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Perspectives and Limitations of Resolutions–Reconstitution Experiments

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Reconstitutions of membranous activities can tell us how many components are required and what their functions are. The mitochondrial proton pump is used as an example. Moreover, the biological activity, such as P_i transport, can be used in reconstituted vesicles as an assay during the isolation of the transporter.

Reconstitution experiments reveal the importance of membrane asymmetry and allow us to study conditions of vectorial assembly.

The mechanism of action of ion pumps has been successfully analyzed in reconstituted liposomes. We can study the movement of ions and the electrogenicity of the system without interference by other unrelated processes.

Based on studies with the resolved Ca^{2+}-ATPase of sarcoplasmic reticulum, we propose a novel formulation of the mechanism of ATP-driven ion pumps in which cyclic binding of Mg^{2+} plays a key role.

Key words: reconstitutions of ion pumps, coupling factors of oxidative phosphorylation, phospholipids, role in ion pump activity, mechanism of ATP-driven Ca^{2+} pump, oxidative phosphorylation, a new hypothesis, ATPases of membranes

It is apparent from this conference that resolution and reconstitution of membrane-linked functions in artificial liposomes have become fashionable. It seems appropriate to take stock and to evaluate what we can expect from such experiments and to point out their limitations. It should be obvious that reconstituted systems do not tell us exactly what happened in the natural membrane. Any biological system separated from the multitude of other intersecting functions is, strictly speaking, an artifact. The primary purpose of resolutions and reconstitutions is indeed the simplification of the system to the minimal number of components required for functions. The price we pay for getting away from the turbulence of metabolic events that take place in natural membranes seems worthwhile to anyone who has been exposed to the frustrations associated with analysis of events in cells or in organelles.

What can we learn from reconstitution experiments? I have outlined in Table I the subjects I shall discuss. In each case I shall stress limitations and deficiencies of the approach of resolution and reconstitution.

Abbreviations: DCCD – N,N'-dicyclohexylcarbodiimide; F_1, F_2, F_6 – coupling factors 1 (ATPase), 2 and 6 respectively; OSCP – oligomycin sensitivity conferring protein.

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TABLE I. What Can We Learn From Reconstitutions?

1. What are the parts required and what are their functions?
2. What are the functions of the phospholipids?
3. What is the role of asymmetry and how do we achieve asymmetrical orientation in reconstitution?
4. What is the mechanism of action of ion pumps?
   a) What ions are moving?
   b) Is it a carrier or a channel?
   c) Is the system electrogenic or electrically silent?
   d) How can we study the molecular mechanism of ion pumps?

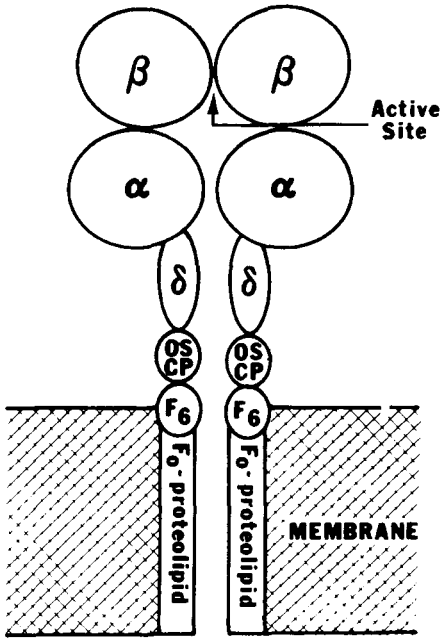

Fig. 1. Relationship between coupling factors in the oligomycin-sensitive ATPase complex.

WHAT ARE THE PARTS REQUIRED AND WHAT ARE THEIR FUNCTIONS?

I have chosen the mitochondrial proton pump to illustrate the reconstitution approach. Almost 20 years ago we resolved from submitochondrial particles the first coupling factor (F_1) for oxidative phosphorylation (1, 2). We learned 2 significant facts: The coupling factor was an ATPase, and it was resistant to oligomycin. At first sight both these facts were confusing. How can an ATPase function as a coupling factor without hydrolyzing the ATP it helps to generate? This puzzle was solved by the discovery of a mitochondrial protein which inhibits ATPase activity without interfering with ATP generation (3). The second puzzle, the resistance of the ATPase activity to oligomycin led to the discovery of a membranous complex that confers oligomycin sensitivity to added F_1 (4). This feature of conferral proved most valuable as an assay during subsequent fractionations and isolations of several additional coupling factors (cf 5).

The scheme of the oligomycin-sensitive ATPase shown in Fig. 1 is incomplete. It is not meant to convey either the number or the positions of the subunits of F_1. It is meant to convey our present knowledge of the broad relationship between the headpiece (F_1) and the other components of the complex. It shows the factors needed for the attachment of F_1 to the membrane and their relationship to the proton channel (F_0). It is partly based on studies on ATPase from E. coli (6) and from spinach chloroplasts (7) which have identified the δ subunit of F_1 as the peptide chain responsible for the binding of F_1 to the membrane. Oligomycin sensitivity conferring protein and F_6 (8) are the membrane components that interact with the subunit of F_1, with OSCP probably located between F_6 and the δ subunit.

What happened to F_2? Its fate exposes one of the limitations of reconstitution experiments. We can readily show that F_2 (factor B) is required for oxidative phorphorylation in defective submitochondrial particles (9). However, in our hands reconstituted liposomes have thus far failed to display a dependency on this factor. For this and other reasons we proposed that F_2 serves as a "sealing factor" which is required to lower the proton permeability of the inner mitochondrial membrane (5). The artificial liposomes made with a large excess of phospholipids appear to be sufficiently impermeable to protons without added F_2.

The function of F_0 as a proton channel was first proposed by Mitchell (10). The reactivity of F_0 with oligomycin and DCCD has been a major aid in the course of its isolation. The highly purified preparations from mitochondria (11), chloroplasts (12), and bacteria (13), which can be used for the reconstitution of liposomes that catalyze DCCD-sensitive proton translocation, contain 2–3 protein bands in addition to the subunits of F_1 and the other known coupling factors. A great deal of attention is now being paid to the proteolipid which was isolated from mitochondria (14, 15) and bacteria (16) based on its reactivity with ^{14}C-labeled DCCD. After extraction with chloroform-methanol, a water soluble proteolipid apoprotein, virtually free of phospholipids, was isolated from mitochondria and shown to enter readily into preformed liposomes suspended in aqueous media (17). The proteolipid apoprotein formed a proton channel which however was not blocked by the addition of DCCD or oligomycin. More gentle procedures (12, 18) are required for the preservation of reactivity with DCCD and for the reconstitution of a DCCD-sensitive proton channel. Further purification and demonstration of capacity to interact with coupling factors is needed for the evaluation of the role of the DCCD-sensitive proteolipid and of other hydrophobic components in the formation of the proton channel.

The reconstitution of the oligomycin-sensitive ATPase into liposomes and the demonstration that these vesicles catalyze oligomycin-sensitive ATP-driven proton translocation (19) was a decisive factor in our acceptance of the chemiosmotic hypothesis and stimulated further attempts to reconstitute oxidative phosphorylation.

The second example which illustrates the value of the reconstitution approach is the respiratory chain of mitochondria. Several complexes of the oxidation chain have been isolated (20) and incorporated with the native mitochondrial orientation into liposomes (21–24). As will be shown later these vesicles exhibit the phenomenon of respiratory control and they generate during oxidation a proton motive force which facilitates the electrogenic inward transport of K^+ in the presence of valinomycin (21). Although these experiments clearly demonstrate that the complexes catalyze proton translocation, the isolation and identification of the individual components which participate in electron transport and in proton translocation, is still a problem for the future.

The reconstitution of the oxidation complexes in the submitochondrial orientation together with the mitochondrial ATPase yielded vesicles which catalyzed oxidative phos-

TABLE II. Reconstitutions of Oxidative Phosphorylation*

Phospholipids	P:2e ratio		
	Site 1	2 + 3	3
Phosphatidylethanolamine + Phosphatidylcholine (4:1)	0.5	0.63	0.4 (0.35)
Phosphatidylethanolamine + Phosphatidylcholine (1:1)	0.8	–	0.2

*The experimental procedures were as described (34, 35).

phorylation. As shown in Table II, when the cholate dialysis procedure was used for reconstitution a phosphatidylethanolamine:phosphatidylcholine ratio of 4 was optimal. The reconstitution of site 2 + 3 yielded with succinate a P:O ratio of 0.63, which was about twice as high as the P:O ratio of 0.35 obtained with the same vesicles with ascorbate-phenazine methosulfate as substrate. The reconstitution of site 3 of oxidative phosphorylation in submitochondrial orientation posed difficulties which illustrate the limitations of our current reconstitution methods. I shall return to this problem when I discuss problems associated with asymmetric reconstitution.

The demonstration (25) that bacteriorhodopsin, which catalyzes a light-driven translocation of protons, can substitute for the mitochondrial respiratory enzymes in the above described experiments, proved that the chemiosmotic mechanism described by Mitchell (10) is operative in a reconstituted system. Since no oxidation-reductions accompany the translocation of protons via bacteriorhodopsin, it was unnecessary to assume that a direct contact takes place between members of the respiratory chain and the ATPase as proposed in the original conformational hypothesis (26). Moreover, the fact that the purple patches of halobacteria contain bacteriorhodopsin as the only protein component excludes the possibility of a direct contact between the ATPase and rhodopsin during light-driven ATP generation in the intact bacteria (27).

In spite of this persuasive evidence we have to agree that mechanisms that operate in model systems or even in bacteria need not be the same in membranes of higher organisms that have evolved and have become more complex and more efficient.

Recent experiments with buffered chloroplasts (28) suggest that the proton flux during light-driven electron transport may not be in equilibrium with the bulk phase of the internal chloroplast water. It is appealing to think in terms of a thin layer of "structured water" associated with the membrane surface, a concept which would eliminate some of the difficulties posed by thermodynamic calculations based on the measured pH differences between the inside of chloroplasts and the external medium. It may be of interest in the future to conduct similar experiments with reconstituted systems since it should be possible to control the surface:volume ratio by varying the size of vesicles, e.g., by fusion (29).

WHAT ARE THE FUNCTIONS OF THE PHOSPHOLIPIDS?

The primary function of the phospholipids is to provide a compartment which is impermeable to ions and other solutes that must be kept within the cell at appropriate concentrations. The phospholipids must also serve as a suitable matrix for the embedding

TABLE III. Restoration of Activities to Delipidated Ca^{2+}-ATPase*

Additions to delipidated enzyme	ATPase μmoles/min/mg	Phosphoenzyme nmoles/mg	Transport nmoles/min/mg
None	0	0.3	0
Phosphatidylcholine	4.0	1.9	0
Phosphatidylethanolamine	2.6	0.8	183
Acetyldilaurylphosphatidylethanolamine	0.12	2.3	(no vesicles)
Phosphatidylethanolamine/Phosphatidylcholine (4:1)	3.3	1.5	344[a]

*The experimental procedure was as described (30).
[a]Phosphatidylethanolamine/phosphatidylcholine (3:1)

of proteins which facilitate the entry of desirable and the exit of undesirable solutes. It seems likely that an asymmetric assembly of phospholipids participates in the asymmetric orientation of transport proteins although experimental evidence for such a relationship is not as yet available. Finally, there are indications that the phospholipids specifically influence the individual catalytic steps involved in the transport process

It can be seen from Table III that a preparation of Ca^{2+}-ATPase from sarcoplasmic reticulum that has been stripped of phospholipids no longer catalyzed the catalytic functions associated with its enzymatic activity (30). Phosphatidylcholine restored to the protein the ability to form phosphoenzyme and to hydrolyze ATP, but did not allow for efficient Ca^{2+} transport. Phosphatidylethanolamine on the other hand was less effective in the first 2 functions, but formed vesicles that catalyzed Ca^{2+} transport. Both phospholipids added together during reconstitution gave the highest values for Ca^{2+} transport. An interesting example is acetyldilaurylphosphatidylethanolamine. It was completely unsuitable for the formation of transport vesicles, it supported very low ATPase activity, but it was the most effective lipid for phosphoenzyme formation. It is apparent from these data that by the appropriate choice of phospholipids we can disect the first step of the catalytic process and study it independently of subsequent events.

The chemical modification of the amino group of phosphatidylethanolamine was explored in experiments conducted in collaboration with Dr. H. G. Khorana (31). As shown in Table IV, acetylation of phosphatidylethanolamine did not impair the ability of the phospholipid to form vesicles with bacteriorhodopsin. Although the kinetics of H^+ pumping were somewhat altered, the overall pump function was not significantly altered by the introduction of the acetyl group. On the other hand, the Ca^{2+} transport activity of reconstituted vesicles was lost completely. Significantly, it could be restored by incorporation of an hydrophobic alkylamine such as stearylamine or oleolylamine. Amines with shorter chain lengths were less effective. Of particular significance is the observation that the ATPase activity was not well supported by acetylated phospholipid as already indicated by the data shown in Table III. Here again, incorporation of stearylamine sustained the catalytic function. Since dicetylphosphate inhibited and could be quantitatively titrated against the stearylamine, it appears that the catalytic function of the ATPase is greatly influenced by the surface charge of the membrane in which it is embedded. Acetylphosphatidylethanolamine was also ineffective for the reconstitution of the mitochondrial ATPase which catalyzes a $^{32}P_i$-ATP exchange. Stearylamine again sustained the activity.

TABLE IV. Acetylphosphatidylethanolamine in Reconstituted Systems*

	Phosphatidyl-ethanolamine (PE)	Acetyl PE	Acetyl PE plus stearylamine
Proton pump	2,360	2,400	–
Calcium pump	114	0	109
Calcium ATPase	890	200	740
$^{32}P_i$-ATP exchange[a]	133	0	112

*The activity of the proton pump is expressed as the extent of n atoms H^+ pumped per mg of bacteriorhodopsin; the other activities are expressed as nmoles/min/mg protein.
[a]Reconstituted with phosphatidylcholine plus the other indicated lipids. The experimental procedure was as described (31).

However, in this case we could show that the acetylphosphatidylethanolamine phospholipid was required in addition to phosphatidylcholine, although the latter was capable by itself of forming impermeable compartments as illustrated by studies with the bacteriorhodopsin proton pump. These studies show how reconstitution can shed light on the contribution of individual phospholipids and allow us to modify the chemistry of the participating components.

Among the limitations of this approach, at least at the present time, is our lack of understanding of the significance of the large variety of lipids present in natural membranes. Some of these components may play a role that we do not appreciate. Thus, reconstitutions with highly purified or even synthetic phospholipids that do not contain the minor components may lead us astray. For example, the role of large quantities of alkylated phospholipids present in many natural membranes is unknown. Reconstitution experiments have thus far not revealed any specific functions for these phospholipids (32), but I have little doubt that there is a good reason for their presence.

Another drawback of reconstitutions is that phospholipid requirements vary with the method used for the formation of liposomes. With the cholate dialysis procedure a phosphatidylethanolamine:phosphatidylcholine ratio of 4 is optimal (see Table II) for most of the systems studied thus far. With the cholate dilution and particularly with the sonication procedure, the lipid requirements are quite different. For example, for the reconstitution of the Ca^{2+} pump by sonication, phosphatidylethanolamine alone was rather ineffective. Transport activity was much more dependent on the presence of phosphatidylcholine than in the case of the dialysis procedure. Differences in procedure must be invoked also in the discrepancies of observations recorded in the literature, such as the effectiveness of phosphatidylcholine as the sole lipid in the reconstitution of the Ca^{2+} pump (33) which is contrary to the data shown in Table III.

WHAT IS THE ROLE OF ASYMMETRY AND HOW CAN WE ACHIEVE IT?

One of the lessons we learned during studies of oxidative phosphorylation is that the orientation of proteins in reconstituted vesicles varies greatly depending on the experimental procedure. It is important in such experiments to devise assays which determine both the percent incorporation and the relative orientations of the protein in the artificial

TABLE V. Uncoupling of Oxidative Phosphorylation by Cytochrome c*

	ATP formation (nmoles/min)
Complete reconstituted systems	9.3
Minus polylysine	5.3
Minus polylysine plus cytochrome c (10 μg)	2.7
Minus polylysine plus cytochrome c (25 μg)	1.8
Plus ETP_H particles	18.8
Plus ETP_H particles plus cytochrome c (25 μg)	19.4

*The reconstituted vesicles (350 μg) were assayed as described (34) with and without 50 μg of polylysine. The ETP_H particles were assayed only without polylysine.

membrane. This is particularly essential in systems that must be oriented unidirectionally to permit measurements of functions as in the case of the third site of oxidative phosphorylation. With all methods of reconstitution used thus far, cytochrome oxidase is preferentially assembled in the mitochondrial orientation with the cytochrome c side of the enzyme facing the medium. Our reconstitution of site 3 of oxidative phosphorylation required however the opposite, namely the inside-out orientation of submitochondrial particles, since under these conditions the system was independent of the transport of P_i and ADP which are not permeant without the aid of specific transporters. With luck and the help of cytochrome c we achieved some submitochondrial orientation of cytochrome oxidase which was masked, however, by an excess of the enzyme in the right-side-out orientation (34). Only when we learned to displace all of the residual cytochrome c from the outside surface of the vesicles, could we demonstrate phosphorylation coupled to electron transport. When cytochrome oxidase was allowed to function in both directions, the membrane potential and the ΔpH was collapsed and no phosphorylation took place. In contrast to natural submitochondrial particles the reconstituted vesicles were thus uncoupled in the presence of small amounts of cytochrome c as shown in Table V.

This story reveals one of the major limitations of reconstitution: our inability to guide the orientation of a protein in a desired manner. Although the incorporation procedure, which I shall describe below, allows for unidirectional orientation, we have no control over its direction: it is right-side out in the case of cytochrome oxidase and inside out in the case of the oligomycin sensitive ATPase. Whether the final outcome suits the experimenter is mainly a matter of luck. In the case of bacteriorhodopsin the preferred inside-out orientation of reconstituted vesicles was a convenient feature which permitted the light-driven formation of ATP by the mitochondrial ATPase which also selects the inside-out orientation during reconstitution by current procedures.

At least in some instances assays can be devised that tell us accurately the quantitative distribution of inside-out and right-side-out orientations. It is also often important to establish the percentage of total protein that is incorporated into the liposomes. Such methods are available for some systems and will be described later for cytochrome oxidase vesicles. In this case a detergent (Tween 80) can be used to open the vesicles without inhibiting enzymatic activity. In the case of reconstituted vesicles catalyzing site 1 of oxidative phosphorylation, spectral change with impermeant reactants were used (35). Specific inhibitors which react with one side of an asymmetric transporter allow us in some instances to determine accurately the inside-out and right-side-out distribution of recon-

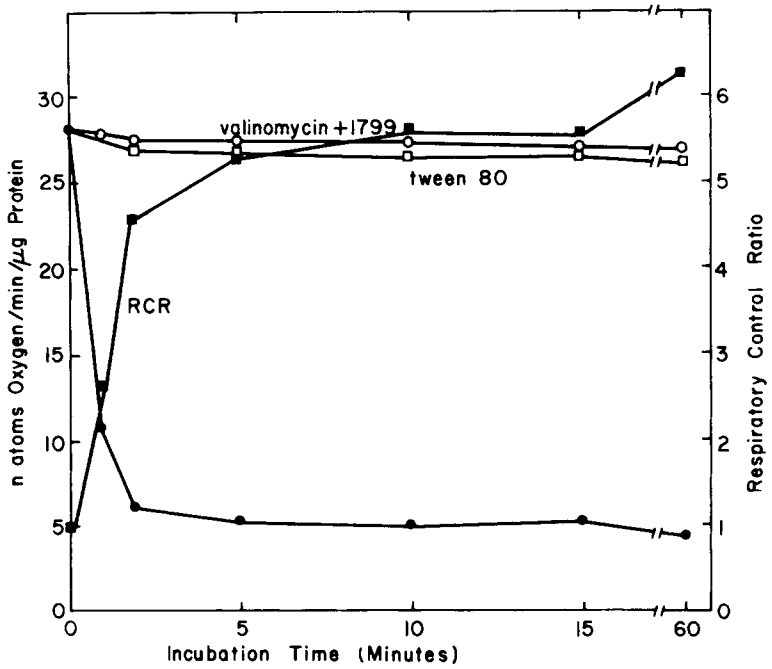

Fig. 2. Incorporation of cytochrome oxidase into performed liposomes. Experimental conditions were as described (39). ●——● oxygen uptake; ■——■ respiratory control ratio (RCR).

stituted vesicles. For example, atractyloside was used for the evaluation of the adenine nucleotide transporter (36), and N-ethylmaleimide for phosphate transport (37). It helps when hydrophobic counterparts of the inhibitors are available such as bongkrekic acid for the nucleotide transporter and N-benzylmaleimide for the P_i transporter. They serve as controls assuring proper operation of the transport system. Moreover, impermeant inhibitors such as atractyloside or ouabain can be included during reconstitution to allow interaction with the sensitive site of the transport system. Such impermeant inhibitors can be useful in establishing the amount of enzyme that has escaped reconstitution, which may be in large excess of the portion that can be incorporated into liposomes. An example for this is the reconstituted Na^+, K^+-ATPase from electric eel (38). Since both ouabain and ATP do not readily permeate through liposomes, all the ATPase activity which is ouabain-sensitive represents the fraction of enzyme that has not been reconstituted either inside out or right-side out.

RECONSTITUTION BY THE INCORPORATION PROCEDURE

We have recently described a method of reconstitution which avoids scrambling of orientation (39). The procedure is simple and consists of preparing liposomes which contain phosphatidylethanolamine and about 30% of an acidic phospholipid. When a hydrophobic protein is added to such vesicles it is rapidly incorporated in an asymmetric and unidirectional orientation. As illustrated in Fig. 2 the oxidation of reduced cytochrome c by cytochrome oxidase takes place at a rapid rate (zero incubation time). When the enzyme is added to phosphatidylserine containing liposomes, the uptake of oxygen

TABLE VI. Sequential Incorporation of Proteins Into Liposomes*

	Respiratory control	
	Expected	Observed
Exp. 1		
Cytochrome oxidase added to liposomes		10
F_0 added to COV plus liposomes	8.2	5.1
Exp. 2		
Cytochrome oxidase added to liposomes		4.7
Cytochrome oxidase added to F_0 liposomes plus liposomes	1.5	4.4

*Cytochrome oxidase or the hydrophobic protein of the ATPase complex (F_0) were added to an equal mixture of protein-free liposomes and protein-containing liposomes as indicated. The expected results were calculated based on random incorporation. Experimental details were as described (42).

drops within a few minutes to 10–20% of the original rate. On addition of valinomycin and 1799 (or nigericin) the original rate of oxidation is restored. This phenomenon, which we look upon as a form of respiratory control, is characteristic for cytochrome oxidase vesicles reconstituted by other procedures (22). The unambiguous interpretation of these observations is that the protein is incorporated asymmetrically into the proton-impermeable liposomes. The collapse of both the ΔpH (e.g., by nigericin) and of the membrane potential by valinomycin is required to release the control mechanism imposed by the proton motive force. When 3% Tween 80 was added to open the vesicles (40) the rate of oxidation was the same as in the presence of the ionophores. As mentioned earlier, if cytochrome c was present during reconstitution, both inside-out and right-side-out orientation of cytochrome oxidase was observed. Stimulation of respiration by ionophores was then only one half or less of the stimulation observed in the presence of Tween 80 (40).

The incorporation procedure illustrated in Fig. 2 was effective with several other membrane proteins, e.g., mitochondrial ATPase, QH_2 cytochrome c reductase, and Ca^{2+}-ATPase (39, 41). The method lends itself to interesting explorations. What effect has one protein already present in the membrane on the incorporation of a second protein? We can give a protein which is to be incorporated a choice between liposomes which are protein-free and liposomes which already contain a protein. We can calculate the results expected from a random distribution provided we have an assay that tells us what proportion of vesicles contain both proteins in the same membrane. The rather unexpected results that were observed in such experiments, opened a field which I faceciously refer to as "molecular psychology."

As shown in Table VI the data show marked deviation from random distribution. The assay used in these experiments is based on the observation that cytochrome oxidase vesicles catalyze a low rate of respiration in the presence of reduced cytochrome c. When a proton channel is present in the same vesicle (22) the rate of oxygen uptake is greatly increased. In these experiments the hydrophobic protein of the oligomycin-sensitive ATPase was used as a proton channel.

The conclusions that can be drawn from these experiments are: 1) Cytochrome oxidase avoids liposomes that contain the proton channel. The enzyme is preferentially incorporated into protein-free liposomes. On the other hand, cytochrome oxidase chooses vesicles that contain QH_2 cytochrome c reductase over protein-free liposomes (41). 2) The

hydrophobic proteins of the oligomycin-sensitive ATPase are incorporated into liposomes that contain cytochrome oxidase in preference to liposomes which contain no protein. Thus, there appears to be considerably specificity in the selection process that takes place during the incorporation of proteins into the liposomes.

Once more I should like to point to the limitations of such reconstitution procedures. Although these experiments may indeed have physiological significance and may shed light on the mechanism of protein incorporation into membranes, as well as on transmembranous protein movements during secretion, it is clear that the assembly of cytochrome oxidase in natural membrane is a much more complex and integrated process of subunit assembly which may take place from both sides of the membrane (42).

WHAT IS THE MECHANISM OF ACTION OF ION PUMPS?

a. What ions are moving? The complexity of natural membranes, such as the mitochondrial inner membrane in which many translocation processes take place simultaneously, makes it difficult to assess the individual translocation events and the compulsory movements of solutes associated with them. Even in much simpler systems, such as in the sarcoplasmic reticulum, the permeability of the membrane to phosphate has precluded the evaluation of a hypothesis (43) that the terminal phosphate of ATP moves together with the Ca^{2+}. This mechanism could be eliminated in experiments with ATP^{32} and reconstituted Ca^{2+} vesicles which are quite impermeable to phosphates (44). Although we admit once more that the reconstituted model does not necessarily represent the events in the natural membrane, the above mentioned experiments demonstrated that a translocation of the terminal phosphate of ATP is not essential for the mechanism. Thus the burden of proof rests on the proponent of the hypothesis who has to show that the mechanism operative in the natural membrane is fundamentally different from that in the reconstituted system.

b. Is it a carrier or a channel? An example for an experimental approach to this question has been described for bacteriorhodopsin which was reconstituted with phospholipids with known transition temperatures. The experiments showed that the pump was operative well below transition temperatures eliminating a mobile carrier mechanism. Rather than describing these experiments in detail I refer the reader to the original paper (45). I want to point out here some of the limitations of this approach. For example. we have attempted similar experiments with the Ca^{2+}-ATPase of sarcoplasmic reticulum. This enzyme would have been particularly suitable for such experiments since complete delipidation with retention of reconstitutive activity has been achieved (30). Such delipidation experiments have yet to be performed with bacteriorhodopsin. Unfortunately in the case of the Ca^{2+}-ATPase we do not have available phospholipids with appropriate transition temperatures that are suitable for Ca^{2+} transport and Ca^{2+}-ATPase activity. The available phospholipids have transition temperatures which are too low, and the rates of Ca^{2+} translocation at these temperatures are too slow to permit an unambiguous interpretation of the data.

c. Is the system electrogenic or electrically silent? The best illustration for the difficulties encountered in the evaluation of the electrogenicity of ion movements in natural membrane is the controversy about the nature of the transport of adenine nucleotides and phosphate in the mitochondrial membrane. Peaks of controversy are reached when various proponents disagree with each other as well as with themselves. In the case of the nucleotide transporter, estimates of electrogenicity range from 0 to 85% (cf 46, 47).

TABLE VII. Reconstitution of P_i Transporter*

Additions	P_i/OH	P_iP_i exchange
	nmoles/min/mg	
Reconstituted vesicles	32	87
" plus valinomycin	64	–
" plus nigericin	58	–
" plus valinomycin plus nigericin	117	99

*The experimental procedure was as described (48).

TABLE VIII. Reconstitution of Nucleotide Transporter*

	ATP/ADP exchange nmoles/min/mg	
Reconstitution	Without ionophores	Plus valinomycin plus nigericin
Nucleotide transporter	102	210
P_i transporter	0	< 20
Nucleotide transporter plus P_i transporter	233	237

*The experimental procedure was as described (49).

Experiments with reconstituted vesicles allow analysis of the transport in isolation. We have reported previously (36) that adenine nucleotide transport is stimulated by ionophores which collapse the membrane potential indicating electrogenicity. Recently we have obtained similar results with reconstituted P_i transporter (48, 49). As shown in Table VII, in reconstituted vesicles the P_i/OH exchange is markedly stimulated by addition of both valinomycin and nigericin. The P_i/P_i exchange is rapid and not stimulated by the ionophores. We proposed that valinomycin is required to collapse the membrane potential when negatively charged phosphate ions move into the vesicles and are electrically not compensated by the charge of OH^- moving out (or H^+ moving in). Nigericin is required to collapse the resulting ΔpH. In the experiment shown in Table VIII the nucleotide transporter was reconstituted alone as well as together with the P_i transporter. The latter stimulated the ATP/ADP exchange in the absence of the ionophores but not in their presence. We therefore suggest that in the reconstituted system both P_i and adenine nucleotide transporter are electrogenic, and that the overall process of P_i and ADP moving in and ATP moving out is essentially electrically neutral. Thus these 2 functionally related processes may be electrically coupled.

These suggestions are based on reconstitution experiments and cannot be applied without reservations to the events in mitochondria where a membrane potential is imposed by the respiratory chain. Moreover, there are data suggesting that in mitochondria P_i transport is electrically neutral (50, 51). It should be remembered, however, that it is virtually impossible to eliminate side reactions in intact mitochondria. Thus H^+/K^+ and H^+/Na^+ antiporter activities were invoked to explain differences in P_i transport depending on the monovalent cations that were used (51). Moreover, the possibility that valinomycin might stimulate the swelling of mitochondria in the presence of ammonium phosphate was not explored (or not recorded). Finally, the possibility that the transporter does indeed

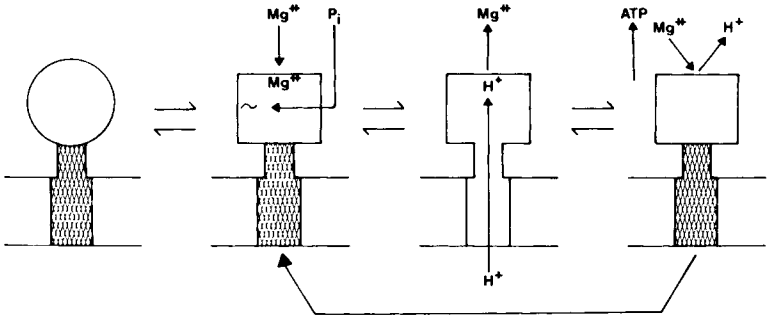

Fig. 3. Proposal for the mechanisms of ATP generation by the proton translocating mitochondrial ATPase.

operate in artificial liposomes by a mechanism that is different from that in natural membranes cannot be ruled out, even though such a notion may be distasteful to some.

d. How can we study the molecular mechanism of ion pumps? Since I have recently reviewed the extensive work that has been performed in numerous laboratories on the mechanism of action of ATP-driven ion pumps (52), I shall restrict the discussion here to our recent calorimetric measurements with various ATPases (53, 54). We have shown that the isolated Ca^{2+}-ATPase catalyzes the net formation of ATP from P_i and ADP in a 2-step reaction (55). The ATP formed was not firmly bound by the enzyme but released into solution. Moreover, the process of ATP formation could be repeated if the protein was precipitated in the presence of EDTA. Finally, formation of a Ca^{2+} gradient across a membrane could be ruled out by performing experiments in the presence of a Ca^{2+} ionophore such as A-23187. All these experiments pointed to an interaction between inorganic ions and the proteins as the driving force for ATP formation. Calorimetric measurements with the Na^+,K^+-ATPase from electric eel (53) or with the Ca^{2+}-ATPase from sarcoplasmic reticulum (54) revealed large enthalphy changes during the interaction of the protein with either Mg^{2+} or P_i (about 40 Kcal/mole of enzyme). These and other findings suggest that major conformational changes of the protein take place during interaction with the ions.

A novel formulation of the mechanism of oxidative and photophosphorylation was proposed based on these experiments (56). Briefly, the following sequence of events is visualized to take place during ATP formation by the mitochondrial ATPase (Fig. 3). The interaction of Mg^{2+} alters the conformation of membranous F_i in such a manner that the protein can now accept a phosphate by either forming a covalent intermediate as in the case of the Na^+, K^+- or Ca^{2+}-ATPases or by forming an activated ionic complex as suggested by Jenks (57). A 2-stage model of 2 conformational states of the enzyme in which the equilibrium is pulled toward the low energy form by addition of Mg^{2+} was suggested in our first publication of the calorimetric measurements (53). In the next step the proton flux generated by the respiratory chain reaches the Mg^{2+} bound to the enzyme and pushes it into the medium. With the discharge of the phosphoryl group, which is transferred to ADP, the enzyme returns to the closed form which excludes the proton flux from the Mg^{2+} site. Now the Mg^{2+} can return to its binding site displacing the proton into the medium and the process recycles. According to this formulation the major function of the proton flux is the displacement of Mg^{2+}, and the major function of the conformational changes is the closing and opening of the proton channel which communicates with the Mg^{2+} site as well as changes in the affinity of the protein to Mg^{2+} and Ca^{2+}.

I need not advertise the limitations associated with conclusions based on experiments with ATPase proteins that are removed from their natural membrane. Yet these experiments have led to the formulation of a new hypothesis which explains at the molecular level how an ion gradient can be utilized to generate the formation of ATP from ADP and P_i. Moreover, this hypothesis suggests experiments that can be conducted with functional membranes which might tell us whether the predicted replacements of Mg^{2+} by H^+ take place during the process of oxidative and photophosphorylation.

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REFERENCES

1. Pullman ME, Penefsky HS, Datta A, Racker E: J Biol Chem 235:3322, 1960.
2. Penefsky HS, Pullman ME, Datta A, Racker E: J Biol Chem 235:3330, 1960.
3. Pullman ME, Monroy GC: J Biol Chem 238:3762, 1963.
4. Racker E: Biochem Biophys Res Commun 10:435, 1963.
5. Racker E: "A New Look at Mechanisms in Bioenergetics." New York: Academic Press, 1976.
6. Smith JB, Sternweiss PC, Heppel LA: J Supramol Struct 3:248, 1975.
7. Younis HM, Winget GD, Racker E: J Biol Chem 252:1814, 1977.
8. Knowles AF, Guillory RJ, Racker E: J Biol Chem 246:2672, 1971.
9. Racker E, Fessenden-Raden JM, Kandrach MA, Lam KW, Sanadi DR: Biochem Biophys Res Commun 41:1474, 1970.
10. Mitchell P: Biol Rev Cambridge Philos Soc 41:445, 1966.
11. Serrano R, Kanner BI, Racker E: J Biol Chem 251:2453, 1976.
12. Winget GD, Kanner N, Racker E: Biochim Biophys Acta 460:490, 1977.
13. Sone N, Yoshida M, Hirata H, Kagawa Y: J Biol Chem 250:7917, 1975.
14. Cattell KJ, Knight IG, Lindop CR, Beechey, RB: Biochem J 125:169, 1970.
15. Stekhoven FS, Waitkus RF, van Moerkerk HTB: Biochemistry 11:1144, 1972.
16. Filingame RH: J Biol Chem 251:6630, 1976.
17. Racker E: In Quagliariello E, Papa S, Palmieri F, Slater EC, Siliprandi N (eds): "Proceeding of International Symposium on Electron Chains and Oxidative Phosphorylation." Amsterdam: North-Holland Publishing Company, 1975, p 401.
18. Nelson N, Eytan E, Notsani B-E, Sigrist H, Sigrist-Nelson K, Gitler C: Proc Natl Acad Sci USA (In press).
19. Kagawa Y, Racker E: J Biol Chem 246:5477, 1971.
20. Hatefi Y, Haavik AG, Fowler LR, Griffiths DE: J Biol Chem 237:2661, 1962.
21. Hinkle PC, Kim J-J, Racker E: J Biol Chem 247:1338, 1972.
22. Racker E: J Membr Biol 10:221, 1972.
23. Ragan CI, Hinkle PC: J Biol Chem 250:8472, 1975.
24. Leung KH, Hinkle PC: J Biol Chem 250:8467, 1975.
25. Racker E, Stoeckenius W: J Biol Chem 249:662, 1974.
26. Boyer PC: In King TE, Mason HS, Morrison M (eds): "Oxidases and Related Redox Systems." New York: John Wiley & Sons, vol 2, p 994, 1965.
27. Danon A, Stoeckenius W: Proc Natl Acad Sci USA 71:1234, 1974.
28. Ort DR, Dilley RA, Good NE: Biochim Biophys Acta 449:108, 1976.
29. Miller C, Racker E: J Membr Biol 26:319, 1976.
30. Knowles AF, Eytan E, Racker E: J Biol Chem 251:5161, 1976.
31. Knowles AF, Kandrach A, Racker E, Khorana HG, J Biol Chem 250:1809, 1975.
32. LaBelle EF, Racker E: J Membr Biol 31:301, 1977.
33. Warren GB, Toon DA, Birdsall NJM, Lee AG, Metcalfe JC: Proc Natl Acad Sci USA 71:622, 1974.

34. Racker E, Kandrach A: J Biol Chem 248:5841, 1973.
35. Ragan CI, Racker E: J Biol Chem 248:2563, 1973.
36. Shertzer H, Racker E: J Biol Chem 251:2446, 1976.
37. Rhodin TR, Racker E: Biochem Biophys Res Commun 61:1207, 1974.
38. Racker E, Fisher LW: Biochem Biophys Res Commun 67:1144, 1975.
39. Eytan GD, Matheson MJ, Racker E: J Biol Chem 251:6831, 1976.
40. Carroll R, Racker E: J Biol Chem (In press).
41. Eytan GD, Schatz G, Racker E: In Abrahamsson S, Pascher I (eds): "Structure of Biological Membranes." New York: Plenum Press, Nobel Symposium 34, Sweden, June 1976, p 373, 1977.
41. Schatz G, Mason TL: Annu Rev Biochem 43:51, 1974.
43. Martonosi A: In "Current Topics in Membranes and Transport." New York:Academic Press, vol 3, p 83, 1973.
44. Knowles AF, Racker E: J Biol Chem 250:3538, 1975.
45. Racker E, Hinkle PC: J Membr Biol 17:181, 1974.
46. Klingenberg M, Rottenberg H: Eur J Biochem 73:125, 1977.
47. Thayer WS, Hinkle PC: J Biol Chem 248:5395, 1973.
48. Banerjee RK, Shertzer HG, Kanner BI, Racker E: Biochem Biophys Res Commun 75:772, 1977.
49. Shertzer HG, Kanner BI, Banerjee RK, Racker E: Biochem Biophys Res Commun 75:779, 1977.
50. Chappell JB: Br Med Bull 24:150, 1968.
51. Mitchell P, Moyle J: Eur J Biochem 9:149 (1969).
52. Racker E: Trends in Biochemical Sciences 1:244, 1976.
53. Kuriki Y, Halsey J, Biltonen R, Racker E: Biochemistry 15:4956, 1976.
54. Epstein M, Kuriki Y, Biltonen R, Racker E: International Symposium on Calcium Binding Proteins, (abstract) June 5–10, Cornell Univ, Ithaca, NY, 1977.
55. Knowles AF, Racker E: J Biol Chem 250:1949, 1975.
56. Racker E: Annu Rev Biochem (In press).
57. Jenks WP: Adv Enzym 43:219, 1975.