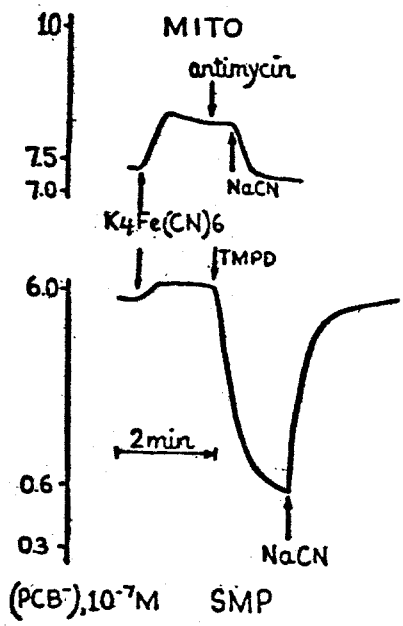

Figure 5. PCB^- transport supported by ferrocyanide oxidation. Incubation mixture (0·25 M sucrose, 0·05 M Tris-HCl buffer) was supplemented with mitochondria (1·6 mg of protein/ml), 8.10^{-3} M ascorbate and 2.10^{-4} M rotenone (upper curve), or with submitochondrial particles (0·9 mg of protein/ml) and 4.10^{-3} M ascorbate (lower curve). Additions: 1.10^{-3} M $K_4Fe(CN)_6$, 2.10^{-4} M antimycin, 2.10^{-3} M NaCN, 8.10^{-3} M TMPD.

The data obtained can be discussed in terms of possible versions of the intramembrane arrangement of the redox chain. Such versions are given in Fig. 8.

Version I describes the type of mitochondrial membrane organization when respiratory chains are localized on both sides of the hydrophobic barrier existing in the internal part of the membrane. According to version II, the respiratory chain is situated in the membrane interior, and two hydrophobic layers separate the chain from the extramembrane space. In version III the membrane is asymmetric, since respiratory carriers are only on one surface of the membrane. As to version IV, respiratory carriers occupy both surfaces of the membrane as in version I but, nevertheless, the membrane is asym-

metric: two membrane surfaces differ in sets of respiratory carriers. It is easy to realize that the presented data are incompatible with versions I, II and III. In the first case non-penetrating electron donors (acceptors) could react in the similar manner with both mitochondria and the "inside-out" particles; in the second case they could react with neither mitochondria, nor particles; in the third case the reaction must take place in mitochondria but not in particles, or in particles, but not in mitochondria.

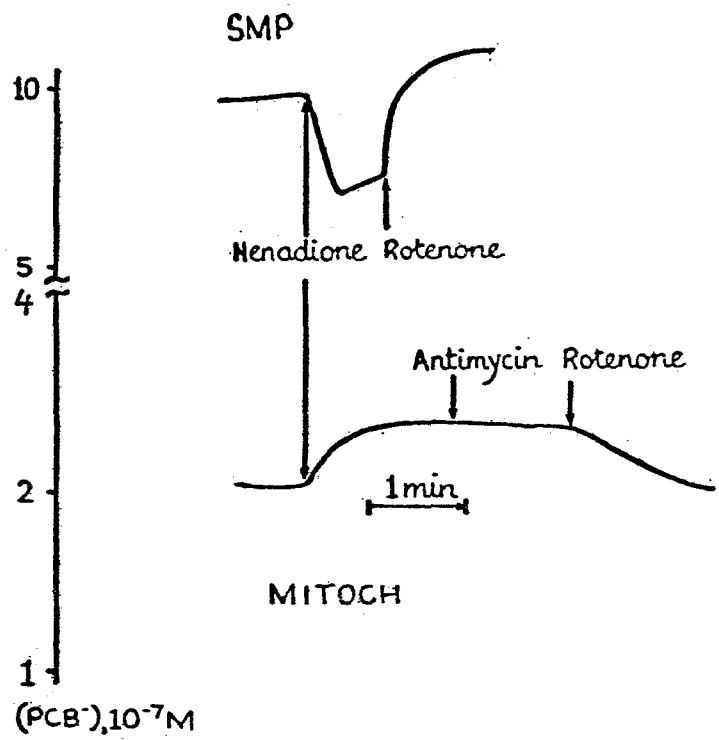

Figure 6. PCB^- transport supported by menadione reduction. Incubation mixture (0·25 M sucrose, 0·05 M Tris-HCl buffer) was supplemented with mitochondria (1·8 mg of protein/ml), 5.10^{-3} M glutamate, 5.10^{-3} M malate and 2.10^{-3} M NaCN (lower curve), or with submitochondrial particles (0·6 mg or protein/ml), alcohol dehydrogenase (0·1 mg/ml), 0·9% ethanol, 5.10^{-4} M NADH, 1.10^{-6} M antimycin and $2{\cdot}5.10^{-3}$ M NaCN (upper curve). Additions: 8.10^{-4} M menadione, 1.10^{-6} M antimycin, 5.10^{-6} M rotenone.

According to version IV non-penetrating donors (acceptors) can be reactive with both mitochondria and particles but their enzymatic partners in those two systems must be different. This was the case.

The simplest explanation of the obtained data consists in that the beginning of the redox chain is localized near the inner surface, the end of the chain—near the outer surface of the membrane of the intact mitochondrion, the middle step(s) of the chain being responsible for the connections between the initial and the terminal steps. A version of such a scheme is shown in Fig. 9.

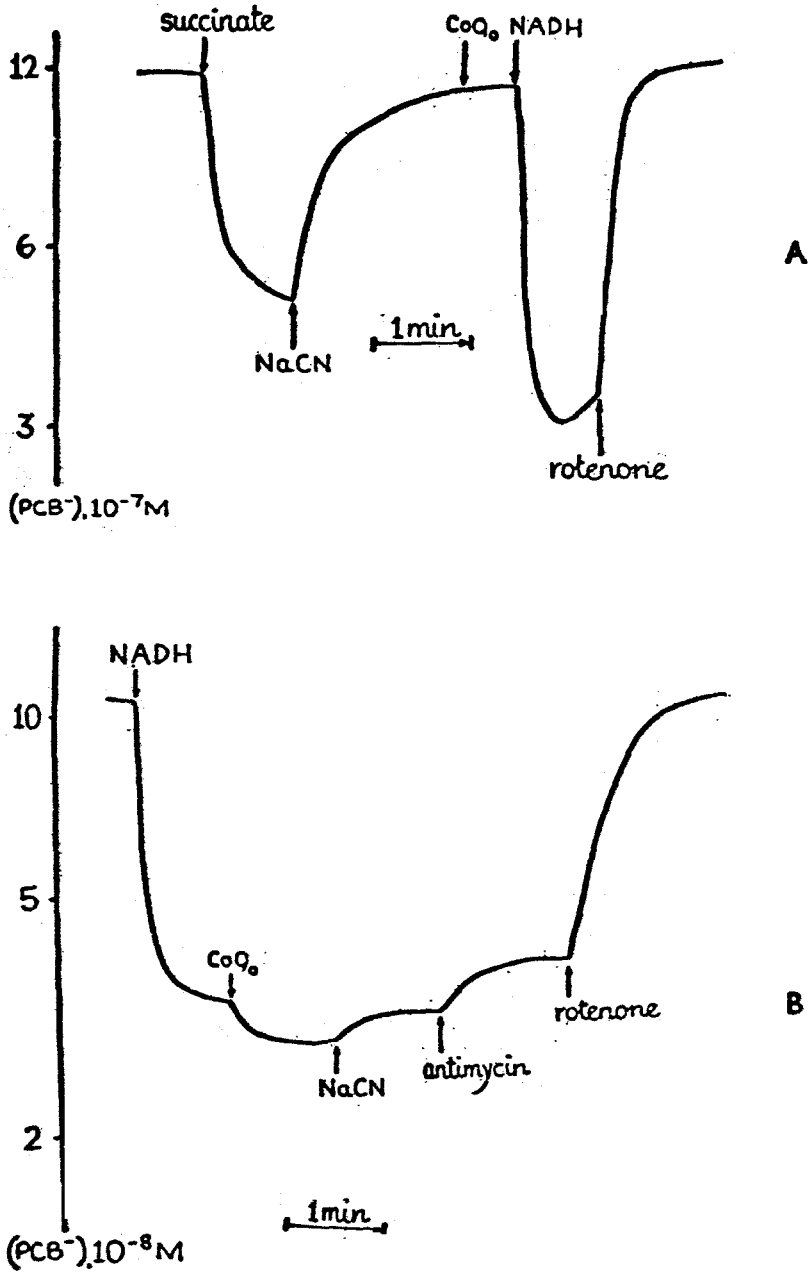

Figure 7. PCB^- transport supported by reduction of the added CoQ_0.

A. Incubation mixture: 0·25 M sucrose, 0·05 M Tris-HCl buffer, submitochondrial particles (0·7 mg of protein/ml). Additions: 0·01 M succinate, 2.10^{-3} M NaCN, 3.10^{-4} CoQ_0, $1{\cdot}10^{-3}$ M NADH, 5.10^{-6} M rotenone.

B. Incubation mixture: 0·25 M sucrose, 0·05 M Tris-HCl buffer, alcohol dehydrogenase (0·1 mg/ml), 0·9% ethanol, submitochondrial particles (1·4 mg of protein/ml). Additions: 1.10^{-3} M NADH, 1.10^{-3} CoQ_0, 0.01 M NaCN, 2.10^{-6} M antimycin, 1.10^{-6} M rotenone.

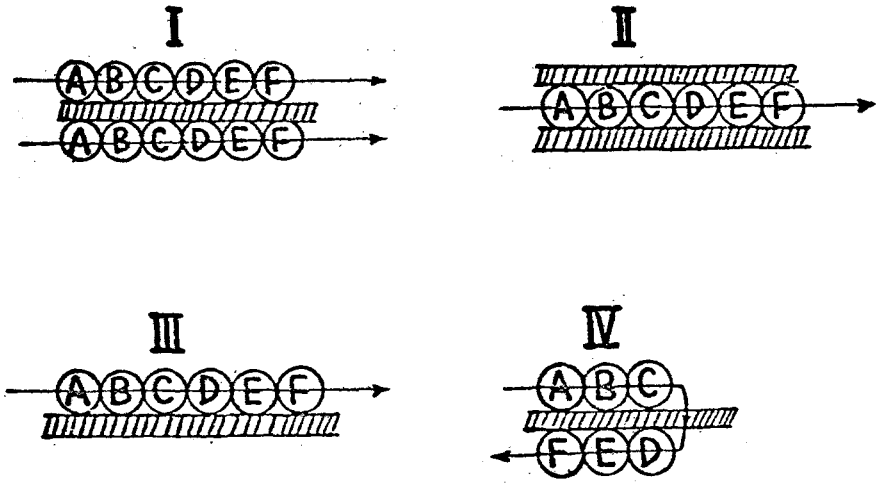

Figure 8. Different versions of the intramembrane arrangement of respiratory carriers. A, B, C, D, E, F—respiratory carriers. Direction of electron and hydrogen transfer is indicated by arrows.

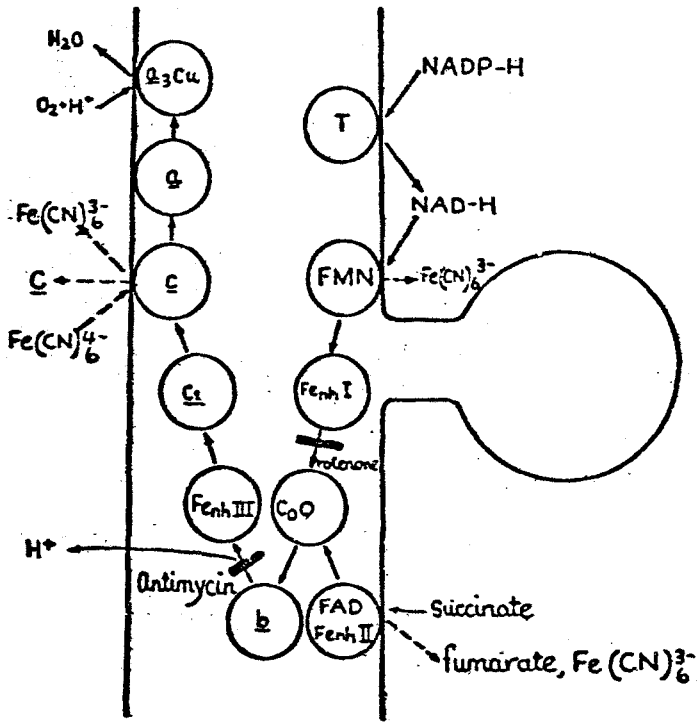

Figure 9. Possible arrangement of the respiratory chain including one step of the transmembrane movement of reducing equivalents. T-transhydrogenase. Stoichiometric coefficients of hydrogen and electron transfer reactions are not indicated.

According to this scheme, the beginning of the respiratory chain including transhydrogenase (T) and flavins is localized in the mitochondrial membrane close to its inner (knob-bearing) surface. The redox couple "CoQ–cytochrome *b*" is engaged in the translocation of reducing equivalents across the membrane. Cytochromes c_1, *c*, *a*, a_3 are localized on the outer surface. The redox chain as a whole is arranged in a helix-like fashion occupying the base piece of the knob which participates in the utilization of the redox chain-released energy.

It is possible, however, that the arrangement of the redox chain can be more complicated than is shown in Fig. 9. So, Mitchell's chemiosmotic scheme[2, 13] includes eight stages of transmembrane movement of reducing equivalents. The above results being discussed in terms of the Mitchellian redox chain necessitates some modifications of the chemiosmotic scheme. One of the modifications concerns the position of CoQ. This carrier seems to be operative in the first "loop" (e.g. between flavins and cytochrome *b*) rather than in the second one since (a) reduction of added CoQ by succinate is not coupled either with the membrane potential generation (see this paper, Fig. 7) or ADP phosphorylation[14] while that by NADH is, and (b) redox potential of endogeneous CoQ is near 0 instead of about + 0·25 V,[15] i.e. it is too negative for a hydrogen carrier involved in the second "loop". In the position occupied by CoQ in Mitchell's scheme, an unknown hydrogen carrier should be postulated.[16] Some complications arise if one tries to apply Mitchell's scheme to the fourth coupling site (transhydrogenase reaction). The process of hydrogen transfer from one nicotinamide nucleotide to another occurs without the H^+ exchange with water.[17] To overcome this difficulty a scheme was put forward suggesting that H^+ ions transferred across the membrane are added to ionized phosphate groups of bound NADH (for details see ref. 18).

Whatever the final solution of the problem of the intramembrane arrangement of respiratory enzymes the available data are sufficient for the conclusion that the reducing equivalents moving along the potential-generating redox chain traverse at least once the inner mitochondrial membrane. This conclusion is supported by experiments on PCB^- transport described above as well as by some other observations suggesting the localization of the respiratory chain flavoproteins and of cytochrome *c* on different sides of the mitochondrial membrane (for review see ref. 16).

If reducing equivalents traverse the membrane in a charged form (e.g. as e^- or H^-), the respiratory chain can charge the membrane without participation of a high-energy intermediate ($X \sim Y$). If it is an electrically neutral component (e.g. H) the respiratory chain *per se* is unable to produce the membrane potential unless the energy of oxidation is transformed into that of $X \sim Y$. This question is now under investigation.

Acknowledgements

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