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CONVERSION OF BIOMEMBRANE-PRODUCED ENERGY INTO ELECTRIC FORM

IV. GENERAL DISCUSSION

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SUMMARY

Possible mechanisms of the energy-dependent charge-specific ion transfer through the membranes of mitochondria, submitochondrial particles and bacterial chromatophores are considered.

It is concluded that penetrating ions move in the electric field orientated across energy-producing membranes and supported by electron and hydrogen transfer or ATP hydrolysis. The following three possibilities for the generation of membrane potential are discussed:

1. Both the respiratory chain and ATPase operate as H^+ pumps thereby creating a potential difference across the membrane (Mitchell's scheme).

2. There exists a special pump driven by hydrolysis of a high-energy intermediate of oxidative phosphorylation.

3. Only the respiratory chain can directly produce the membrane potential, whereas ATP energy can be utilized for this purpose *via* reverse electron transfer.

In the first case, the function of the membrane potential should be the coupling of oxidation and phosphorylation. In the second and the third cases the membrane potential would be functioning as an 'energy buffer' and (or) as a mechanism for transport of penetrating compounds through the energy-producing membranes against concentration gradients.

The transfer of synthetic penetrating ions as a probe for membrane potential

Experimental data presented in the three previous communications[1-3] can be summarized as follows:

(1) Sonicated submitochondrial particles, intact mitochondria and chromatophores of *Rhodospirillum rubrum* are capable of the energy-dependent accumulation of synthetic ionized compounds.

(2) The mechanism of ion accumulation is operative with ionized compounds of various structure which penetrate across phospholipid membranes.

(3) It is the sign of the charge of the penetrating compound, rather than other

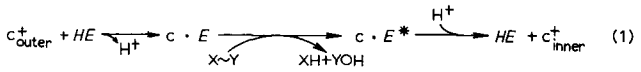
details of its structure, that determines the direction of movement of the ion across the membrane. Cations and anions move through the energy-producing biomembranes in opposite directions.

(4) Reversal of the orientation of the membrane results in a change in direction of the ion movement. On transition from a nonenergized to an energized state, mitochondria extrude penetrating anions into the external solution, whereas sonicated submitochondrial particles take up these anions.

(5) Electron transfer *via* any of the coupling sites of the redox chain, or hydrolysis of ATP (and of inorganic pyrophosphate in chromatophores), can supply the energy for the charge-specific ion transfer process.

(6) Accumulation of penetrating anions is coupled to an alkalinization of the suspending medium, accumulation of cations to acidification.

All these findings are in agreement with MITCHELL'S hypothesis of oxidative phosphorylation. According to MITCHELL, the redox chain and ATPase are able to separate charges in the membrane, thereby generating a potential difference between the outer and inner spaces of mitochondria, submitochondrial particles or chromatophores. MITCHELL[4–6] has summarized a number of indications suggesting the generation of an electric field in mitochondrial membranes at the expense of respiration and ATP hydrolysis. These indications were derived mainly from studying the active transport of metallic ions into mitochondria as well as the accompaning pH changes; these phenomena could be easily explained by the assumption that the electric field orientated across the inner mitochondrial membrane is the motive force for the accumulation of ions. However, it was difficult to rule out the alternative explanation that the active transport of cations in mitochondria is carried out by special 'translocases' localized in the membrane. It was suggested[7] that a complex of translocase with the transported ion undergoes some energy-linked conformational transition which is coupled to the hydrolysis of the intermediate of oxidative phosphorylation (Eqn. 1).



(1)

where C^+ is a transferred cation, H*E* is a translocase, the enzyme capable of exchanging C^+ for H^+, and *E**, the translocase in its altered conformation. It is proposed that the translocation of the bound cation C^+ from the outer side of the membrane to the inner side takes place when the conformational transition $E \longrightarrow E^*$ occurs. Each step of the process (1) can be illustrated by certain biological precedents from the fields of the biochemistry of antibiotics, contractile proteins and others.

The results obtained in the experiments with synthetic ions are inconsistent with the conformational hypothesis of active transport. Eqn. 1 does not explain why cations and anions are translocated in opposite directions. Furthermore, it is hardly possible that synthetic compounds of very different structure can be exchanged for H^+ (or OH^-) and then transferred across the membrane, being bound to a translocase adapted for the transfer of some natural ion. For this reason the 'translocase' scheme cannot be saved by the hypothesis postulating the existence of two translocases, cationic and anionic, operating in opposite directions. On assuming this hypothesis, one is compelled to propose that the 'cation translocase' is able to transfer

compounds as different as Ca^{2+}, the K^+ – valinomycin complex, dibenzyl dimethyl ammonium, tetrabutyl ammonium and triphenyl methyl phosphonium. By the same token, the 'anion translocase' ought to transport phenyl dicarbaundecaborane, tetraphenyl boron, picrate and I^- anions.

Rejecting the concept of 'the everything translocase', we must consider the electrophoretic scheme of ion transport. The latter hypothesis suggests that ion accumulation against a concentration gradient is due to the movement of ions down the electrical gradient across the membrane.

The above-mentioned properties of the ion transport system across the mitochondrial membrane, incompatible as they are with the 'translocase scheme', have proved to be the direct and indispensable consequence of the electric nature of the observed phenomenon. Indeed, cations and anions, if transferred in an electric field, should move in opposite directions. The system of ion translocation supported by the energy of a membrane potential should be nonspecific for the ion structure; it should be operative with any ionized compound capable of penetrating through the membrane. This explains the striking (from any other points of view) adequacy of artificial membrane models for describing the process of ion transport in mitochondria and the related phenomenon of the uncoupling of oxidative phosphorylation.

We have studied about 40 compounds of various structure capable of increasing artificial membrane conductance. In all cases, without a single exception, the compounds affecting the membranes display a definite and predictable effect on the mitochondrial functions. If the compound investigated penetrated across the phospholipid membrane as a cation, then it was accumulated in an energy-dependent way in mitochondria but not in particles. If it was an anion, it accumulated in particles but not in mitochondria. If the compound increased the conductance of membranes by inducing H^+ permeability, it proved to be an efficient uncoupler in both mitochondria and particles. By using an artificial membrane model the action of a number of agents on mitochondria and particles was predicted. It is in this way that synthetic penetrating ions as well as a new class of strong uncouplers (derivatives of borane) were discovered.

It should be stressed that the data obtained cannot be explained by the assumption that a transition to the 'energized' state is accompanied by the appearance of chemical groups capable of binding the penetrating ions in the membrane. If this were the case, the effect would not depend on the orientation of the mitochondrial membrane and would reveal itself in a similar way on addition of penetrating cations (or anions) to both mitochondria and 'inside out' submitochondrial particles. Should we choose to explain the above data in terms of ion binding rather than ion transfer, we would be compelled to accept a number of improbable postulates, such as (1) ions which penetrate through the phospholipid membrane are unable to move through the mitochondrial membrane; (2) ions which cannot be absorbed by either artificial membranes or by natural membrane under a deenergized state, for instance iodide acquire the ability to combine with the natural membrane under its transition to the energized state; (3) for effect (2) the corresponding ion carrier, capable of transfering this ion across phospholipid membranes, is necessary (see p. 25 of the previous paper[3]); (4) penetrating cations such as dibenzyl dimethyl ammonium (*plus* phenyl dicarbaundecaborane anion) or K^+ (*plus* valinomycin) accumulate not in the matrix but in the membrane phase of mitochondria, and so on.

It is noteworthy that the uptake of synthetic cations by mitochondria is accompanied by a swelling of the mitochondrial matrix, similar to what is observed during Ca^{2+} accumulation (see ref. 2, Fig. 7). This observation indicated that synthetic cations are accumulated in the matrix rather than in the mitochondrial membrane. Swelling of the matrix suggests also that cations are pumped into mitochondria against a concentration gradient. To cause swelling, the concentration of ions accumulated in the matrix should be sufficient to increase appreciably the intramitochondrial osmotic pressure. The ion concentration inside mitochondria is not less than 0.3 M. In the experiment mentioned above, dibenzyl dimethyl ammonium cations were added to the sample up to a final concentration of 0.001–0.005 M. To induce swelling, the concentration of these cations in mitochondria had to be increased many times.

Assuming movement in the electric field to be the only possible explanation for the effects of the penetrating ions described above, we are bound to accept Mitchell's postulate that mitochondria can transform the energy of respiration (or ATP hydrolysis) into the electric energy of a membrane potential.

Measurement of the movement of penetrating ions across the mitochondrial membrane is evidently the direct and the most reliable probe for a membrane potential in objects as small as intracellular organelles. In this case the electric field, *i.e.* the possibility to carry out the work of charge translocation, can be detected by the appearance of a concentration gradient of penetrating ions between the inner and outer spaces of the organelle.

It is particularly profitable to use ions, such as phenyl dicarbaundecaborane, which possess a very high penetrating ability. The concentration of this ion can be measured applying the phospholipid membrane method down to a level of 10^{-9} M. Using low concentrations of phenyl dicarbaundecaborane one can measure the active anion transport process without any significant changes in internal pH.

The properties of phenyl dicarbaundecaborane are not unique. Very similar responses were found with tetraphenyl boron, but we prefer to use the former since, unlike tetraphenyl boron, it does not bind K^+. The high penetrating ability of these two anions is apparently due to delocalization of the negative charge of the ionized atom and its screening by hydrophobic substituents. The membrane potential can also affect the distribution of some other ions, such as picrate, I^- anions in the presence of di(pentafluorophenyl) mercury, dibenzyl dimethyl ammonium, tetrabutyl ammonium, and triphenyl methyl phosphonium cations, whose penetrating ability, while still measurable, is not as high as that of the two anions mentioned above. It is probable that some hydrophobic derivatives of sulfonic acid used by CHANCE[8,9], such as bromothymol blue and *N*-anilino naphthalene sulfonate, might also move across the mitochondrial membrane in the electric field which should affect the distribution of bromothymol blue and *N*-anilino naphthalene sulfonate between the inner and outer spaces and, as a consequence, their concentration within the mitochondrial membrane. In fact, MITCHELL[10] observed the efflux of bromothymol blue from mitochondria and JACKSON AND CROFTS[11] the bromothymol blue uptake by chromatophores on transition to the energized state. Discharge of the energized state was accompanied by restoration of the original level of bromothymol blue in the outer solution.

TUPPER AND TEDESCHI[12,13] tried to measure the potential difference across the mitochondrial membrane using microelectrode technique. This approach would seem to be very difficult, however, because of the rather high resistance of the inner mitochondrial membrane, which is evaluated at about 10^7–10^9 $\Omega \cdot cm^2$ (see refs. 4, 5, 14 and 15). It is hardly possible to insert an electrode into such a small space as a mitochondrion without causing some decrease in resistance, so high initially, at the point where the membrane is pierced. Besides, the charging electric capacity of the most sensitive measuring instrument with the electric current produced by a mitochondrion would require considerably more than the lifetime of a mitochondrion under these experimental conditions. It is not surprising that the results of microelectrode measurements proved to be quite different from what had been expected. The resistance of mitochondrial membrane was very low (several Ω), potential about 10 mV, the sign '*plus*' inside the mitochondrion[12,13]. It is impossible to rule out the explanation that these data reflect the peculiarity of the experimental system (insect muscle mitochondria in a medium of high viscosity were used). However, it seems most probable that significant damage of mitochondrial integrity by the electrode took place. Another possibility exists that the electrode was inserted into the intermembrane space and all the data must be referred to the outer membrane.

For the quantitative estimation of the potential difference across mitochondrial membranes one may apply the method used for detecting the membrane potential, namely incubation with synthetic penetrating ions. The only complication is the precise estimation of the penetrating ion concentration in the water phase inside the particle. The synthetic ions used in our experiments readily penetrate into the lipid phase. An increase in ion concentration in the 'inner water' might result in a rise in ion concentration within the membrane. If this is the case, then the increase in the amount of ions in the 'inner water' should be smaller than the measured decrease in the 'outer water'. Therefore, for quantitative measurements of the membrane potential it is expedient to use ions of low affinity for lipid and small amounts of the carrier ('ionophore').

This method was used by MITCHELL AND MOYLE[6] in experiments with mitochondria. The membrane potential was determined by measurement of a K^+ gradient arising in the energized state between the outer and intramitochondrial spaces in the presence of valinomycin.

For qualitative detection of a membrane potential one should better use a range of structurally different synthetic cations and anions. In this way one can minimize the possibility that the ion gradient formation is due to the activity of enzymes, 'translocases', specialized in the active transport of natural ions. The existence of translocases operating according to the conformational principle and localized in mitochondrial membranes is not excluded by experiments with synthetic ions. The only points that we would like to emphasize are that (a) energy-dependent translocases, if they exist in energy-producing membranes, are not the only mechanism for ion transfer against a concentration gradient and (b) energy-dependent transfer of synthetic penetrating ions is carried out, without the participation of translocases, at the expense of electric field energy.

The detection of a membrane potential with ions which do not penetrate across the lipid membrane *per se* but are able to combine with 'ionophores' was restricted because of the absence of satisfactory anion carriers. Our experiments showed that

the role of an 'ionophore' can be performed by some synthetic compounds. For instance, di(pentafluorophenyl) mercury and some other nonionized mercury compounds containing electrophilic substituents were found to be good I^- carriers. These compounds also transported Cl^- but not as effectively as I^-. This difference can be simply explained by a lower affinity of mercury for Cl^- than for I^-.

It is important that penetrating compounds used in the above experiments are completely ionized at neutral pH, being strong acids or bases. The study of the distribution of weak acids and bases existing at pH 7 as a mixture of ionized and nonionized forms cannot be applied to detecting a membrane potential. The distribution of these compounds inside and outside of the mitochondria can be affected by transmembrane diffusion of the uncharged form and its subsequent dissociation inside the particle, where the pH value may differ from that in the outer phase.

Let us consider, for instance, the distribution of a weak acid whose undissociated form (AH) can penetrate across the membrane, whereas its anion (A^-) cannot.

Under nonenergized conditions the equilibrium concentrations of AH inside and outside of the mitochondria should be equal. Transition of mitochondria to the energized state leads to the charging of mitochondrial membrane so that the '*minus*' should be on its inner side and the pH of the mitochondrial interior should rise. The latter effect causes dissociation of AH to A^- and H^+ inside the mitochondria. A decrease in the concentration of the form AH within the mitochondria leads to the uptake of a new portion of weak acid from the outer solution. As a result, the total concentration of A^- inside the mitochondria should increase. This effect might be erroneously interpreted as an indication of the orientation of the electric field (the '*plus*' being inside), if it is assumed that A^- rather than AH is the penetrating species (see *e.g.* ref. 16). Apparently, many uncertainties regarding anion transfer across mitochondrial membranes arise from the use of anions of weak acids.

The application of penetrating anions of strong acids such as phenyl dicarbaundecaborane allows the missing link in the chain of evidence for a membrane potential to be supplied. The energy-dependent accumulation of K^+ in mitochondria on addition of a K^+ carrier (valinomycin) was established 6 years ago[17]. It was then found that under the same conditions 'inside out' submitochondrial particles do not accumulate K^+ but rather extrude these ions[18–22]. JACKSON *et al.*[23] showed K^+ extrusion after the transition of valinomycin-treated chromatophores to the energized state. Experiments with penetrating anions of strong acids[1–3] demonstrated that the direction of anion flow across the mitochondrial or chromatophore membrane was opposite to that of K^+ flow in the presence of valinomycin.

A potential difference across a membrane can also be detected by measuring certain parameters which respond to the appearance of a membrane potential. JACKSON AND CROFTS[24] measured the carotenoid spectra in chromatophores of photosynthetic bacteria. Their experiments showed that generation of a potential difference across membranes of chromatophores on the addition of H^+ and a proton carrier, or K^+ *plus* valinomycin, induces a characteristic shift in the spectra of chromatophore carotenoids. The same spectral changes were observed on the transition to the energized state — on illumination or addition of ATP. The authors concluded that there is a membrane potential, the '*plus*' being inside, when chromatophores are in the energized state.

JACKSON AND CROFTS[24] tried to calculate the absolute value of potential difference across the chromatophore membrane under energized conditions and obtained the value of about 200 mV. Values of the same order of magnitude were obtained after calculating the gradient of penetrating ions in our experiments with submitochondrial particles and chromatophores.

Our data indicate that the measurement of the uptake of penetrating anions is a very sensitive probe for a membrane potential and for the energized state of chromatophores and submitochondrial particles. This approach allowed the energy conservation process on reversal of the energy-requiring transhydrogenase reaction to be shown, whereas other methods were ineffective in this respect[25]. This is not so surprizing since no threshold value of the membrane potential can exist for the process of ion accumulation caused by an electric field. In contrast, other energy-linked functions, such as ATP synthesis and reversed electron transport, if they are supported by the membrane potential, should cease as soon as the potential decreases below some minimum level. In fact, the values for the potential difference generated on the reversal of the energy-requiring transhydrogenase reaction were usually smaller than those generated by respiration or by ATP hydrolysis. There may be two reasons for this difference. Firstly, the energy yield of the reversal of the energy-requiring transhydrogenase reaction could be too small to maintain the membrane potential at the high level. Secondly, the rate of the oxidative reaction in question is too low to create a high potential and to compensate for a leakage of H^+ through the membrane of submitochondrial particles or chromatophores.

Mechanism of generation of the membrane potential

The transport of synthetic penetrating ions into mitochondria, their particles and chromatophores, was associated with characteristic pH changes of the incubation medium[1–3]. Cation uptake was accompanied by acidification of the outer solution, while accumulation of anions induced alkalinization. The efflux of previously accumulated ions upon cessation of the energy supply was accompanied by the reversal of pH changes induced by active transport of ions.

These findings confirm MITCHELL'S idea[4] that the generation of a potential difference across the mitochondrial membrane is the result of proton translocation.

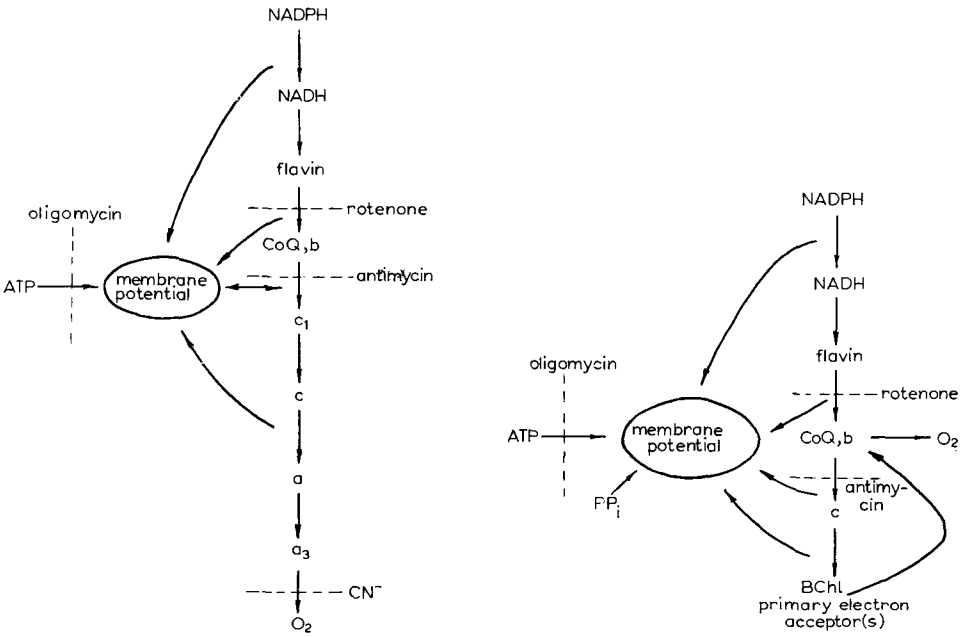
However, certain complications should be mentioned which arise from comparing changes in pH and penetrating anion concentration in experiments with submitochondrial particles or chromatophores. At low concentrations of penetrating anions the uptake of H^+ was greater than the uptake of anions. The discrepancy diminished when the concentration of the added anion was increased. Using rather high concentrations of phenyl dicarbaundecaborane it was possible to demonstrate an [anion]: [H^+] ratio equal to 1 (see, for instance, the experiment with light-induced anion and H^+ transport in *R. rubrum* chromatophores, ref. 3, Fig. 9). The extra uptake of H^+ on accumulation of low amounts of penetrating anions might be due to an increase in the electric capacity of the membranes which have taken up anions.

As a matter of fact, the experiments with synthetic penetrating ions can be considered as a direct confirmation of Mitchell's postulate of a membrane potential. These results could be predicted from the chemiosmotic scheme of oxidative phosphorylation. This, however, does not mean that these data confirm the chemiosmotic concept *in toto*. Analysis of relationships between electron transfer, hydrolysis (and

synthesis) of ATP and the system for generating of the membrane potential reveals several possible versions of energy transformation, only one being in accord with Mitchell's concept of coupling.

A general outline of the potential producing systems in mitochondrial and chromatophore membranes is given in Schemes 1 and 2, respectively.

Scheme 1 summarizes the results of experiments with mitochondria and submitochondrial particles. It is shown that a membrane potential generation can be supported by each of the following five processes: (1) ATP hydrolysis; (2) electron transfer *via* coupling sites in the regions of the NADH dehydrogenase; (3) between cytochromes *b* and *c*; (4) between cytochrome *c* and O_2; (5) between NADPH and NAD^+.



Scheme 1. Pathways of membrane potential generation in mitochondria and submitochondrial particles.

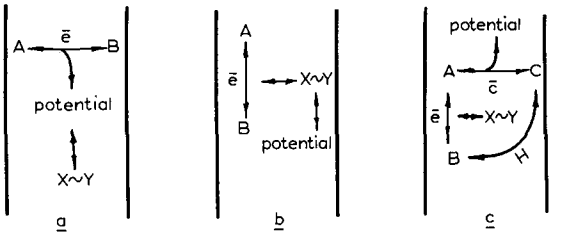
Scheme 2. Pathways of membrane potential generation in chromatophores of *R. rubrum*.

For chromatophores (Scheme 2), there are six energy-supplying processes: (1) hydrolysis of ATP; (2) hydrolysis of inorganic pyrophosphate; (3) electron transfer at the NADH dehydrogenase step; (4) between cytochromes *b* and *c*; (5) between cytochrome *c* and the 'bacteriochlorophyll–primary electron acceptor(s)' system; (6) between NADPH and NAD^+.

According to MITCHELL[4], each of the sites of coupled electron transfer and the system of ATP hydrolysis, operating independently, are able to generate the membrane potential. This means that five types of generators of electric energy are coexisting in the same mitochondrial membrane. It is possible, however, that the variety of potential generating mechanisms is not as large. For example, according to the scheme of CHAPPELL AND CROFTS[26], the energy of electron transfer and ATP

is always unified as some high-energy intermediate (X ~ Y), being converted into the membrane potential by means of the only type of specialized proton pump. The scheme of Mitchell suggests that the electron transfer is coupled to a high-energy intermediate *via* the membrane potential (redox chain ⟷ potential ⟷ X ~ Y).

According to the scheme of CHAPPELL AND CROFTS[26], the role of the intermediate is performed by the X ~ Y component (redox chain ⟷ X ~ Y ⟷ potential). To exhaust all possible versions one should consider one more scheme, not discussed previously. According to this scheme, the energy of the X ~ Y component is transduced into the membrane potential *via* the electron transfer chain (potential ⟷ redox chain ⟷ X ~ Y).



Scheme 3. Possible interrelationships of electron transfer, oxidative phosphorylation and membrane potential.

The above three possible mechanisms for generating the membrane potential are presented in Scheme 3, where A, B, and C are the components of the redox chain. It is seen that the three versions differ in the position of electron carriers in the membrane. According to Mitchell's scheme (3a), electron and hydrogen are transferred across the membrane from one side to another. According to Scheme 3b, the redox chain is orientated along the membrane. Scheme 3c suggests the existence of two pathways for electron transfer; one of them orientated along the membrane is responsible for the formation of the high-energy intermediate X ~ Y; a second one, orientated across the membrane, generates the membrane potential. According to Scheme 3c, ATP energy is utilized for charging the membrane *via* reversed electron transfer from a respiratory chain carrier B to A with the subsequent oxidation of reduced A by C.

The available information concerning the mechanism of potential generation does not allow a choice between the three versions given in Scheme 3. Version (a) seems to be rather attractive because it promises to explain the mystery of the mechanism of oxidative phosphorylation. Furthermore, it should be noted that the possibility of conversion of chemical energy into electric energy in the mitochondrial membrane, which is now a certainty, was suggested by MITCHELL in the form of one of the postulates of the concept represented in Scheme 3a. One is tempted to believe that other postulates of Mitchell will also be proven.

Scheme 3b views the electric generator as a special enzyme system, *i.e.* a proton pump hydrolyzing the X ~ Y intermediate. According to this scheme the respiratory chain and ATP synthetase *per se* do not directly participate in production and utilization of electric energy. The disadvantage of this viewpoint is the necessity of postulating a mechanism for the reversible generation of the membrane potential at the expense of hydrolysis of some high-energy intermediate. The rever-

sibility of interconversion of membrane potential energy and that of the X ~ Y intermediate seems to be the most vulnerable postulate in Mitchell's concept of oxidative phosphorylation. Only this point is retained in Scheme 3b, while other and very attractive features of chemiosmotic hypothesis are lost.

Comparing two possible mechanisms for membrane charging, X ~ Y hydrolysis and electron transfer, one can easily be convinced that the later process much better serves the function of a proton pump. In fact, hydrogen atoms cleaved from oxidation substrates are always separated into e^- and H^+ by respiratory chain enzymes. The pathways for the electrons and protons formed are enzymatically different, which would be useful for charge separation on the membrane. For this purpose it should be enough to place a hydrogen carrier (*e.g.* flavin) in the vicinity of one side of the mitochondrial membrane and an electron carrier (*e.g.* a cytochrome) close to the other side.

Scheme 3c postulates that the function of a proton pump and an electric generator is performed by the redox chain but not by X ~ Y hydrolysis. According to Scheme 3c, there is a special system for electron and hydrogen transfer in the membrane engaged in generating the membrane potential. Another redox chain takes part in oxidative phosphorylation which in Scheme 3c (as well as in 3b) is described in terms of a chemical coupling mechanism[27] (for more details see ref. 28). In order to distinguish Scheme 3c from the other two, inhibitor analysis might be used. According to Scheme 3c, complete inhibition of electron transfer at all sites of energy coupling should prevent membrane charging at the expense of ATP energy. It is difficult to perform such experiments because of the lack of specific inhibitors of the transhydrogenase and of the cytochrome *c*–cytochrome *a* step. In terms of Scheme 3c, the ATP utilization for generation of the membrane potential would be retained as long as at least one of the coupling sites of the redox chain is operative. If any of the coupling sites is intact, the generation of electric energy at the cost of ATP hydrolysis may be provided by means of cyclic electron transfer in the loop B→A→C→B (see Scheme 3c).

In this connection, the data by HEMKER[29] should be mentioned. This author described inhibition of mitochondrial ATPase by amytal and antimycin A. These inhibitors, arresting electron transfer in the first and the second coupling sites, appreciably decreased the rate of ATP hydrolysis in the presence of DNP, the effects of the two poisons being additive. The amounts of the agents necessary for the inhibition of ATPase and of respiration were found to be the same. In this laboratory, I. Severina and S. Smirnova have confirmed this finding and have also shown that the degree of inhibition of ATP utilization processes in mitochondria is highest if three inhibitors are added together: rotenone, antimycin and cyanide. A very convenient system for studying the effect of the three inhibitors was mitochondrial swelling caused by the addition of ATP in the presence of phosphate or acetate and any penetrating cation (Ca^{2+}, K^+ *plus* valinomycin, and dibenzyl dimethyl ammonium were tested). A decrease in the rate of the 2,4-dinitrophenol-activated ATPase reaction, caused by inhibitors of the respiratory chain, could also be shown with submitochondrial particles. Rather high concentrations of inhibitors are necessary for suppression of ATP-supported accumulation of penetrating anions in particles. Amounts of rotenone, antimycin and cyanide, sufficient for the inhibition of anion transport supported by oxidation of NADH, succinate and reduced *N*, *N*, *N'*, *N'*-

tetramethyl-*p*-phenylenediamine ($TMPDH_2$), respectively, have little effect on anion uptake induced by ATP. It is not excluded that, in this case, the membrane potential is generated by cyclic electron transfer in the coupling site localized as the levels of transhydrogenase and (or) cytochrome *c–a*, if cyanide inhibits the chain after the third site of energy conservation.

Results of experiments with ferricyanide restrict the number of possible variants of Schemes 3b and 3c. It was shown[1,2] that ferricyanide can serve as an electron acceptor supporting ion accumulation in mitochondria but not in 'inside out' particles (succinate was used as the substrate, respiration being inhibited by cyanide). The same relationships were found in experiments in which ferrocyanide was used as the electron donor. These results suggest that cytochromes c_1 and *c*, reducing ferricyanide and oxidizing ferrocyanide in mitochondria, are situated on the inner side of the particle membrane and are therefore inaccessible to hydrophilic electron carriers. It is probable that the cytochrome segment of the respiratory chain, if stretched along the membrane (see Schemes 3b and 3c), is shifted to the outer space of mitochondria and to the inner space of submitochondrial particles. This conclusion is supported by the data of MITCHELL AND MOYLE[30] who measured pH changes during oxidation of ferrocyanide by O_2 in mitochondria.

Some attention should be paid to the possibility that the mechanisms for the generation of the membrane potential of different coupling sites are not identical. This compromise allows one to overcome a number of complications arising from the attempt to describe all coupling sites in terms of any single concept.

Mitchell's scheme satisfactorily describes the mechanism of membrane potential formation in the flavin region of the respiratory chain. In trying to extend his scheme to the coupling sites localized in the cytochrome system, Mitchell was compelled to place ubiquinone between cytochrome *b* and cytochrome c_1. This required the assumption that the redox potential of ubiquinone is about 250 mV more positive than that of cytochrome *b*. We failed to confirm this postulate. No anion accumulation was coupled with succinate oxidation by added ubiquinone. Under the same conditions, oxidation of NADH by ubiquinone was accompanied by anion uptake, the process being sensitive to rotenone but not to antimycin. (Similar relationships were demonstrated previously for phosphorylation coupled with reduction of added CoQ (ref. 31).) If we agree, nevertheless, with Mitchell's speculation concerning the role of ubiquinone, it remains obscure why the last step of transmembrane electron transfer requires a redox chain consisting of four cytochromes (c_1, *c*, *a*, a_3) and copper. The respiratory chain appears to include too many electron carriers and too few hydrogen carriers to obey Mitchell's scheme of energy coupling.

Another weak point in Mitchell's scheme is the energy-linked transhydrogenase. Here Mitchell has to introduce an unknown hydride carrier.

It should be kept in mind that the hydrogen atom carried by the energy-linked transhydrogenase is translocated from one nicotinamide nucleotide to another without exchanging with water[32]. Consequently, even if directed across the membrane, this process cannot produce the transmembrane proton gradient *per se*. Thus, it is necessary to resort to a number of second-order hypotheses to extend Mitchell's scheme (and either of the two other schemes) to all sites of energy coupling in the redox chain.

Membrane potential functions

It is remarkable that membranes of such experimentally distant objects as particles of beef heart mitochondria and chromatophores of photosynthetic bacteria are capable of generating electric energy and that they do it in a very similar manner. This fact suggests a fundamental significance of this type of energy conversion for the cell's economy. Apparently, the possibility of membrane potential generation is a common property of energy-producing biological membranes.

The universal significance of a membrane potential is quite understandable if we follow Mitchell in assuming that oxidation and phosphorylation are coupled by charge transfer through the membrane.

However, energy coupling is not the only possible function of membrane potentials, just as Mitchell's scheme is not the only possible concept of a potential-producing mechanism.

Considering the mechanisms of Schemes 3b and 3c, we could conceive functions for the membrane potential other than those of coupling oxidation and phosphorylation. One of these functions might be ion transfer across the mitochondrial membrane resulting in a steady unequal distribution of some compounds between the extra- and intra-mitochondrial spaces of the cell. There might be quite a number of such compounds. They may include not only natural penetrating ions but also non-ionized molecules able to combine with some charged carriers localized in the mitochondrial membrane.

Concentration gradients created by a membrane potential could play a part in the regulation of metabolic processes in mitochondria and cytoplasm as well as in energy storage by the principle of an 'energy buffer'. Under conditions of energy deficiency, the equalization of transmembrane concentration gradients results in a release of a significant amount of energy. This energy could be accumulated in the form of ATP by reversal of the electrophoretic mechanism of ion transport.

The membrane potential could also serve as a transportable form of energy in intracellular compartments separated by membranes. The system of hydrophobic membranes and cristae may hinder the energy transfer by such a hydrophilic energy carrier as ATP. The problem would be simplified if energy could be transmitted along the membrane in the form of an electric field. In this way it would be possible, for example, to unite in the common system that multitude of energy-producing individual enzyme complexes which are fitted into spatially distant areas of the mitochondrial membrane. It is well known that mitochondrial and other energy-producing membranes possess one more form of energy unification besides ATP. The component in question is the so-called high-energy ATP precursor whose formation from ATP (but not from the redox chain) is arrested by oligomycin. Energy stored in this form can be carried from one coupling site of the respiratory chain to another. It is rather improbable that the function of such a mobile energy carrier is performed by a high-energy coupling factor of protein nature corresponding to one of the chemical intermediates of ATP synthesis. A protein molecule would be too large to move easily within the membrane. The membrane potential would qualify much better for this role.

Electric energy transfer along the membrane can be effective only if the resistance of the energy-transferring membrane is high. Bimolecular phospholipid membranes, whose resistance can reach $1 \cdot 10^9\ \Omega \cdot cm^2$, could be models for such a system.

Membranes of similar structure, if they exist *in vivo*, would play the role of intracellular 'power grids', transferring energy from one part of a mitochondrion to another, between two (or many) mitochondria, or from mitochondria to some other components of the cytoplasm.

We ought to pay attention to these and related problems, since the definition of the mitochondrion as an electric power station of the cell now seems to be more than a metaphor.

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